S H O R T T I T L E.

PIASMA PROGESTERONE LEVELS IN HUMAN PREGNANCY.

A B S T R A C T.

TITLE: "PLASMA PROGESTERONE LEVELS IN HUMAN PREGNANCY. A STUDY BY COMPETITIVE PROTEIN BINDING".

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DEPARTMENT: EXPERIMENTAL MEDICINE.

DEGREE: M.Sc.

Plasma progesterone levels in normal and abnormal human pregnancy have been determined by Competitive Protein Binding, utilizing the affinity of progesterone for corticosteroid binding globulin. The technique is rapid (8 hours), sensitive (to 1 microgram/100 ml.), and requires small sample volumes (1 ml.). The sensitivity, specificity, accuracy and precision of the assay have been determined.

The literature on progesterone in pregnancy has been reviewed concerning; synthesis and metabolism, reported plasma levels and alternative assay techniques.

Plasma progesterone levels,(in microgram/100 ml.), are reported throughout normal pregnancy to delivery, (early; 1 - 5, late; 5 - 25), postpartum (0), and fetal levels (umbilical vein; 40.8, artery; 18.7). There is poor correlation between progesterone levels and the "progesterone effect" by vaginal cytology. In threatened abortion, abnormally low progesterone levels (below 1), carry a poor prognosis. With intrauterine fetal death, levels varied from abnormally low to normal values, (1.1 - 15.8).

PLASMA PROGESTERONE LEVELS IN HUMAN PREGNANCY. A STUDY BY COMPETITIVE PROTEIN BINDING.

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I N D E X.

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I

A C K N O W L E D G E M E N T S.

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II

I N T R O D U C T I O N

Progesterone is recognized as a steroid hormone having a unique association with reproduction and pregnancy. While this hormone is produced as an intermediate compound in the synthesis of other steroids by the adrenal cortex, testis and ovarian stroma, these amounts are infinitesimal compared with the quantities of progesterone produced by the corpus luteum and the placenta. Abundant experimental and clinical evidence indicates that abnormalities of progesterone production may be associated with reproductive problems. It is therefore reasonable that measurement of plasma progesterone levels could be of prognostic or therapeutic significance in the management of clinical problems associated with pregnancy.

Previous methods of measuring plasma progesterone levels, such as by double isotope derivative assay, have been much too difficult and time consuming to permit their use for routine clinical studies. Clinical assessment of progesterone metabolism has been by indirect methods such as twenty-four hour urinary pregnanediol, vaginal cytology, basal body temperature, absence of "ferning" in cervical mucous and endometrial biopsy.

The principle of "Competitive Protein Binding" [1] first applied to progesterone in this laboratory in 1967, [2] has been applied for the rapid determination of plasma progesterone concentrations. Apart from a liquid

scintillation counter, only simple laboratory techniques and equipment are required. One technician can handle approximately twenty-five samples, in duplicate, in an eight hour day, using one milliliter or less of plasma per assay. Briefly, the method consists of preliminary petroleum ether extraction of the plasma to separate the progesterone from the corticosteroids. (The latter can be assayed simultaneously from the same plasma sample if desired). Progesterone is determined in a system using dog corticosteroid-binding-globulin and tritiated corticosterone. The assay determines the entire "progestin" group of related steroids, but by paper chromatography, we have demonstrated that, in practice, this consists almost entirely of progesterone.

The assay is very similar to "radio-immunoassay" [3], utilized for protein (nitrogen-containing) hormones. In competitive protein binding, a steroid hormone is bound to a naturally occurring carrier protein. Radio-immunoassay however utilizes a protein antibody produced by animals by active immunization. The purified protein hormone is injected and acts as an antigen.

Plasma progesterone levels will be described in a number of clinical situations associated with normal and abnormal pregnancy.

III

TRIVIAL NAMES OF STEROID HORMONES

STEROID HORMONES.

TRIVIAL NAMES.	STRUCTURE.
Progesterone	Pregn-4-ene-3, 20-dione
17α hydroxy-progesterone	17α-hydroxy-pregn-4-ene-3, 20-dione
20α dihydro-progesterone	20α-hydroxy-pregn-4-ene-3-one
Corticosterone	11β,21-dihydroxy-pregn-4-ene-3, 20-dione
Cortisol	11β,17α,21-trihydroxy-pregn-4-ene-3, 20-dione
Desoxycorticosterone	21-hydroxy-pregn-4-ene-3, 20-dione
Pregnenolone	3β-hydroxy-pregn-5-en-20-one
17α hydroxy-pregnenolone	3β,17α dihydroxy-pregn-5-en-20-one
Pregnanediol	5β-pregnane-3α, 20α-diol
Testosterone	17β hydroxy -androst-4-ene-3-one
16α hydroxy-dehydroisoandrosterone	3β ,16α-dihydroxy-androst-5-ene-17-one
Estriol	estra-1,3,5 (10)triene-3,16α ,17β -triol

IV

HISTORICAL REVIEW

A. REVIEW OF PROGESTERONE SYNTHESIS, CONVERSION AND METABOLISM DURING PREGNANCY.

The corpus luteum had been described for nearly three hundred years before a role was ascribed to it. In 1910, Fraenkel showed that the maintenance of pregnancy required the presence of the corpus luteum. Also in 1910, Ancel and Blouin demonstrated that the corpus luteum was required to produce secretory changes in the endometrium[4]. In 1929, a crystalline hormone, originally called "progestin" ("for pregnancy") was isolated from the corpus luteum of sows by Corner and Allen[5]. The hormone was soon synthesized and the name changed to progesterone. In 1936, Venning and Brown described the metabolism of progesterone to pregnanediol and the excretion of the latter in the urine[6].

Early pregnancy. (Corpus luteum function).

In early pregnancy, progesterone production is due to persistence of corpus luteum function beyond the luteal phase of the menstrual cycle. In response to chorionic gonadotrophin hormone (CGH), produced by the cytotrophoblast (Langhan's Layer) of the chorionic villi of the young placenta, the luteinized granulosa cells of the corpus luteum continue progesterone synthesis. Maternal plasma progesterone levels

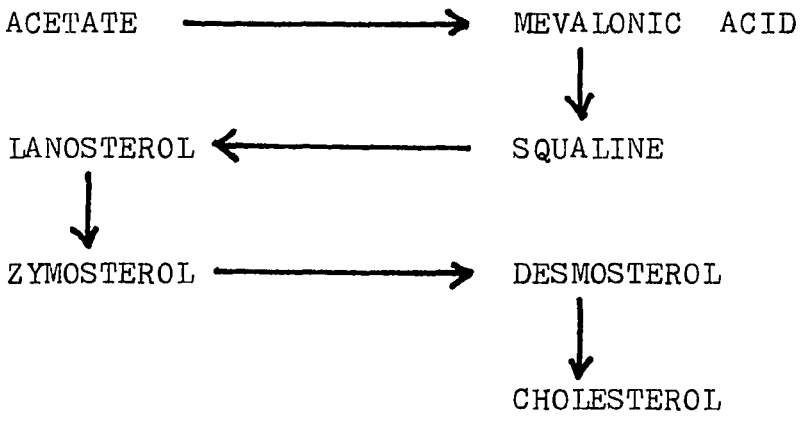
reflect this continuation of corpus luteum function. The role of progesterone appears to be that of decidualization of the uterine endometrium and inhibition of myometrial contractility, thus producing an intrauterine environment favorable for the developing pregnancy. Synthesis of progesterone by the corpus luteum is from acetate via mevalonate, squaline, cholesterol and pregnenolone (Figure 1). By approximately 80 days gestation, corpus luteum progesterone production has gradually declined despite persisting levels of CGH[7,8]. The placenta itself gradually assumes progesterone synthesis in the syncitiotrophoblast. However, *in vitro* studies[9], have shown that the corpus luteum is capable of synthesizing progesterone throughout pregnancy and up to four days postpartum.

Late pregnancy. (Placental function)

Circulation between the fetus and placenta is established as early as the 17th day of gestation (from the last menstrual period) and organogenesis is complete in the embryo at 8 weeks gestation[10]. From this time, the interplay of steroid synthesis and conversion between the two parts of the "feto-placental" unit[11, 12] becomes a possibility.

Figure 1.

BIOSYNTHESIS OF CHOLESTEROL FROM ACETATE.*



* After Cornforth, J.W.. J. Lipid Research, October, 1959.

However, due to technical problems, most *in vivo* experimentation has been performed at a later gestational age, that is, usually at mid-pregnancy, when therapeutic abortion must be terminated by hysterotomy rather than by uterine curettage. Information on steroid hormone metabolism during human pregnancy has been obtained from a large variety of experimental models, usually involving the introduction of radioactive labelled tracer steroid precursors and the later isolation and identification of radioactive metabolites. Techniques have included: *in vivo* injection or perfusion of precursor into maternal and/or fetal circulations; *in vitro* studes in isolated organs, including placenta, maternal adrenal, corpus luteum, fetal liver, adrenal, testis, ovary and other tissues; incubation studies with tissue slices, homogenates, specific cell fractions, such as mitochondria, and tissue cultures. Discrepancies have sometimes been noted between the results of *in vitro* and *in vivo* studies. However, the latter are usually considered to more accurately represent physiological situations and are therefore probably more valid[13]. Steroid levels have also been measured in a variety of clinically abnormal pregnancies, such as in adrenalectomized or oophorectomized (after conception) women, or with an anencephalic fetus with atrophic adrenal cortical tissue.

While placental progesterone synthesis de novo from acetate has been demonstrated in vitro, the large amounts of progesterone produced in late pregnancy, estimated in excess of 250 milligrams per day [14], are most consistent with synthesis from preformed cholesterol obtained from the maternal circulation [15, 16]. The contribution of fetal cholesterol to this process appears to be small [17]. Progesterone synthesis in the corpus luteum and placenta is summarized in Figure 2.

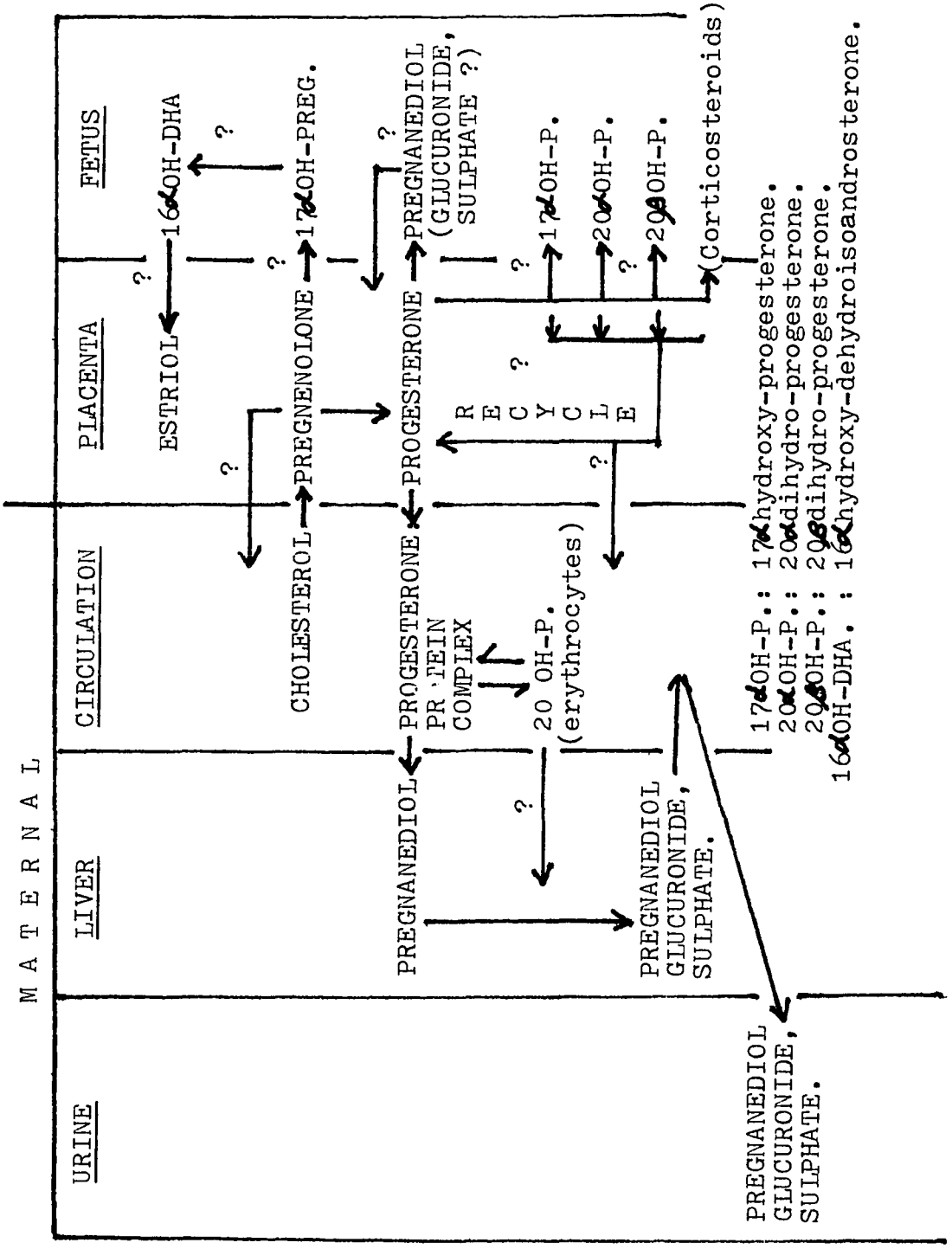
A reasonable explanation for the large amounts of progesterone produced by the placenta is to produce a high myometrial concentration of the progesterone. This inhibits myometrial contractility due to a hyperpolarizing effect on the cell membrane of the smooth muscle fibers [18]. This would prevent the onset of uterine contractions and maintain pregnancy despite increasing uterine volume and myometrial stretch, (the "Progesterone Block" theory of Csapo [19, 20].) The concentration of progesterone in placental tissue is very low, indicating that there is no storage of the hormone in the placenta itself.

Progesterone passes from the placenta into both the maternal and fetal circulations. Here it is carried in the plasma almost entirely bound to various plasma proteins. The hormone is bound non-specifically and with low affinity

Figure 2.

PROGESTERONE BIOSYNTHESIS AND METABOLISM

(Modified from Solomon, S.[62]).



to albumin. However, due to the large concentration of albumin in the plasma, the amount of albumin-bound progesterone is a large proportion of the total. Progesterone is also bound with high affinity by alpha-1 acid glycoprotein (orosamucoid) which because of its very low plasma concentration actually binds only a small proportion of the progesterone [21,22]. The third plasma protein, corticosteroid binding globulin (CBG) has a high affinity for progesterone (that exceeds its affinity for cortisol [23]) and appears to be the major carrier protein for progesterone in the plasma [24]. The level of CBG rises in pregnancy due to its increased synthesis, presumably in the liver, under the stimulus of the high estrogen levels of pregnancy. Elevated CBG levels can be produced by estrogen administration to men and non-pregnant women [25].

In the liver, progesterone is metabolized to pregnanediol, conjugated as the glucuronide or sulphate, and excreted in the maternal urine. Progesterone lacks a free hydroxyl group and cannot be conjugated directly with glucuronic or sulphuric acid. It has been shown [26] that all urinary pregnanediol does not originate from progesterone. Pregnenolone and 20α-hydroxy-4-pregnene-3-one are also precursors of urinary pregnanediol.

The high maternal levels of progesterone appear to act as an aldosterone antagonist competing with the latter at

receptor sites. The high maternal aldosterone levels noted during pregnancy appear to be a compensatory activity by the maternal adrenal cortex[27].

The markedly higher progesterone levels in the umbilical vein compared to those in the umbilical artery indicate that there is marked utilization of progesterone by the intrauterine fetus. The metabolic fate of this progesterone is at present partly uncertain. Some progesterone appears to be metabolized to pregnanediol in the fetal liver and some reduced to 20α dihydro-progesterone (see below). The fetal adrenal cortex, with its fetal or "X" zone, has a structure distinct from the adult adrenal cortex. This fetal organ appears capable of synthesizing, from basic precursors, such steroids as 5-pregnenolone, 17 hydroxy-pregnenolone, dehydroepiandrosterone, and 16α hydroxy-dehydroepiandrosterone. However, in vitro, the gland cannot convert these steroids to the more biologically active 4-3 ketone types due to the lack of the enzyme 5-3-beta-hydroxy-steroid-dehydrogenase. However, steroids such as progesterone, already possessing the 4-3 ketone configuration, could be used to synthesize the corticosteroids in the fetal adrenal cortex[28].

Other progestational steroids.

There appear to be two derivatives of progesterone that might be significant, namely, 17α hydroxy-progesterone (17α OH-P)

and 20α-dihydro-progesterone (20αOH-P). The former, 17αOH-P, appears to be produced by the corpus luteum in early pregnancy but does not appear to be produced in the placenta which lacks the ability for 17α-hydroxylation[28]. Progesterone appears to be metabolized by the fetus to both 17αOH-P and 20αOH-P, both steroids being found in greater amounts in the umbilical artery than in the umbilical vein. (The reverse of the levels of progesterone). It also appears that progesterone can be metabolized to 20αOH-P by erythrocytes in the maternal (and presumably fetal) peripheral circulations[29]. However, these two "progestins" appear to have only a slight degree of biologic progestational activity in humans, although the same may not be true for other animal species. For example, in rabbits, the predominant "progestin" appears to be 20α-dihydro-progesterone[59].

B. REVIEW OF THE LITERATURE OF PLASMA PROGESTERONE LEVELS DURING PREGNANCY.

A detailed review of the published data on plasma progesterone levels has recently been presented by van der Molen and Aakvaag[30]. The following discussion summarizes that publication together with original articles.

I. Plasma progesterone levels in early pregnancy.

There have been relatively few results published for plasma progesterone values in early, as compared to late, pregnancy. This is mainly because the low levels in early pregnancy are often at the limits of accuracy of the physiochemical methods previously used for progesterone determinations. Levels ranging from 2 - 7 microgram per 100 ml. plasma have been found by most investigators[31,32]. The lower values are in the range found in the luteal phase of the menstrual cycle[33]. Levels as high as 20 microgram per 100 ml. plasma were reported by Yoshimi[8], in cases where ovulation was induced artificially by exogenous gonadotrophin administration. These high levels appear to represent excessive stimulation of the corpus luteum (or perhaps multiple corpora lutea) rather than a physiological situation.

II. Plasma progesterone levels in early pregnancy compared with hormonal assessment by vaginal cytology, (a bioassay).

No previous reports of comparison of plasma progesterone levels with simultaneously performed vaginal cytology were found in the literature. However in studies [34,35] comparing vaginal cytology with urinary pregnanediol excretion, the correlation was sufficiently poor as to prevent vaginal cytology being utilized as a reliable assessment of the hormonal status of pregnancy in the first trimester.

III. Plasma progesterone levels in first trimester threatened abortion.

Peripheral plasma progesterone levels were reported in 21 cases of threatened abortion by Eaton and Short [31]. They found that of 11 cases that aborted only three had low plasma progesterone levels. Of the 10 cases in which pregnancy continued, 2 had abnormally low levels. Their conclusion was that abortion could occur despite normal progesterone values. No mention is made of their case selection to eliminate cases of criminal abortion. Johansson [32] reported abnormally low plasma progesterone levels (0.22 to 0.80 microgram per 100 ml. plasma) in 5 of 9 patients aborting in the first trimester. Again no

mention is made of case selection. Acevido et al [36] reported on urinary pregnanediol excretion in 24 cases of threatened abortion. The 15 patients who subsequently aborted showed abnormally low pregnanediol excretion, while normal levels were found in the patients whose pregnancies continued.

IV. Plasma progesterone levels in mid and late pregnancy.

There have been a relatively large number of reports of plasma progesterone levels during the latter half of pregnancy [8, 31, 32, 37, 38, 39, 40, 41]. Most studies have reported single determinations in individual subjects but a few have reported serial determinations during the same pregnancy. The reported progesterone levels range from 4 to 40 microgram per 100 ml. plasma, depending on the period of gestation. Table I, modified from van der Molen and Aakvaag [30], summarizes the levels reported by various investigators.

V. Plasma progesterone levels in late pregnancy, at delivery and postpartum.

Various investigators [31, 38, 42, 43] have reported plasma progesterone levels at the end of pregnancy. Their

TABLE I.

PLASMA PROGESTERONE LEVELS IN PREGNANCY.

REFERENCE.	WEEKS GESTATION.		
	0 - 12	13 - 26	27 - 40
	(microgram/100 ml.)		
ZANDER [46]			4 - 27
ETON & SHORT [31]	2 - 6	2 - 10	6 - 28
GREIG ET AL [37]		3 - 9	2 - 27
KUMAR ET AL [38]			3 - 25
HARBERT ET AL [39]			0 - 39.5

findings may be summarized as follows:

(1) Progesterone levels at the onset of labour may be either higher or lower than previously determined levels.

(2) Progesterone levels during labour did not appear to fall by statistically significant amounts.

(3) Within 24 hours postpartum, progesterone levels had declined to nonpregnant values.

VI. Plasma progesterone levels - comparison between maternal vein and fetal umbilical vein and umbilical artery levels at delivery.

Investigators[37, 39] have uniformly found highest progesterone levels in fetal umbilical artery plasma, intermediate values in fetal umbilical vein plasma and lowest levels in maternal vein plasma. These findings are summarized in Table II.

VII. Plasma progesterone levels in cases of intrauterine fetal death and high risk pregnancy.

Plasma progesterone levels in clinically abnormal pregnancy were reported by Eaton and Short[31]. Normal values were found in 3 Rhesus immunized pregnancies and only 1 of 15 patients with pre-eclamptic toxemia had low

TABLE II

Levels of plasma progesterone in umbilical cord plasma.

(microgram/100 ml. plasma)

Author		Umbilical vein	Umbilical artery.
Greig et al [37]		40 - 187	22 - 80
	mean	102	57
Harbert [39]		31 - 169	0 - 99
	mean	72.4 ± 29.5	43.6 ± 32.9

values. Abnormally high plasma progesterone levels were noted in 7 of 17 diabetic pregnancies and 3 of these 7 terminated in fetal death. Greig et al[37] concluded that plasma progesterone levels followed urinary estriol levels rather than urinary pregnanediol. In general, clinically normal pregnancies with large babies had high plasma progesterone levels, while clinically abnormal pregnancies with small babies had low maternal plasma progesterone levels. Johansson[32] found low plasma progesterone levels in 3 of 5 cases of intrauterine fetal death, 2 of these cases were pre-eclamptic toxemia.

Assessment of placental progesterone synthesis by urinary pregnanediol excretion is also uncertain. Thus Acevido[36] found evidence of "biochemical progestational insufficiency" in 11 cases of toxemia of pregnancy. However, Bell et al[44] reported generally normal pregnanediol excretion in 5 high risk pregnancies.

C. REVIEW OF ALTERNATIVE METHODS OF PLASMA PROGESTERONE ASSAY.

Bioassays.

A number of bioassays using different end points have been suggested for the estimation of progesterone. Such methods have generally employed the effect of progesterone on the uterine endometrium or the capacity to maintain pregnancy following the removal of the ovaries (containing the corpora lutea) in a variety of test animals. An example of the former type, the Hooker-Forbes test [45], depends on hypertrophy of stromal nuclei in the endometrium of ovariectomized mice, after intrauterine injection of progesterone containing extracts. The bioassay technique usually lacks specificity for progesterone in that progesterone derivatives, such as 17α-hydroxy-progesterone or 20β or 20α-dihydro-progesterone or synthetically produced progestins, may exert synergistic or antagonistic effects. Also variations in specificity and reactivity of different strains of the same animal species may influence the results of the bioassay.

The cost and time requirements for biological testing make these methods generally unsatisfactory. However, bioassays remain indispensable for assessing the large number of synthetic progestins being developed. Here, biological

(progestational) activity must be determined rather than the concentration of the compound.

Physiochemical methods for blood progesterone.

These may be classified according to the method of final detection of progesterone (or a progesterone derivative).

Spectrophotometric techniques.

Ultraviolet absorption of progesterone, the first reliable method for the determination of progesterone levels in blood, was introduced by Zander in 1954[46]. The absorption of the ultraviolet radiation at 240 mμ resulting from the presence of the 4-3 keto structure in the progesterone molecule. The sensitivity of the method (0.3 - 0.5 μg progesterone) limits the application for reasonable plasma volumes (10 ml. plasma, progesterone concn. 5 μg/100 ml.) to analysis of human blood during the second half of pregnancy.

Spectrophotometric methods to measure coloured derivatives of progesterone, such as progesterone-bis-dinitrophenylhydrazone, utilizing the oxo groups of the molecule, have a sensitivity of 0.1 - 0.2 μg progesterone. (Equivalent to 10 ml. plasma, progesterone concn. 1 - 2 μg/100 ml.).

Isotope labelling. (Double isotope dilution derivative assays).

The use of radioactive labelled reagents that will react with progesterone, has permitted the preparation of labelled progesterone derivatives. After purification of such derivatives, the amount of radioactivity in the final residue will be proportional to the amount of progesterone or its derivative. These techniques have involved preparation of derivatives such as 20 α-dihydro-progesterone[47] or progesterone-thiosemi-carbazide-diacetate[48]. The sensitivity of such techniques depends on the sensitivity of estimations of radioactivity and the specific activity of the labelling reagents. To ensure accuracy and precision the isotope derivative procedure has been applied predominantly in combination with the isotope dilution procedure to correct for losses during purification of the sample. - The double isotope dilution derivative assay. The sensitivity for the dihydro-progesterone method is 0.1 μg (equivalent to 10 ml. plasma, progesterone concn. 1 μg/100 ml.) and for the thiosemicarbazone technique is 0.002 μg (equivalent to 0.2 ml. plasma, progesterone concn. 1 μg/100 ml.).

Gas chromatography.

In combination with gas-liquid chromatography, a number of physical methods for detection of compounds have become available. Flame ionization[49] and electron capture[50] have proved to be sensitive enough for estimation of the small

amounts of progesterone (or derivatives) isolated from biological sources. The detection systems are generally non-specific and require separation of progesterone (or a derivative) from other compounds before reaching the detector. Sensitivity varies from 0.001 to 0.1 μg progesterone per sample, (0.01 to 10 ml. plasma, progesterone concn. 1 μg/100 ml.).

It is important to note that Yannone's method[49] requires three days to complete plasma progesterone assays on ten samples. This long duration, which is common to all the above sensitive methods, makes them impractical for routine measurements in clinical situations.

V.

METHODOLOGY

A. GENERAL COMMENTS ON COMPETITIVE PROTEIN BINDING.

The basic principles of Competitive Protein Binding Analysis were described by Murphy in 1964[1] and their application to the determination of plasma progesterone in 1967[2]. The important points concerning this method of steroid hormone assay are summarized below.

Steroid Hormones in Blood.

Because steroid hormones are not associated with the cellular component of the blood (at least in the human) it has become accepted practice to record steroid concentrations per volume of plasma or serum rather than per volume of whole blood.

The steroid hormones are present in the plasma in various forms:

1. The free (unconjugated) hormone, which is biologically active.
2. The conjugated hormone (sulphate and/or glucoronide), lacking biologic activity.

This study is concerned with the free (unconjugated) hormones which exist in the plasma:

1. Bound to albumin, a high capacity, low affinity, non-specific system.

2. Bound to orosomucoid (α-acid glycoprotein)[21,22] - of little significance because of its low plasma concentration.
3. Bound to a specific carrier protein, present in a relatively low concentration. For progesterone, this protein is a corticosteroid-binding-globulin (CBG), which has a higher affinity for progesterone than for cortisol itself[23].

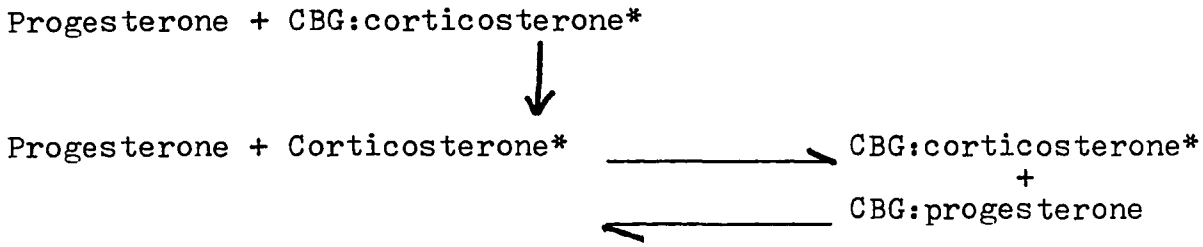
Thus, in the plasma, there is a dynamic equilibrium between:

Free hormone + Carrier Protein $\rightleftharpoons$ Protein: Hormone Complex

Principles of the Competitive Protein Binding Assay.

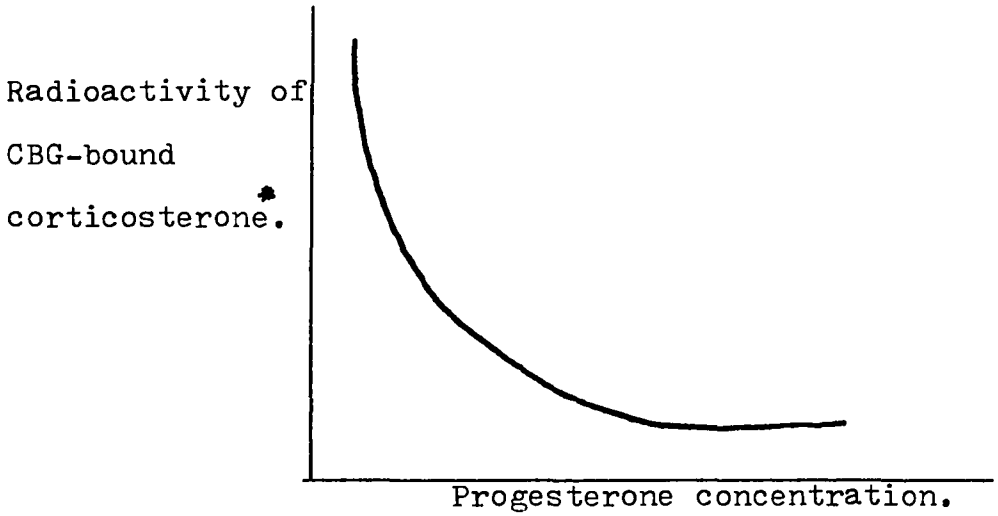
In performing the assay, a "Protein Binding Solution" is made up in distilled water, containing a tracer amount of tritiated hormone (H*), just sufficient to saturate a small amount of carrier protein (P). For the progesterone assay, tritiated corticosterone* (compound B) is used with dog CBG (see below).

When amounts of non-radioactive progesterone are added to the system, there is displacement of the tritiated corticosterone* from the CBG in direct proportion to the amount of progesterone added.



Separation of free Corticosterone* from the CBG-bound fraction is achieved by Florisil adsorption of the former. The radioactivity of an aliquot of the CBG-bound fraction is then added to modified Bray's solution[51] (see below) and the radioactivity is determined in a liquid scintillation counter.

A standard curve is constructed using a series of known amounts of progesterone.



The identical procedure is carried out with samples containing unknown amounts of progesterone. The radioactivity of the unknown sample is determined and is used to read off the progesterone concentration from the standard curve. In practice, the unknown samples and the known values for the Standard Curve are all processed simultaneously.

Additional Notes

The large capacity, low specificity binding of steroid hormone to albumin is reduced by the marked dilution with distilled water (99 volumes to 1). The effect of other plasma proteins is minimized by using corticosterone* as the tracer. Dog CBG is used, rather than human, as its greater affinity for progesterone increases the sensitivity of the assay.

The volume of plasma varies from 0.05 to 0.4 ml. per assay depending on whether a high (late pregnancy) or low level (early pregnancy) of progesterone is expected.

Blanks are run with male pooled plasma (MPP) which has a progesterone value of zero in this assay.

B. COLLECTION, PREPARATION AND STORAGE OF PLASMA SAMPLES.

1. Maternal venous blood samples were collected in my private office, and in the obstetrical clinics, emergency department, wards and obstetrical case rooms of Saint Mary's Hospital, Montreal.
2. Fetal blood samples were collected, immediately following delivery of the fetus, from the umbilical artery and vein. Collection was from a 6 to 12 inch segment of the umbilical cord which was clamped with surgical forceps at both ends to keep the vessels filled with blood.
3. All samples were collected in preheparinized "Vacutainers", 10 or 20 ml. size. They were refrigerated at 4 - 10 ^{o} centigrade, never longer than 48 hours, until centrifuged. Following centrifugation, the plasma was removed, and stored frozen at -4 ^{o} centigrade until assayed.

Other techniques.

1. The weight of fetus and placenta were recorded at delivery.
2. Routine vaginal cytology was performed at first pregnancy visits and the slides were stained by

the standard Papanicolou technique. A maturation index (differential cell count) as a "bioassay" of the hormonal status of the pregnancy was recorded when the smears were screened for neoplastic or other atypical cells by the author.[52,53]

(C) MATERIALS AND EQUIPMENT

Materials.

Dog plasma (for dog CBG) was obtained from adult mongrel dogs at the Experimental Surgical Laboratory, Donner Building, McGill University.

Radioactive steroids, Corticosterone 1,2- ^{3}H, and Progesterone 1,2- ^{3}H, were obtained from the New England Nuclear Corporation, Boston, Mass.

Nonradioactive steroids were obtained from the Sigma Chemical Company, St. Louis, Mo.

Solvents, reagents for liquid scintillation counting and paper chromatography were obtained from the Fisher Scientific Company, Montreal and Picker Nuclear Company, Montreal.

Florisil, 60-100 mesh, was obtained from the Floridin Company, Talahassee, Fla.

Equipment

The Liquid Scintillation Counter (720 series) was manufactured by the Nuclear Chicago Corporation, Chicago, Ill.

The Eberbach Testtube Shaker was made by the Eberbach Corporation, Ann Arbour, Michigan.

The plastic measuring spoon for Florisil and the test tube Evaporating Manifold were obtained from the Endocrine Laboratories, Queen Mary Veterans' Hospital, Montreal.

D. PROTOCOL OF PROGESTERONE ASSAY.

1. Prepare protein binding solution:

   To dog plasma 2.5 ml.

   Add 0.4 m ^{3}H - Corticosterone

   Add distilled water to total volume of 100 ml.

2. Pipette volume* of plasma into centrifuge tube.

   * Early pregnancy: 0.4 ml.

   Late pregnancy: 0.1 ml.

   Umbilical vein or artery 0.05 ml.

   (All samples in duplicate)

3. Extract progesterone with petroleum ether, (approx. 1 ml) x 3.

4. Preparation of Standard Curve.

   Pipette duplicate samples of the following concentrations of progesterone (µgm/100 ml. ethanol): 0,4,8,12,20,40 into centrifuge tubes.

5. Evaporate extracts of all samples to dryness, simultaneously with Standard Curve using manifold.

6. Add 1 ml. protein binding solution to each test tube.

7. Equilibrate at approx. 45 degrees centigrade in water bath for 5 minutes.

8. Cool in ice bath for 30 minutes or longer.

9. Add 1/8 spoon (40 milligram) Florisil to each test tube.

10. Shake for 2 minutes in Eberbach Shaker.

11. Cool in ice bath 10 minutes.

12. Pipette an aliquot of each sample (0.5 ml.) into Bray's solution 10 ml. (see below) in scintillation counter vial.
13. Cool all samples for approx. 15 minutes in Liquid Scintillation Counter before starting count.
14. Usual count: Time to reach 4000 counts.
15. Modified Bray's Solution:

    To one U.S. gallon of Dioxane in a large bottle add:

    400 gms Naphthalene
    28 gms PPO (2,5-diphenyloxazole)
    1.2 gms Dimethyl-POPOP (1,4-bis-2-(4-methyl-5-phenoxyloxazolyl)-benzene)

    Shake well. Allow to stand overnight.

E. SEPARATION OF PROGESTERONE FROM THE CORTICOIDS IN THE PLASMA. SOLVENT EXTRACTION OF PROGESTERONE BY PETROLEUM ETHER.

Because cortisol competes with progesterone for binding by the CBG in the assay solution, it is necessary to separate these two steroids before performing the progesterone assay. This is achieved by extracting the progesterone into petroleum ether, the cortisol remaining in the plasma. This separation was assessed throughout the range of progesterone concentrations of the Standard Curve: 0,4,8,12, 20 and 40 micrograms progesterone per 100 ml. plasma. Tritiated progesterone (0.01 microcuries per test tube) was used as the tracer.

It was found that progesterone recovery ranged from 64.4 to 56.2 percent as summarized in Table III. That is, the mean progesterone recovery is approximately 60 percent. The recovery was tested with petroleum ethers with six different boiling point ranges between 30 and 160 degrees centigrade and was always found to be at the 60 percent level.

The results reported in the clinical portions of this thesis are uncorrected for the 60 percent efficiency of extraction. That is, the absolute plasma progesterone concentration would be: reported value X $\frac{100}{60}$.

It was previously determined[2] that the petroleum ether

TABLE III

Progesterone Concentration (microg./100 ml.)	Percent recovery of H^3 - Progesterone by petroleum ether extraction.
0	64.4
4	67.0
8	60.2
12	58.5
20	58.5
40	56.2

extraction would remove 2.4 percent of the cortisol and that 97.6 percent remained in the plasma.

This degree of separation of progesterone from cortisol is quite satisfactory for a routine clinical laboratory procedure.

F. (1) SENSITIVITY OF THE ASSAY.

It was established that, using 0.1 ml. of plasma per assay, the technique was sensitive to detect a progesterone concentration of 1 microgram progesterone/100 ml. plasma (equivalent to 1 millimicrogram progesterone per 0.1 ml. plasma assay volume).

However, to increase the sensitivity of the assay and keep the determinations on the most sensitive portion of the Standard Curve, the volume of plasma used in the assay was varied as follows:

First trimester pregnancy and postpartum (low values expected)	:	0.2 or 0.4 ml. plasma per assay and result divided by 2 or 4 respectively.
Mid & late pregnancy delivery	:	0.1 ml. plasma per assay.
Umbilical artery umbilical vein (very high values expected)	:	0.05 ml. plasma and result multiplied by 2.

F. (2) SPECIFICITY OF THE PROGESTERONE ASSAY.

(i) Competition by Natural Steroids.

It was established by Murphy[2] that the following steroids have an extremely low affinity for CBG and therefore would not compete with progesterone in the assay: 17β-estradiol, estrone, estriol, pregnanediol, cholesterol, androsterone, androstenedione, dehydroepiandrosterone.

Testosterone.

Testosterone was found to have an affinity for dog CBG one eighth that of progesterone. This lower affinity, together with the normally low level of plasma testosterone (non-pregnant 49; pregnant 114 millimicrograms per 100 ml. plasma[54]) would result in a neglible effect on the progesterone assay.

Pregnenolone.

The affinity of pregnenolone for dog CBG was tested by a Standard Curve using pregnenolone concentrations of 0, 10, 20, 30 and 40 microgram/100 ml. The resulting curve was extremely "flat", indicating that pregnenolone will not compete significantly in the progesterone assay (Figure 3).

20α dihydro-progesterone (20αOH-P).

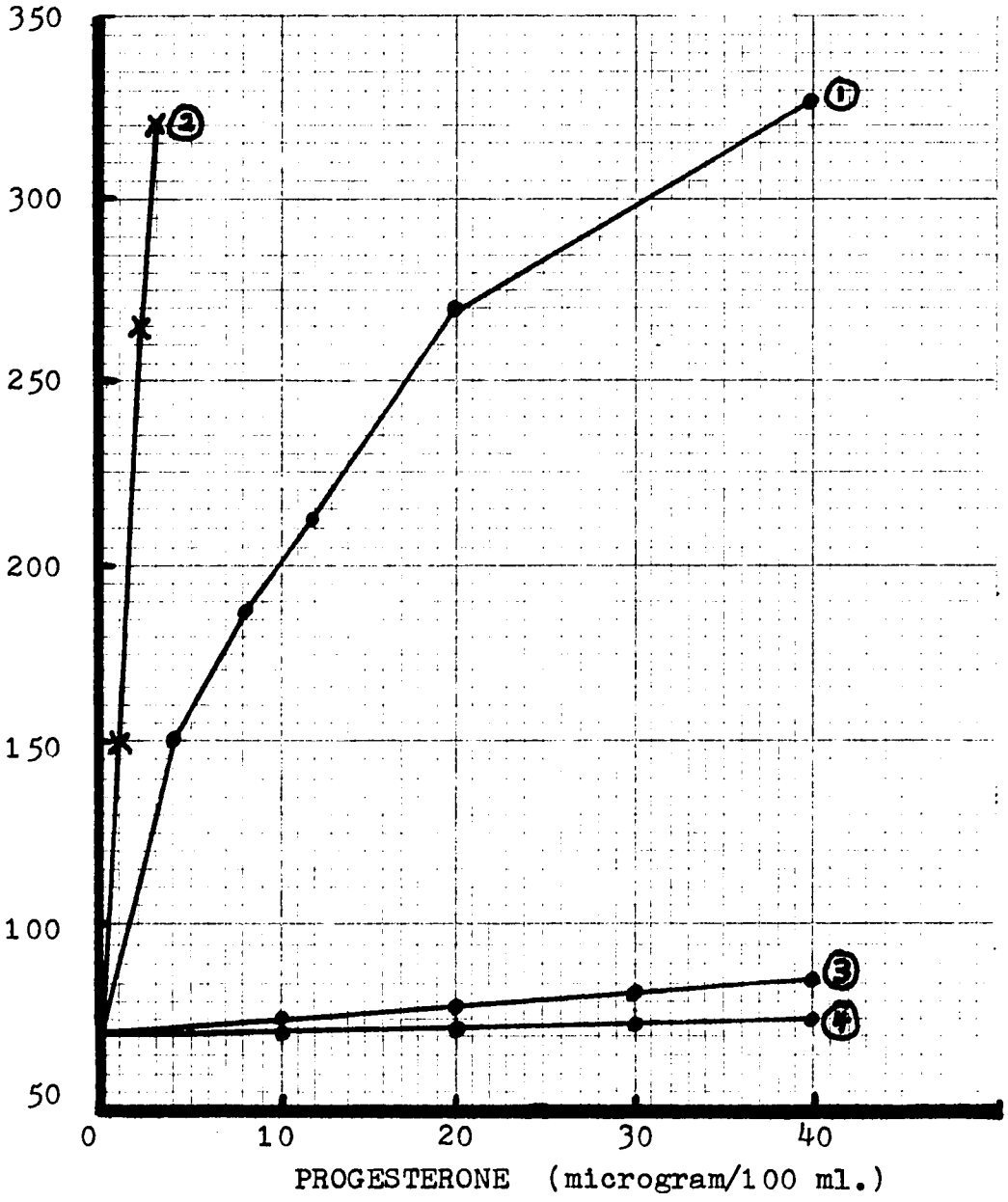
A Standard Curve of 20α dihydro-progesterone was tested by a Standard Curve of 20αOH-P at concentrations of 0, 10, 20, and 30 microgram per 100 ml. The resulting "flat" curve indicates a very low affinity of 20αOH-P for dog CBG and that this steroid will not compete significantly in the progesterone assay (Figure 3).

17α hydroxy-progesterone (17αOH-P).

A Standard Curve for 17αOH-P demonstrates that this steroid has a very high affinity for dog CBG greatly exceeding that of progesterone. However, Yoshimi et al[8] reported that the plasma levels of 17αOH-P during early pregnancy are approximately one tenth of the plasma progesterone levels (maximum 1.8 mg/100 ml. plasma) and disappear entirely after the 10th week. Thus this steroid is present in too small amounts to significantly affect the accuracy of the progesterone assay (Figure 3).

Figure 3.

TYPICAL STANDARD CURVE FOR PROGESTERONE AND RELATED NATURAL STEROIDS.



Curve 1; Progesterone
Curve 2; 17α hydroxy-progesterone
Curve 3; 20α dihydro-progesterone
Curve 4; Pregnenolone

F. (2) SPECIFICITY OF THE PROGESTERONE ASSAY.

(ii) Competition by synthetic steroids and hormones.

A group of 18 drugs, of the types in frequent therapeutic use in obstetrics and gynecology, and containing synthetic steroids, were investigated for their affinity to bind with CBG and thus compete with natural progesterone in the assay system. These drugs, together with their trade names and steroid components are listed in Table IV. They include: 10 oral contraceptives, of both the "sequential" and combined types; 3 synthetic estrogens; 1 progestational agent; 1 "pregnancy test"; 2 androgens and 1 anabolic agent.

In each case, 1 tablet of each drug, that is 1 dose, was dissolved in 100 ml. of 50/50 ethanol and water and 0.1 ml. of solution was used in each assay. These concentrations are far in excess of actual physiological concentrations of these drugs in the body.

In Table V the amount of each steroid measured in the assay is compared with the amount of compound actually present in the sample and the former is expressed as a percentage of the latter. It can readily be seen that, for this group of steroids, the maximum amount assayed is 1 percent (for "Ovex") or less of the actual amount of steroid present in the sample.

This indicates neglible interference by any of these compounds with the assay.

TABLE IV

Drug, Trade Name	Steroid Constituents	Therapeutic Use.
1. Oracon (white)	E.E.*0.1 mg.	Oral
Oracon (pink)	E.E. 0.1 mg. + Dimethisterone 25 mg.	contraceptive (Sequential)
2. Miniquen (pink)	Mest. 0.1 mg.	Oral
Miniquen (blue)	Mest. 0.1 mg. + Ethynodiol diacetate 0.5 mg.	contraceptive (Sequential)
3. C-Quens (yellow)	Mest. 0.1 mg.	Oral
C-Quens (pink)	Mest. 0.1 mg. + chlormadinone acetate 1.5 mg.	contraceptive (Sequential)
4. Ovex (white)	E.E. 0.1 mg.	Oral
Ovex (pink)	E.E. 0.1 mg. + Megestrol acetate 5 mg.	contraceptive (Sequential)
5. Norlestrin 1	E.E. 0.05 mg. + Norethindrone acetate 1 mg.	Oral contraceptive (combined)

Drug, Trade Name	Steroid Constituents	Therapeutic Use.
6. Ovulen 1	Mest. 1 mg. + Ethynodiol diacetate 1 mg.	Oral contraceptive (combined)
7. Ovral	E.E. 0.05 mg. + d-Norgestrel 0.25mg.	Oral contraceptive (combined)
8. Norinyl 1	Mest. 0.05 mg. + Norethindrone 1 mg.	Oral contraceptive (combined)
9. Orthonovum 1	Mest. 0.05 mg. + Norethindrone 1 mg.	Oral contraceptive (combined)
10. Enovid 5	Mest. 0.15 mg. + Norethynodrel 9.85 mg.	Oral contraceptive (combined)
11. Neo-estrone 1.25	Conjugated estrogens, equine.	Estrogen replacement.
12. Estinyl 0.05	17 Estinylestradiol 0.05 mg.	Estrogen replacement.

Drug, Trade Name	Steroid Constituents	Therapeutic Use.
13. Stilbestrol 25 mg	Stilbestrol 25 mg	Estrogen replacement Neoplastic therapy
14. Neo-mens	E.E. 0.05 mg + Ethisterone 50 mg	"Pregnancy test" (withdrawal bleeding)
15. Provera 5	Medroxyprogesterone acetate 5 mg.	Progestational therapy
16. Methyl testosterone	Methyl testosterone 10 mg.	Androgen
17. Halotestin	Fluoxymesterone 5 mg.	Androgen (neoplasm)
18. Dannabol	Methandrostenolone 5 mg.	Anabolic agent.

*E.E. = Ethinyl estradiol

Mest = Mestranol = Ethynylestradiol 3-methyl ether.

TABLE V.

Effect of synthetic steroids in Progesterone Assay

No.	Drug Name	Actual amt. in sample (micrograms) (X)	Assayed amt. (micrograms) (Y)	Percent of actual amt. $\left(\frac{(Y) \times 100}{(X)}\right)$
1.	Oracon, white	0.1	0	0
	Oracon, pink*	25	0.0011	0.004
2.	Miniquen, blue	0.10	0.0002	0.200
	Miniquen, pink*	0.50	0.0002	0.040
3.	C-Quens, yellow	0.01	0.0003	0.300
	C-Quens, pink*	1.50	0.0024	0.160
4.	Ovex, white	0.10	0.0010	1.000
	Ovex, pink*	5.00	0.0032	0.060
5.	Norlestrin 1	1.00	0	0
6.	Ovulen 1	1.00	0.0002	0.020
7.	Ovral	0.25	0.0004	0.160
8.	Norinyl 1	1.00	0.0003	0.030
9.	Orthonovum 1	1.00	0.0005	0.050
10.	Enovid 5	5.00	0.0008	0.016

No.	Drug Name	Actual amt. in sample (micrograms) (X)	Assayed amt. (micrograms) (Y)	Percent of actual amt. ((Y) x 100 / (X))
11.	Neo-estrone	1.25	0.0003	0.024
12.	Estinyl	0.05	0.0001	0.200
13.	Stilbesterol	25.00	0.0004	0.016
14.	Neo-mens	50.00	0.0047	0.009
15.	Provera	5.00	0.002	0.040
16.	Methyl testosterone	10.00	0.040	0.400
17.	Halotestin	5.00	0.0033	0.060
18.	Dannabol	5.00	0.0040	0.080

Note: Numbers 1*, 2*, 3*, 4*, 5, 6, 7, 8, 9, 10, 11, consider only amount of progestational steroids for calculation. (Amount of estrogen is ignored.)

F. (2) SPECIFICITY OF THE PROGESTERONE ASSAY.

(iii) Paper chromatography. Identification of progesterone.

The progesterone in duplicate 0.1 ml. plasma samples from mother at delivery and fetus' umbilical venous blood were extracted x 3 with petroleum ether and evaporated to dryness in centrifuge tubes.
The steroids were redissolved in approx. 0.5 ml. ethanol and each applied to a strip of chromatography paper 50 cm. long.
Chromatography was carried out in a descending Bush System, EB-4[55], until the solvent front reached the end of the paper (5 hours).
A standard strip, to which had been applied approx. 20 micrograms each of progesterone, 17α-hydroxy-progesterone (17αOH-P) and desoxycorticosterone (DOC), was run with each duplicate sample pair.
Identification of the steroids on the standard strip was made under ultra-violet light, and the three corresponding steroid containing areas marked on the test strip.
Each of the test strips was cut into ten 5 cm. lengths, three of which correspond to the individual steroid-containing areas of the standard strip.
Each 5 cm. length was eluted, using methanol, into test-tubes and evaporated to dryness.

Protein Binding Solution, 1 ml. was then added to each test-tube and the progesterone assay carried out for each 5 cm. length.
A standard (progesterone) curve was run simultaneously.

The values obtained for assay of the duplicate samples of maternal plasma after paper chromatography are expressed numerically in Table VI and graphically in Figure 4. Equivalent figures for umbilical venous plasma are given in Table VII and Figure 5.

It can readily be seen that there is a marked peak in the assay values in the strips, number 6, corresponding to the position of progesterone. This confirms that, in practice, the assay is measuring almost entirely progesterone.

TABLE VI

MATERNAL PLASMA

Strip Number	Length (cm)	Steroid position	Progesterone level Duplicate samples (g/100 ml. plasma)		
			1	2	Mean
1	5	-	3.8	5.4	4.4
2	5	-	1.0	2.0	1.5
3	5	DOC	1.5	2.8	2.1
4	5	17αOH-P	3.5	1.0	2.25
5	5	-	4.5	4.0	4.25
6	5	Prog.	14.0	19.0	16.5
7	5	-	1.0	1.0	1.0
8	5	-	0.8	0.8	0.8
9	5	-	1.0	0.8	0.9
10	5	-	3.5	2.0	2.75

Figure 4.

PAPER CHROMATOGRAPHY.

MATERNAL PLASMA.

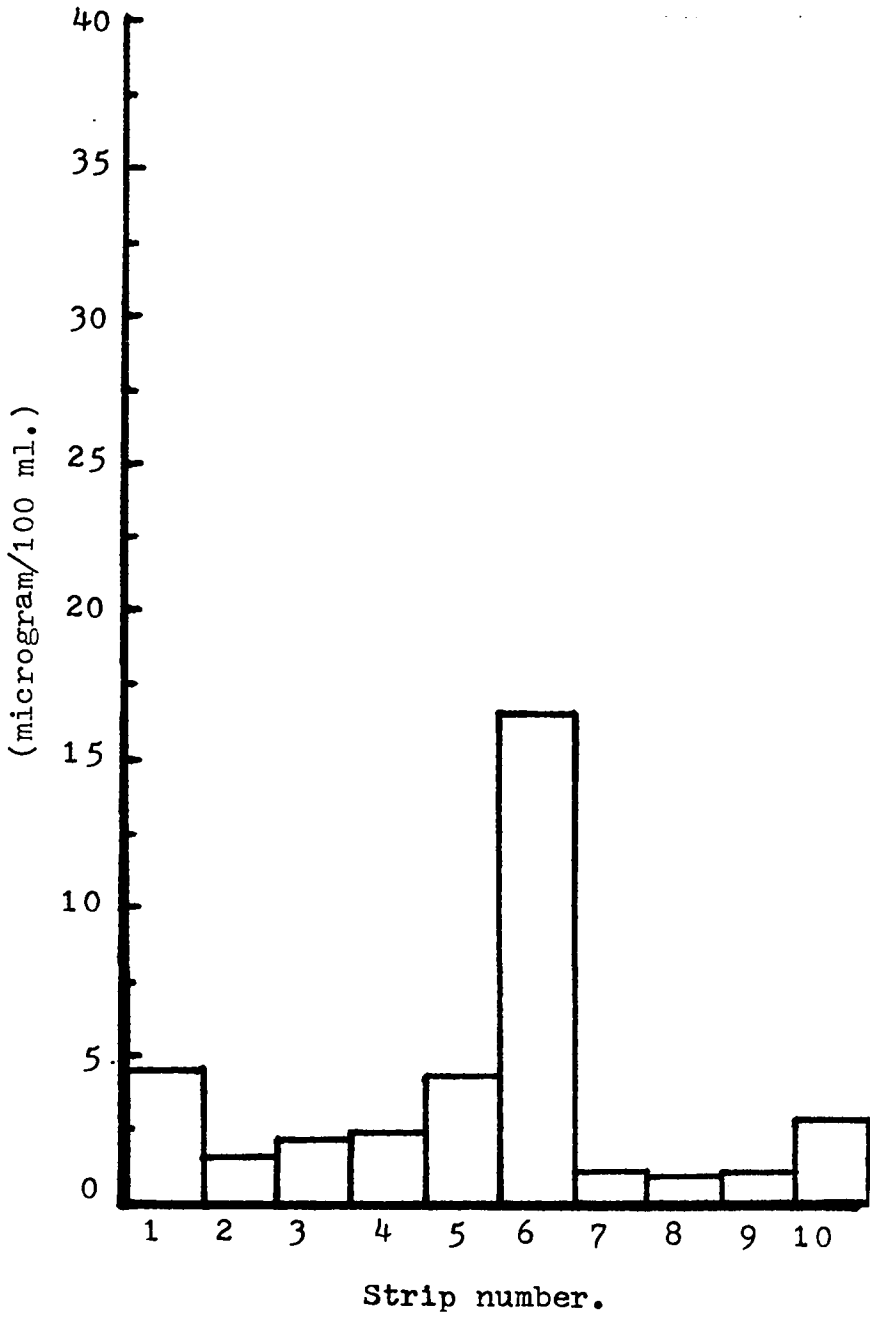


TABLE VII

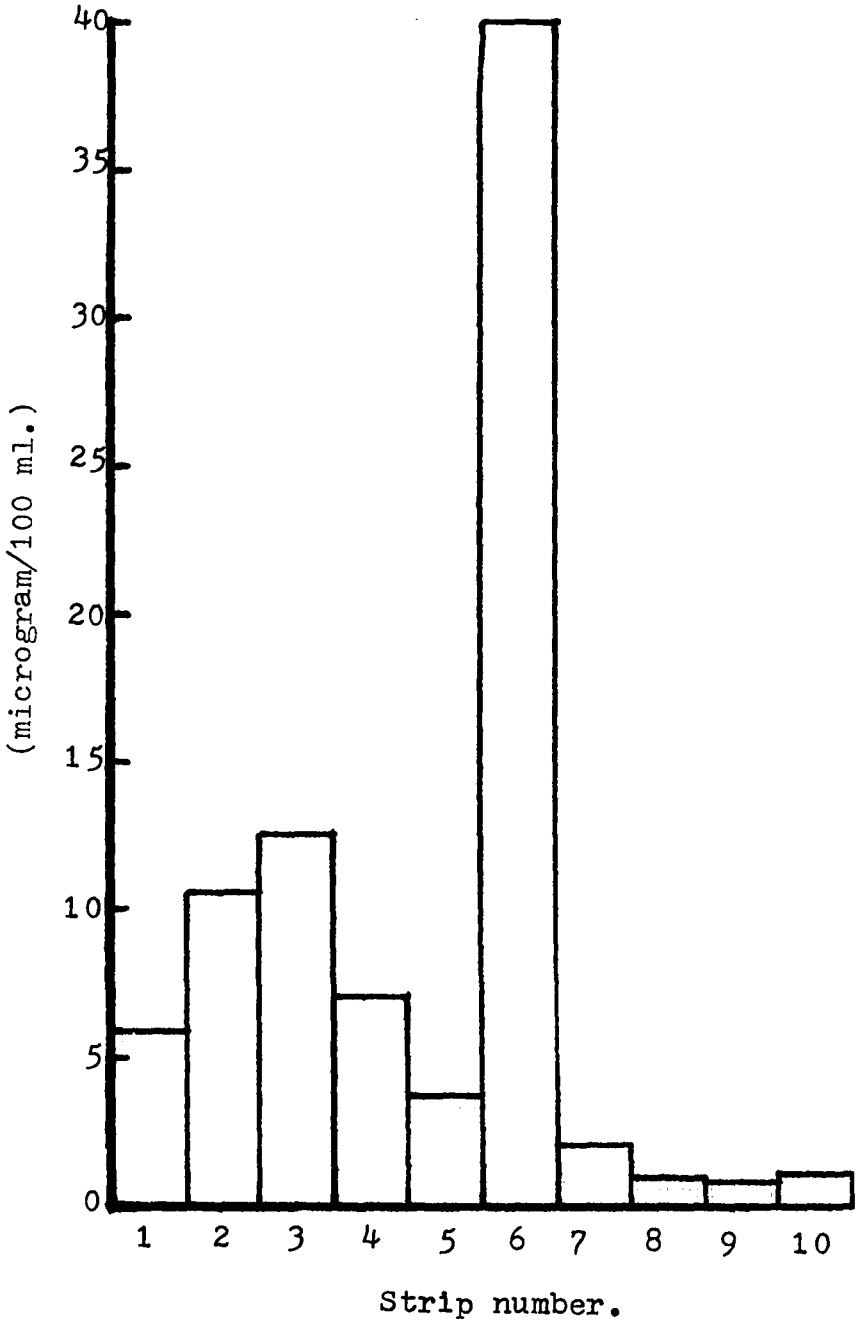
UMBILICAL VENOUS BLOOD

Strip Number	Length (cm)	Steroid position	Progesterone level Duplicate samples (g/100 ml. plasma)		
			1	2	Mean
1	5	-	4.0	7.5	5.85
2	5	-	2.5	18.5	10.5
3	5	DOC	15.0	10.0	12.5
4	5	17αOH-P	8.0	6.0	7.0
5	5	-	3.5	3.8	3.65
6	5	Prog.	40.0+	40.0	40.0+
7	5	-	1.0	3.0	2.0
8	5	-	1.0	0.8	0.9
9	5	-	1.0	0.5	0.75
10	5	-	0.5	1.5	1.0

Figure 5.

PAPER CHROMATOGRAPHY.

UMBILICAL VEIN PLASMA.



F. (3) ACCURACY OF THE PROGESTERONE ASSAY

The accuracy of the plasma progesterone assay was determined by assaying known amounts of progesterone (equivalent to known concentrations) using the standard assay technique.

The results (Table VIII) indicate that the assay is accurate for a wide range of progesterone concentrations.

TABLE VIII

ACCURACY OF THE PROGESTERONE ASSAY

Actual Progesterone Concentration (microgram/100 ml.)	Measured Progesterone Concentration (microgram/100 ml.)	Recovery %
1.0	2.0	(200)
5.0	4.9	98
8.0	7.0	87.5
10.0	9.7	97

F. (4) PRECISION OF THE PROGESTERONE ASSAY.

The precision of the progesterone assay was calculated for ten duplicate pairs of samples in each of four groups of progesterone concentrations throughout the assay range, namely: 0 - 5.0; 5.1 - 10.0; 10.1 - 20.0; and 20.1 - 40.0 (microgram/100 ml.). The Standard Deviation was calculated for each group of ten pairs using the formula:

$$S.D. = \sqrt{\frac{\Sigma \Delta^2}{2n}}$$

where Δ = difference between duplicate pairs.

n = number of samples per group. (10).

The Standard Deviation was calculated as a percentage of the mean value of each of the four groups as follows: 2.5 ($\pm$ 10%); 7.5 ($\pm$ 6.0%); 15 ($\pm$ 6.8%); 30 ($\pm$ 4.7%) microgram/100 ml.

The detailed results are expressed numerically in Table IX and graphically in Figure 6.

TABLE IX

PRECISION OF THE PROGESTERONE ASSAY

Group (1). Progesterone 0.0 - 5.0 (microgram/100 ml.)

Sample	Duplicate pair		Δ	Δ^2
	#1	#2		
1	2.6	2.6	0.0	0.0
2	2.8	3.0	0.2	0.004
3	0.4	0.5	0.1	0.01
4	3.8	3.2	0.6	0.36
5	1.8	1.7	0.1	0.01
6	0.6	0.5	0.1	0.01
7	2.0	1.5	0.5	0.25
8	3.5	3.4	0.1	0.01
9	2.3	2.9	0.6	0.36
10	3.0	2.6	0.4	0.16

$$\Sigma \Delta^2 = 1.21$$

$$\text{S.D.} = \sqrt{\frac{1.21}{2 \times 10}}$$

$$= \sqrt{0.06}$$

$$= 0.25$$

$$\text{Mean} = 2.5 \pm 0.25$$

$$= 2.5 \pm 10\%. \text{ (microgram/100 ml.)}$$

Group (2). Progesterone 5.1 - 10 (microgram/100 ml.)

Sample	Duplicate pair		Δ	Δ^2
	#1	#2		
1.	9.5	9.0	0.5	0.25
2.	6.4	7.4	1.0	1.0
3.	7.0	8.4	1.4	1.96
4.	10.0	10.0	0.0	0.00
5.	6.0	5.6	0.4	0.08
6.	5.4	5.8	0.4	0.08
7.	5.1	5.4	0.3	0.09
8.	7.5	7.0	0.5	0.25
9.	5.1	5.7	0.6	0.36
10.	7.0	6.7	0.3	0.09

$$\Sigma \Delta^2 = 4.16$$

$$\text{S.D.} = \sqrt{\frac{4.16}{20}}$$

$$= \sqrt{0.208}$$

$$= 0.45$$

$$\text{Mean} = 7.5 \pm 0.45$$

$$\doteq 7.5 \pm 6\%$$

Group (3). Progesterone 10.1 - 20 (microgram/100 ml.)

Sample	Duplicate pair		Δ	Δ^2
	#1	#2		
1.	17.1	20.0	2.9	8.40
2.	11.0	11.1	0.1	0.01
3.	17.0	18.2	1.2	1.42
4.	13.8	15.4	1.6	2.55
5.	10.5	10.7	0.2	0.04
6.	15.5	14.7	0.8	0.16
7.	10.2	10.5	0.3	0.09
8.	11.4	10.1	1.3	1.69
9.	14.4	12.0	2.4	5.75
10.	22.2	22.6	0.4	0.16

$$\Sigma\Delta^2 = 20.27$$

$$\text{S.D.} = \sqrt{\frac{20.27}{20}}$$

$$= \sqrt{1.01}$$

$$= 1.01$$

$$\text{Mean} = 15.0 \pm 1.01$$

$$= 15.0 \pm 6.8\%$$

Group (4). Progesterone 20.1 - 40 (microgram/100 ml.)

Sample	Duplicate pair #1	Duplicate pair #2	Δ	Δ^2
1.	23.0	21.0	2.0	4.00
2.	34.0	35.4	1.4	1.95
3.	23.6	23.4	0.2	0.04
4.	40.0	38.8.	1.2	1.44
5.	38.0	39.2	1.2	1.44
6.	28.5	28.5	0	0.00
7.	38.0	34.5	3.5	12.20
8.	20.1	24.5	4.3	18.40
9.	28.1	28.1	0	0
10.	27.2	27.1	0.1	0.01

$$\Sigma \Delta^2 = 39.48$$

$$\text{S.D.} = \sqrt{\frac{39.48}{20}}$$

$$= \sqrt{1.97}$$

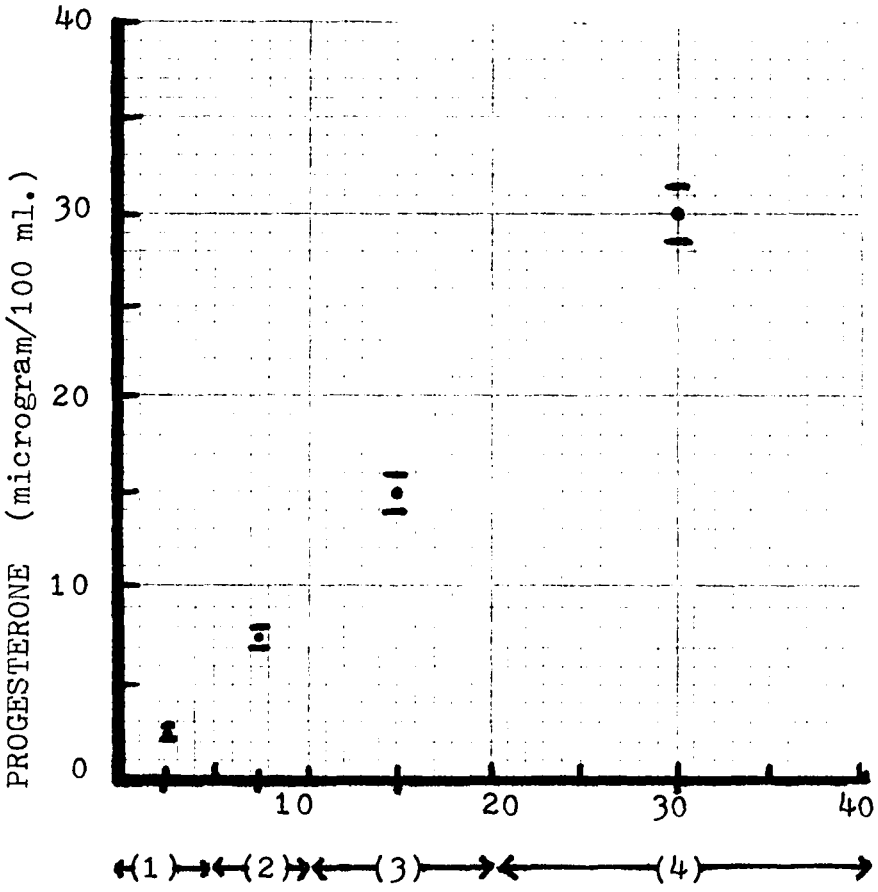
$$= 1.4$$

$$\text{Mean} = 30 \pm 1.4$$

$$= 30 \pm 4.7\%$$

Figure 6..

PRECISION OF THE PROGESTERONE ASSAY



Mean and Standard Deviation noted for each group:

VI.

RESULTS

RESULTS

(A) PLASMA PROGESTERONE LEVELS IN EARLY PREGNANCY.

Table X lists 28 patients seen in obstetrical clinics at the first prenatal visit, when blood was taken for plasma progesterone determination and route vaginal cytology was performed. The cases are listed in order of increasing gestational age (weeks from last menstrual period) and the plasma progesterone levels and percentage of superficial cells in the cytological smear are recorded. Figure 7 is a graphic representation of plasma progesterone levels vs. weeks gestation. All pregnancies in this series proceeded to normal full term delivery.

TABLE X

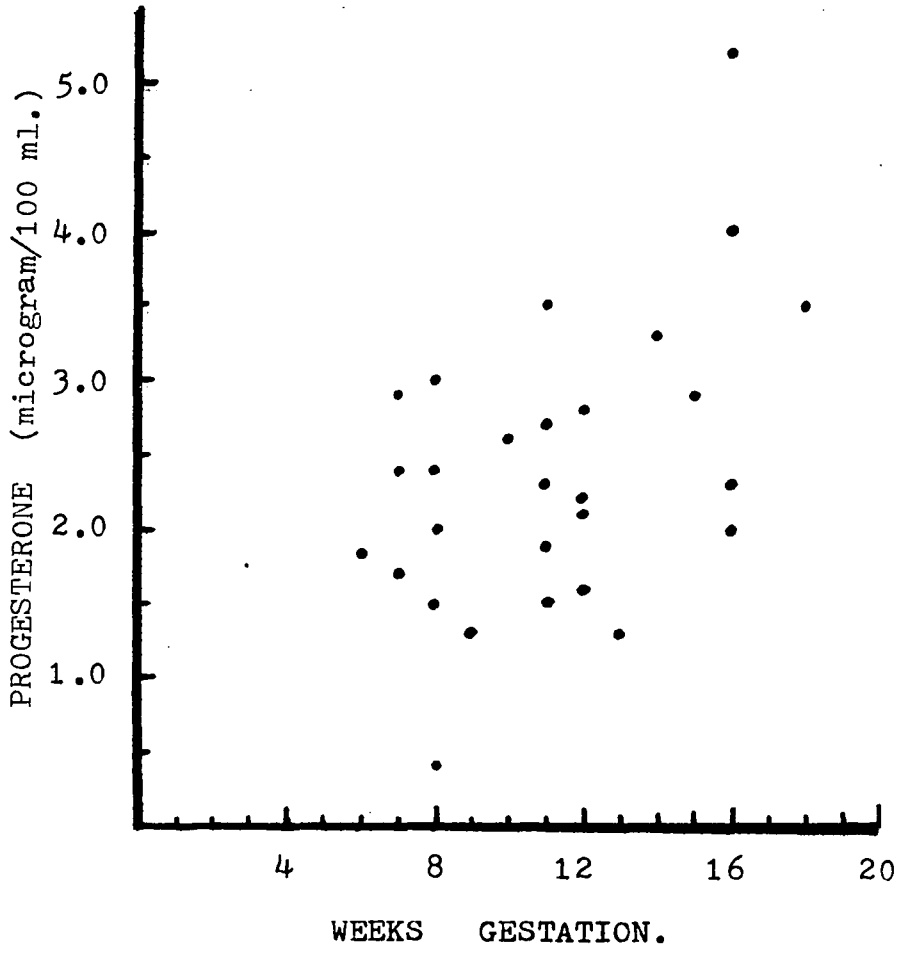
PLASMA PROGESTERONE LEVELS IN EARLY PREGNANCY AND COMPARISON WITH VAGINAL CYTOLOGY. (AT FIRST PRENATAL VISIT.)

Name	Weeks Gestation	Plasma Progesterone (microgram/100 ml. plasma)	Vaginal cytology % superficial cells
1. I--------i	6	1.85	5
2. M-----e	7	2.40	20
3. M-----s	7	2.90	20
4. V------u	7	1.70	-
5. W------k	8	2.00	10
6. T----r	8	0.40	5
7. G------r	8	2.40	35
8. S---k	8	1.50	40
9. F----g	8	3.00	25
10.C------e	9	1.30	40
11.B----r	10	2.60	60
12.C---f	11	2.70	10
13.N----n	11	2.30	5
14.M-------d	11	1.50	-
15.B----c	11	3.50	35
16.S---h	11	1.90	30

Name	Weeks Gestation	Plasma Progesterone (microgram/100 ml. plasma)	Vaginal cytology % superficial cells
17. H--------n	12	2.10	10
18. P-o	12	1.60	35
19. L---i	12	2.20	50
20. T---i	12	2.80	5
21. L----t	13	1.20	35
22. P--e	14	3.30	40
23. Y--e	15	2.90	35
24. M----n	16	4.00	30
25. T--------u	16	2.30	40
26. M---e	16	5.20	10
27. R--------o	16	2.00	10
28. R-y	18	3.50	45

Figure 7.

PLASMA PROGESTERONE LEVELS IN EARLY PREGNANCY.



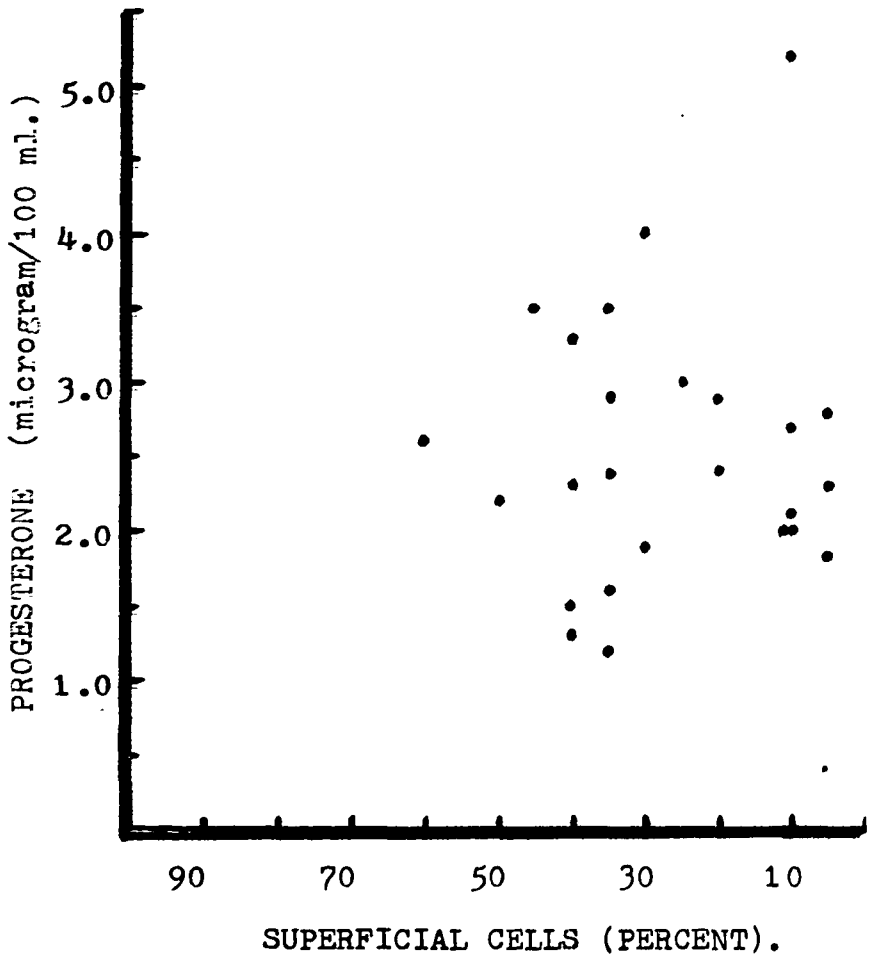
RESULTS

(B) PLASMA PROGESTERONE LEVELS IN EARLY PREGNANCY COMPARED WITH HORMONAL ASSESSMENT BY VAGINAL CYTOLOGY, A BIOASSAY.

Figure 8 is a comparison of plasma progesterone levels vs percentage of superficial cells in the vaginal cytology smears (maturation index) in the early pregnancy cases listed in Table X.

Figure 8.

PLASMA PROGESTERONE LEVELS IN EARLY PREGNANCY COMPARED WITH VAGINAL CYTOLOGY. (MATURATION INDEX).



(C) PLASMA PROGESTERONE LEVELS IN CASES OF FIRST TRIMESTER THREATENED ABORTION.

Table XI lists 21 cases of pregnancy with the diagnosis of "threatened abortion", that is, spontaneous uterine bleeding in the first trimester of pregnancy. These cases were all my private patients, thus eliminating cases that could come through the hospital's emergency department, where a possibility of criminal abortion would exist in an appreciable number. Blood samples were drawn when the patients first presented with vaginal bleeding. The cases are arranged in increasing order of plasma progesterone values. Also recorded is the "maturation index" of the vaginal cytology, the outcome of the pregnancy and the occurrence of positive urine pregnancy tests (for chorionic gonadotrophin).

TABLE XI

PROGESTERONE LEVELS: FIRST TRIMESTER THREATENED ABORTION

Case	Progesterone (microg./100 ml)	Cytology (% superficial cells)	Positive urine pregnancy test	Out-come
1. B-----t	0	25		AB
2. M----n	0	35	+	AB
3. H----s	0	-	+	AB
4. Q---d	0	-	+	AB
5. L-----l	0.10	55		AB
6. C---e	0.20	-		AB
7. J-----n	0.25	-		AB
8. K-------n	0.50	-		AB
9. R-y	0.50	-	+	AB
10.R------n	0.70	-		AB
11.B-----i	0.75	-		AB
12.B---e	1.00	10		N
13.N---k	1.00	30		N
14.L----f	1.20	30		N
15.R-----s	1.3	35		AB
16.O---a	1.4	60		AB
17.B-------e	1.5	35		AB
18.G---r	1.8	55		N
19.G-----r	1.8	45		N

Case	Progesterone (microg./100 ml)	Cytology (% superficial cells)	Positive urine pregnancy test	Outcome
20. P----r	2.3	50		N
21. M----r	2.4			N

AB = spontaneous abortion

N = normal full term pregnancy

(D) SERIAL DETERMINATIONS OF PLASMA PROGESTERONE IN MID AND LATE PREGNANCY.

In 5 cases, plasma progesterone was determined serially on samples taken at successive prenatal clinic visits, intervals ranging from one to four weeks. All pregnancies terminated in normal labour and delivery. The results are presented numerically in Table XII and graphically in Figure 9.

TABLE XII

SERIAL DETERMINATIONS OF PLASMA PROGESTERONE LEVELS IN MID AND LATE PREGNANCY.

Case 1. M----n, #90480

Weeks gest.	Progesterone (microgram/100 ml.plasma)
11	1.09
15	0.70
28	1.80
31	6.90
39	11.9

Case 2. P---e, #122795

16	3.65
20	2.70
24	4.27
28	5.10
32	6.75
36	7.46
37	5.56
39	2.58

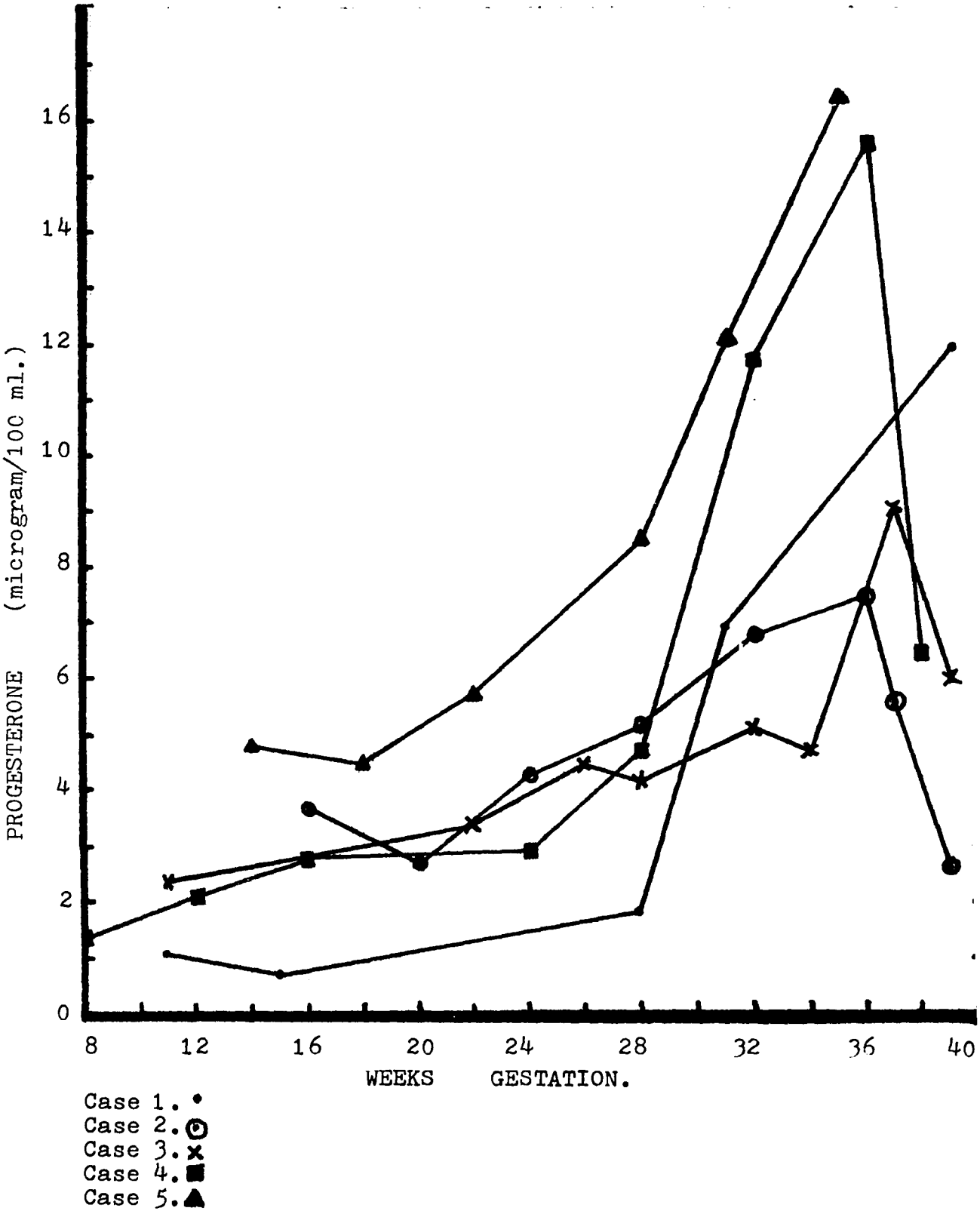
Case 3, S---h #122413

11	2.39
22	3.32
26	4.45
28	4.14
32	5.10
34	4.68

Weeks gest.	Progesterone (microgram/100 ml. plasma)
Case 3, S---h #122413 (continued)	
37	9.08
39	6.00
Case 4, N-------u # 122385	
8	1.35
12	2.10
16	2.75
24	2.89
28	4.68
32	11.70
36	15.60
38 (delivery)	6.40
Case 5, K---y # 121844	
14	4.78
18	4.50
22	5.72
28	8.48
31	12.10
35	16.40

Figure 9.

SERIAL DETERMINATIONS OF PLASMA PROGESTERONE LEVELS IN MID AND LATE PREGNANCY.



(E) SERIAL PLASMA PROGESTERONE LEVELS IN LATE PREGNANCY, AT DELIVERY AND POSTPARTUM.

In 8 cases, serial plasma progesterone levels were determined at weekly prenatal clinic visits during the last six weeks of pregnancy, at delivery and postpartum. The results are listed numerically in Table XIII and graphically in Figures 10 and 11.

TABLE XIII

SERIAL PLASMA PROGESTERONE LEVELS IN LATE PREGNANCY, AT DELIVERY AND POSTPARTUM

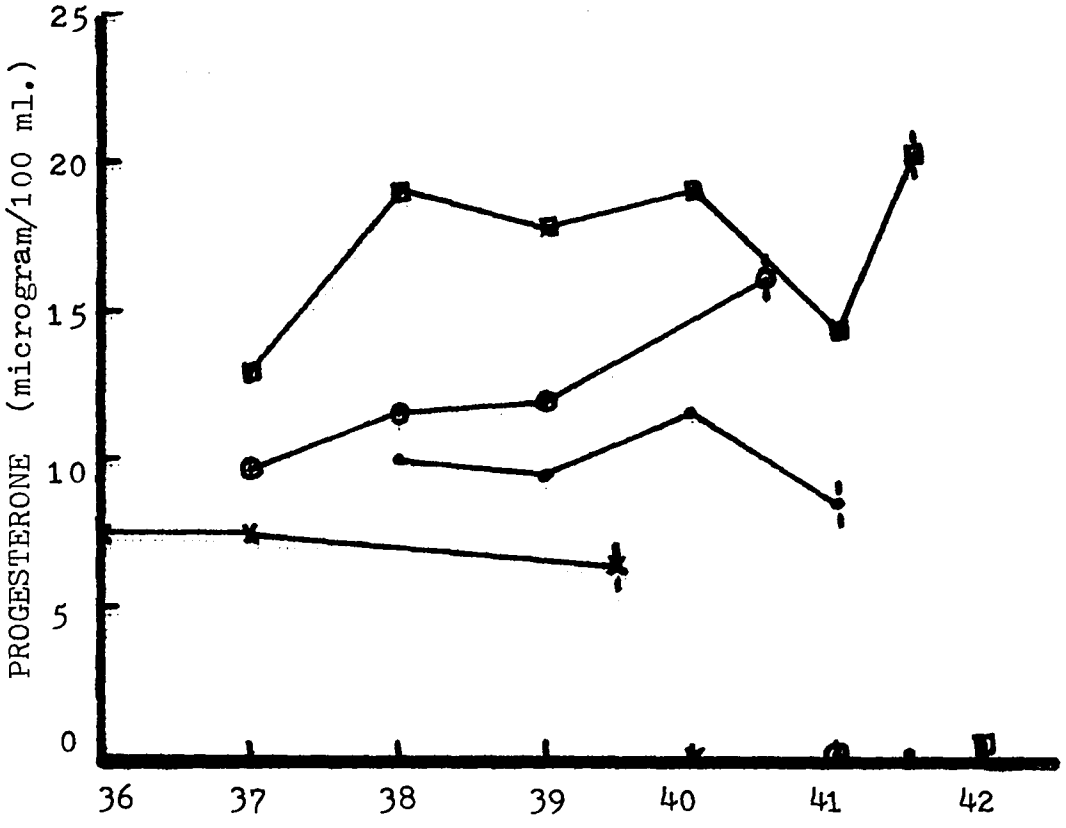
	Weeks Gest.	Progesterone (microgram/100 ml.)
Case 1, W-------n		
	38	10.0
	39	9.5
	40	11.6
Delivery	(41)	8.5
Postpartum (day 3)		0.05
Case 2, G------d		
	36	7.4
	37	7.5
Delivery	(39½)	6.4
Postpartum (day 3)		0.0
Case 3, M--------y		
	37	9.7
	38	11.5
	39	11.7
Delivery	(40½)	15.1
Postpartum (day 3)		0.05

	Weeks Gest.	Progesterone (microgram/100 ml.)
Case 4 E---s		
	37	12.8
	38	19.0
	39	17.8
	40	19.1
	41	14.4
Delivery	(41½)	20.4
Postpartum	(day 3)	0.5
Case 5 G----y		
	36	10.6
	37	12.1
	38	16.0
Delivery	(38½)	10.2
Postpartum	(day 3)	1.4
Case 6 V--------h		
	37	8.4
	38	8.8
	39	12.3
Delivery	(39½)	8.3
Postpartum	(day 3)	0.0
Case 7, A-----y		
	36	19.7
	37	19.1
	38	22.0

Weeks Gest.	Progesterone (microgram/100 ml.)
Case 7, A-----y (Continued)	
39	18.1
40	22.2
41	19.0
Delivery (41½)	14.6
Postpartum (day 1)	0.2
Case 8, P--r	
37	7.3
38	10.6
39	9.0
41	6.6
Delivery (42)	9.6
Postpartum (day 1)	1.3

Figure 10.

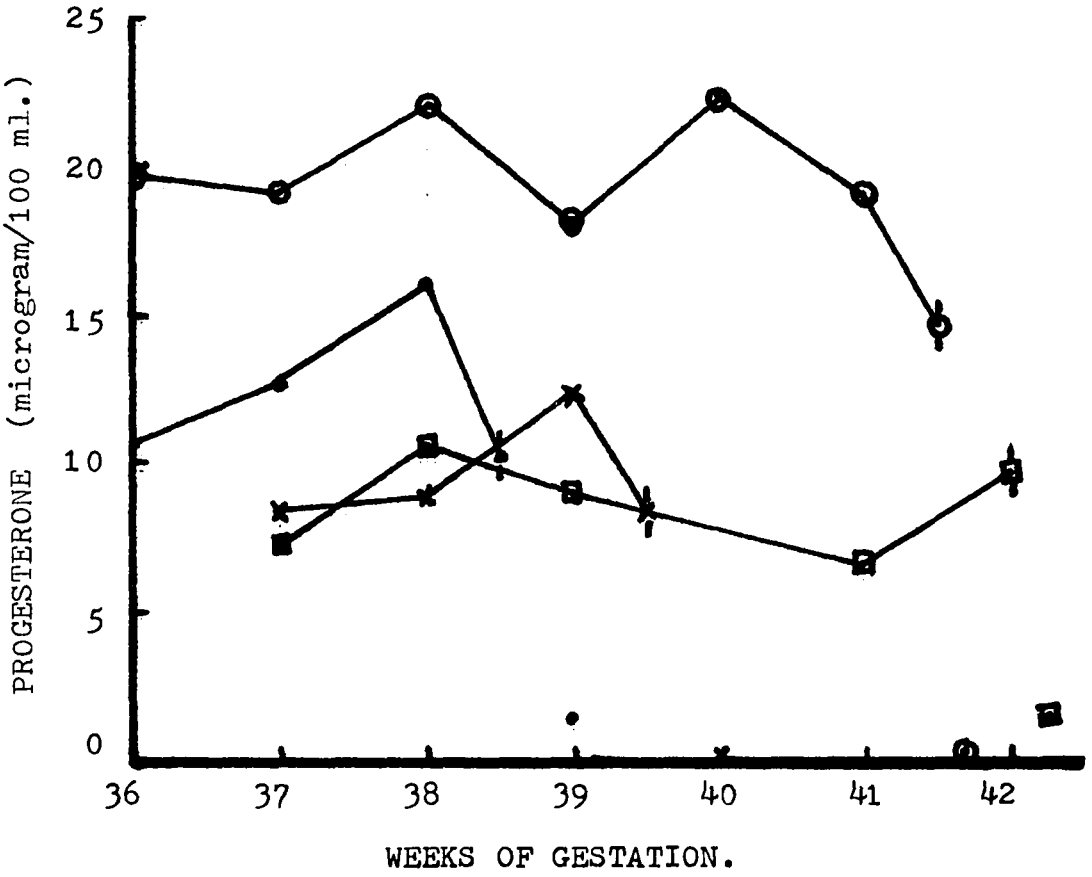
SERIAL PLASMA PROGESTERONE LEVELS IN LATE PREGNANCY, AT DELIVERY AND POSTPARTUM.



Case 1; •
Case 2; ×
Case 3; ⊙
Case 4; ⊡

Figure 11.

SERIAL PLASMA PROGESTERONE LEVELS IN LATE PREGNANCY, AT DELIVERY AND POSTPARTUM.



Case 5; •
Case 6; ×
Case 7; ⊙
Case 8; ⊡

(F) COMPARISON OF MATERNAL VENOUS PLASMA PROGESTERONE LEVELS WITH FETAL UMBILICAL VEIN AND ARTERY LEVELS AT DELIVERY.

In 14 cases of normal pregnancy and delivery, maternal venous and fetal umbilical artery and umbilical vein plasma progesterone levels were determined. The fetal sex, weight and placental weight were recorded. This data, for individual cases, is presented in Table XIV, together with umbilical vein - umbilical artery progesterone difference (PD) and the PD per 100 gram of fetus and per 100 gram of placenta. The mean progesterone values for maternal venous, umbilical artery and umbilical vein plasma arelisted in Table XV.

TABLE XIV

PROGESTERONE LEVELS AT DELIVERY

Name	Sex	Wt. (grams)	Placenta Wt.	Progesterone (microgram/100 ml. Maternal Vein	Umbilical Artery.	Umbilical Vein
1. F-----r	F	3855	595	5.4	26.4	44.8
2. P---e	F	3061	680	18.7	13.4	46.2
3. G-----e	F	3968	566	7.5	11.2	36.1
4. Q---d	F	3260	538	9.0	10.3	28.5
5. D--------o	F	3203	595	7.6	22.2	49.0
6. L-----e	M	3316	595	11.0	14.0	36.2
7. C-------i	M	3175	652	16.4	18.6	44.6
8. L------e	M	3061	566	13.4	13.0	36.0
9. D-----o	M	3940	708	7.7	13.8	39.6
10. A-----r	M	3260	453	10.1	11.2	37.8
11. J-------c	M	3175	595	5.9	17.8	32.0
12. D--------s	M	2834	566	14.4	35.2	51.6
13. T------t	M	3345	625	17.9	44.4	68.6
14. B----w	F	3203	625	7.3	11.0	21.4

)gram/100 ml.)

ıl —	Umbilical Vein	Fetal prog. difference Umb. vein - umb. artery	Prog. difference 100 gm fetus	Prog. difference 100 gm placenta
	44.8	18.4	0.476	3.10
	46.2	32.8	1.07	4.82
	36.1	24.9	0.628	4.40
	28.5	18.2	0.550	3.38
	49.0	26.8	0.837	4.51
	36.2	22.2	0.670	4.37
	44.6	26.0	0.821	4.0
	36.0	23.0	0.754	4.05
	39.6	25.8	0.654	3.65
	37.8	26.6	0.815	5.88
	32.0	14.2	0.449	2.39
	51.6	16.4	0.580	2.90
	68.6	24.2	0.725	3.87
	21.4	10.4	0.325	1.67

TABLE XV

PROGESTERONE MEAN MATERNAL & FETAL LEVELS AT DELIVERY.

	Maternal Vein	Umbilical Artery	Umbilical Vein	U.A. - U.V.
	10.9	18.7	40.8	22.1 *
S.D.=	(±5.3)	(±9.3)	(± 11.4)	

* No relationship to fetal sex or weight or placental weight.

S.D.; Standard Deviation.

(G) PLASMA PROGESTERONE LEVELS IN CASES OF INTRAUTERINE FETAL DEATH.

Plasma progesterone levels were determined in 8 cases of intrauterine fetal death after 28 weeks gestation (the age of theoretical viability). Blood samples were drawn as soon as the patient was admitted to hospital and the diagnosis suspected. The results are shown in Table XVI, arranged in order of increasing plasma progesterone levels.

TABLE XVI

PLASMA PROGESTERONE LEVELS: INTRAUTERINE FETAL DEATH (THIRD TRIMESTER)

Case	Progesterone (microg./100 ml. plasma)
1	1.1
2	1.6
3	3.4
4	4.9
5	5.1
6	5.7
7	9.9
8	15.8

VII.

D I S C U S S I O N

(A) PLASMA PROGESTERONE LEVELS IN EARLY PREGNANCY

From the results it can be seen that:

i) All values, with a single exception, lie above 1 microgram per 100 ml. plasma. This single low value of 0.40 microg./100 ml. (case 6) is unexplained as the pregnancy proceeded uneventfully.

ii) There is great variation between values for different pregnancies at the same gestational age. For example, at 8 weeks gestation, values range from 0.40 to 3.00 micrograms per 100 ml. and at 12 weeks gestation from 1.16 to 2.8 micrograms per 100 ml.

iii) There is a general trend to rising levels of plasma progesterone with advancing gestational age.

The plasma progesterone levels in early pregnancy initially lie within the range of values found in the luteal phase of the menstrual cycle (1+ micrograms/100 ml.).[33] The levels tend to rise slowly during the first trimester but there is considerable variation between progesterone levels of pregnancies at the same gestational age. These findings are in agreement with recent publications.[30, 31, 32]

(B) PLASMA PROGESTERONE LEVELS IN EARLY PREGNANCY COMPARED WITH HORMONAL ASSESSMENT BY VAGINAL CYTOLOGY, A BIOASSAY.

It has long been considered that there is a "progesterone effect" on the maturation of vaginal epithelial cells during pregnancy which results in a partial depression in the "Maturation Index". This is seen as a marked preponderance (90 percent) of intermediate type vaginal epithelial cells with a corresponding decrease in superficial cells [52]. It could be considered that there should be less than 25 percent superficial cells in a pregnancy with normal hormonal status. However, it can be seen in Figure 8 that there is very poor correlation between plasma progesterone level and hormonal assessment by vaginal cytology. Thus abnormally high percentages of superficial cells, above 30 percent, were found with plasma progesterone levels ranging from 1.20 to 4.0 micrograms per 100 ml. plasma, all considered to be in the normal range, and all in pregnancies with successful outcome. These cases were chosen to exclude any with a vaginal infection, such as trichomonas vaginalis, which can modify vaginal cytology.

There are no previous publications comparing plasma progesterone levels with simultaneously performed vaginal cytology for assessment of "progesterone effect". However

the present study is in agreement with published reports of poor agreement between vaginal cytology and urinary pregnanediol [34,35]. This lack of correlation is probably explained if vaginal cytology is considered a bioassay of the combined effects of estrogen and progesterone rather than an assessment of progesterone levels alone. Of course, the plasma estrogen levels are unknown.

(C) PLASMA PROGESTERONE LEVELS IN CASES OF FIRST TRIMESTER THREATENED ABORTION.

The following may be noted from the cases of threatened abortion listed in Table XI:

i) All of the 11 cases with plasma progesterone levels below 1 microgram per 100 ml. plasma terminated in spontaneous abortion. This outcome is not surprising considering that normal luteal phase progesterone levels are above this value. These low levels indicate impaired corpus luteum function.

ii) Abortion occurred in 4 of these 11 patients despite positive urine pregnancy test for chorionic gonadotrophin hormone (CGH). This indicates that the placenta was still capable of CGH production with deficient progesterone production by the corpus luteum. However it is impossible to say whether the low progesterone levels were the cause or result of the abortion (due to other causes such as a blighted ovum).

iii) Normal plasma progesterone levels (above 1 microgram /100 ml. plasma) could still be found in pregnancies ending in spontaneous abortion (Cases 15,16, 17). This indicates that there are factors other than progesterone levels that affect the outcome of early pregnancies.

An interesting study would be to correlate the plasma progesterone level with pathologic examination of the aborted material in an attempt to determine whether abnormally low progesterone levels were the cause or the result of the abortion. In the former, it is theoretically possible that the administration of exogenous progestational drugs could have prevented abortion.

iv) The vaginal cytology could not always be performed due to heavy bleeding. However, when smears were obtained, hormonally abnormal smears (with high percentages of superficial cells) could still be found in cases proceeding to a normal pregnancy outcome (Cases 18, 19, 20). The findings of poor agreement between hormonal assessment by vaginal cytology and pregnancy outcome are similar to those of McLennan and McLennan[60] who were previously supporters of this approach.

This study is the most detailed concerning plasma progesterone levels in cases of threatened abortion. The finding that low levels of plasma progesterone carry a poor prognosis for successful pregnancy outcome is in agreement with the reports of a smaller number of cases in the literature[31,32].

(D) SERIAL DETERMINATIONS OF PLASMA PROGESTERONE IN MID AND LATE PREGNANCY.

The following may be noted from the serial determinations recorded in Table XII and Figure 9:

i) There is a marked variation between plasma progesterone levels between cases at the same stage of pregnancy and between serial determinations in individual pregnancies.

ii) Before 20 weeks gestation, progesterone levels tend to rise slowly and remain below 5 micrograms per 100 ml. plasma.

iii) At some point between 20 and 28 weeks gestation, which varies with each individual case, there is a marked and accelerating increase in plasma progesterone levels. This appears to be out of proportion to the rate of placental growth as measured by the parameters of placental diameter, volume, surface area or weight [56, 61].

iv) Three of 5 cases showed decreasing progesterone levels in the last few weeks of pregnancy, with increasing levels in the other two cases.

The findings of this study are in general agreement with previous reports [8, 31, 32, 37, 38, 39, 40, 41], Table I.

(E) SERIAL PLASMA PROGESTERONE LEVELS IN LATE PREGNANCY, AT DELIVERY AND POSTPARTUM.

The following may be noted from the results in Table XIII and Figures 10 and 11:

i) There is marked variation between progesterone levels in cases at the same gestational age and marked variation between serial determinations in individual cases.

ii) Values at delivery could either be higher (3 cases) or lower (5 cases) than the last value prior to delivery.

iii) Postpartum values showed a rapid fall, approaching zero within 24 hours postpartum.

These findings indicate that a decrease in circulating (peripheral) plasma progesterone levels do not play a significant role related to the onset of parturition, and are in agreement with recent reports [31, 38, 42, 43]. It is more likely that the onset of labour is related to a decrease in the local myometrial progesterone concentration [19, 20].

The virtual disappearance of progesterone from the peripheral circulation confirms that the placenta is the source of progesterone synthesis during pregnancy.

(F) COMPARISON OF MATERNAL VENOUS PLASMA PROGESTERONE LEVELS WITH FETAL UMBILICAL VEIN AND ARTERY LEVELS AT DELIVERY.

i) The highest plasma progesterone levels were found in the umbilical vein with the mean value (40.8 microgram per 100 ml. plasma S.D. $\pm$ 11.4) approximately twice the intermediate mean value of the umbilical artery (18.7 micrograms S.D. $\pm$ 9.3) and approximately four times the mean value for the maternal peripheral plasma (10.9 micrograms S.D. $\pm$ 5.3).

ii) The umbilical venous-arterial difference (mean 22.1 micrograms) represents the progesterone metabolized by the fetus per 100 ml. of plasma circulating through the fetus, probably to pregnanediol and 20αdihydro-progesterone in the fetal liver.

iii) No relationship could be detected between the maternal venous, fetal umbilical venous, umbilical arterial progesterone levels or umbilical venous-arterial progesterone difference (P.D) and the fetal sex, weight or placental weight. The progesterone difference per 100 grams of fetus

varied from 0.476 to 1.07 and per 100 grams of placenta from 2.39 to 4.82. This independence of plasma progesterone levels from fetal or placental size does not appear to have been noted previously.

(G) PLASMA PROGESTERONE LEVELS IN CASES OF INTRAUTERINE FETAL DEATH.

It can be seen from Table XVI that in cases of intrauterine fetal death plasma progesterone values vary from grossly abnormal values (1.1, 1.6 microgram/100 ml. cases 1 and 2) to high normal values (15.8 microgram/100 ml. case 8). The etiology of fetal death is unknown in all cases except case 8 where the patient was a severe juvenile diabetic.

It appears that a single plasma progesterone determination, unless abnormally low, would be of no value in the prediction or diagnosis of intrauterine fetal death.

At present there is insufficient data concerning the role of plasma progesterone in high risk pregnancy. It can be postulated that serial plasma progesterone levels could be of prognostic significance in conditions that might interfere with maternal placental circulation and function. Toxemia of pregnancy should fulfill this requirement as the arterial spasm and reduced maternal placental blood flow shown to occur in this condition, could theoretically interfere with placental oxygenation and progesterone metabolism[57,58].

Thus it is possible that serial plasma progesterone determinations can be an additional useful parameter in assessing the intrauterine status of the feto-placental unit.

VIII

C O N C L U S I O N S.

This thesis has studied the application of an assay based on Competitive Protein Binding to measure the plasma progesterone levels in human pregnancy.

The method appears to be very satisfactory for routine clinical use. Multiple samples (approximately 20) can be processed, in duplicate, in an eight hour day, by a single technician, using a plasma sample of less than 1 ml. The assay is highly specific for progesterone and is unaffected by a wide range of closely related natural steroids and commonly used synthetic progestational drugs.

The plasma progesterone levels throughout normal human pregnancy are reported. They are in agreement with the results obtained by other investigators using much more tedious methods.

It is shown that the commonly used bioassay for progesterone, namely the "maturation index", determined by the vaginal cytology, bears no relationship to the actual circulating plasma progesterone level. Therefore, the failure of this bioassay to predict pregnancy outcome is understandable.

Plasma progesterone levels appear to be of prognostic significance in the management of cases of threatened abortion. Levels below 1 microgram/100 ml. carry a uniformly poor prognosis, even if the standard urine pregnancy test (urinary chorionic gonadotrophin) is positive. With further

assessment, it may be possible to determine which cases of threatened abortion may be maintained by exogenous synthetic progestin administration.

There is no correlation between the onset of parturition and a decrease in the plasma progesterone levels.

The reasons for the large quantities of progesterone being metabolized by the fetus, as evidenced by the differences in progesterone levels in the umbilical vein and umbilical artery, and the metabolic end products are subjects worthy of further study.

The value of plasma progesterone levels in the management of high risk pregnancies approaching term is uncertain. It is possible that serial determinations would be useful in conditions affecting maternal placental circulation such as toxemia of pregnancy.

The author is already pursuing further studies of progesterone metabolism in humans and animals, with particular reference to reproduction, using Competitive Protein Binding Analysis.

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