STUDIES ON PROGESTERONE LEVELS IN THE PERIPHERAL BLOOD OF COWS DURING THE ESTROUS CYCLE

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CHAPTER I

INTRODUCTION

The continued appearance of new and modified techniques in the literature for the determination of progesterone in biological materials underscores the problem of isolation as well as quantitation of very small amounts of progesterone while retaining adequate accuracy and specificity. The generally low levels of progesterone in body fluids, the result of the high metabolic clearance rate, has been a major stumbling block to quantitative measurements. Thus knowledge of normal physiological levels of progesterone in biological fluids has been of an uncertain nature, consisting of isolated determinations in individual animals. One basic problem of biological research, variation among individuals, has obscured meaningful relationships in many instances.

The first quantitative estimates of progesterone in tissues were possible due to the development of a bioassay technique by Corner and Allen (1929). This work also opened the way for structural identification of the hormone several years later. This technique required approximately 1 mg of progesterone to elicit a positive response; this, as it turned out, precluded estimation of progesterone in body fluids as well as in most tissues because the amount of hormone present was usually much below the minimum sensitivity level.

Later, Hooker and Forbes (1947) were able to increase greatly the sensitivity of the bioassay technique. Their technique, unfortunately,

was very laborious as several dilutions of the unknown substance had to be injected into mice until no response was obtained. Also, with the emergence of chemical techniques, it became apparent that the bioassay methods overestimated progesterone levels. It was subsequently shown that the bioassay response was influenced by the presence of other compounds having a progesterone-like action, for example, 20α-hydroxypregn-4-en-3-one (20α-OH) and 20β-hydroxypregn-4-en-one (20β-OH); estrogens also increased the response. The importance of bioassay methods should not be discounted, however, as they were essential to early progress in the field, and, in the final analysis, the biological response is the critical test of a hormone.

During the interim between the development of the bioassay technique and more specific spectrophotometric techniques in the 1950's, urinary metabolites of progesterone were used by many investigators as an indicator of progesterone levels. One of the early obstacles to the use of these metabolites was their inability to elicit a response that could be quantitated with the bioassay technique. As suggested by Short (1961), urinary metabolite assays were disappointing because:

(1) Only about 15% of exogenously administered progesterone could be accounted for in pregnanediol and this was subject to considerable variation.

(2) Progesterone metabolites were found to be excreted in large amounts via the gastro-intestinal tract. Sandberg and Slaunwhite (1958) found 30% of progesterone-^{14}C in bile but only 13% in feces, thus indicating the possibility of resorption of progesterone metabolites.

(3) The lag time between the presence of progesterone in blood and its appearance as pregnanediol in the urine may be as long as several

days and transient daily changes may be unobserved.

The measurement of the ultraviolet (UV) absorption of progesterone at 240 mμ was the first important physicochemical technique (Reynolds & Ginsberg, 1942); it greatly reduced the amount of steroid necessary for reliable quantitation. Later, gas-liquid chromatography (GLC), double isotope derivative methods and competitive protein-binding radioassay were to play important roles in progesterone estimation in biological tissues and fluids. In general, the physicochemical techniques have proven to be very laborious. This is due in part to the presence of large amounts of interfering substances in biological materials which necessitates the use of involved isolation procedures. In addition, the structural configuration of progesterone (i.e., a diketone) with its lack of polarity rendered isolation more difficult. Scarcity of functional groups on the molecule limits the derivatives one can prepare of progesterone and thus derivative formation has limited usefulness as an aid to purification.

While the importance of progesterone in preparation of the uterus for the nutrition of the zygote, implantation, blastocyst development and fetal maintenance is well documented, most of the information on progesterone has been qualitative. Knowledge of the concentration of progesterone in the peripheral plasma is important because it is the best indicator of the amount delivered to the main target organs such as the uterus and mammary gland. This is because progesterone produced by corpora lutea moves via the ovarian venous effluent into the general circulation before reaching the general body tissues. There is no evidence at the present time to support a direct influence of ovarian venous progesterone on the uterus. It appeared that a better understanding of

the dynamics of reproductive physiology as it pertained to progesterone might evolve from the ability to make a number of determinations in the peripheral blood of an animal over a relatively short time span. The development of sensitive quantitative methods for progesterone afforded the opportunity to ask: Do peripheral plasma progesterone levels reflect the functional status of progesterone-producing endocrine organs such as the corpus luteum?

In order to answer this question, a technique was required that had excellent sensitivity, good specificity and reliability. Also, supplemental identification of the hormone in question was desirable in order to insure that the findings obtained by the experimental procedure were valid. This is of particular significance since information as to the concentration of the hormone (progesterone) is limited and because the concentration is estimated under conditions which have not been thoroughly investigated (bovine estrous cycle).

At the onset of this study, the most sensitive analytical technique available was the progesterone chloroacetate derivative method of van der Molen and Groen (1965) (see Table I). Also, once the chloroacetate derivative of progesterone was prepared it could be used for supplemental identification by such methods as the establishment of a constant specific activity and by mass spectrometry. Supplemental identification was precluded by use of the other basic techniques with similar sensitivity, namely, fluorescence and double isotope derivative methods. Thus it was decided to attempt to adapt the chloroacetate derivative technique for the determination of progesterone in the peripheral blood of domestic animals and in particular, the cow.

This dissertation presents details of the adaption of the

chloroacetate derivative technique for the daily estimation of peripheral plasma progesterone levels during the estrous cycle of the cow.

CHAPTER II

REVIEW OF THE LITERATURE

The Role of the Corpus Luteum

The corpus luteum was first described by de Graaf in 1672, but it was not until more than two hundred years later that Prenant (1898) suggested its function as an organ of internal secretion. Prenant's suggestion was based on microscopic observations. At approximately the same time, Fraenkel (1903, 1910) began to develop concepts given to him by his teacher, the Breslau embryologist, Gustav Born (Corner, 1923). Born observed that corpora lutea of placentalial animals were associated with the development of young. Born also associated corpus luteum development with embryo attachment to the uterus. He even went so far as to suggest the corpus luteum as the source of a secretion which prepared the uterus for reception and implantation of the zygote. Prior thought had been that inception of uterine progestational development began only with the presence of the embryo. Born's argument hinged on the development of decidual changes in the uterus even when the pregnancy was tubal.

Fraenkel (1903) tested Born's hypothesis with a series of experiments employing pregnant rabbits. It had been established that implantation of the zygote occurred at about the seventh day after ovulation. Knowledge, however, was limited concerning the preimplantation changes in the uterus which would serve to facilitate nidation. Fraenkel (1903) removed both ovaries in some experiments, unilateral ovariectomies

serving as controls. In other experiments, corpora lutea were removed by cauterization; in controls, corpora lutea were left intact while the rest of the ovary was destroyed. All control animals (unilateral ovariectomy or intact corpora lutea) of a large series became pregnant while all those in which corpora lutea were absent or destroyed failed to become pregnant. These results established the requirement of the corpus luteum for implantation of the fertilized ovum.

The work of Leo Loeb (1907) added further understanding to the role of the corpus luteum in the sensitization of the uterine endometrium. Loeb (1907) found that a foreign body placed in the uterine cavity of a guinea pig elicited a marked proliferation of endometrial tissue on the seventh day following an infertile mating. He could prevent the decidуoma reaction by the removal of ovaries or early cauterization of corpora lutea.

The morphological support for Fraenkel's and Loeb's work was reported by Bouin and Ancel (1910). They used rabbits mated to vasectomized males to induce corpus luteum development. Uteri became enlarged and hyperemic. Epithelial proliferation as well as glandular development were noted histologically. These changes, interpreted by Bouin and Ancel (1910) as preparative for implantation, reached their maximal development at the eighth day after ovulation. If zygotes were not present, regressive changes started at the tenth postovulatory day and continued until the twenty-fifth day when regression was complete.

Fraenkel (1910) laid the foundation for understanding the human reproductive cycle. Through direct observation of ovaries of women undergoing laparotomy for reasons other than gynecological, Fraenkel (1910) found recent corpora lutea only during the second half of the menstrual

cycle. He believed the corpus luteum reached its height of development eight days after ovulation, began to regress at menstruation, and disappeared at the end of this period. On the basis of his observations, Fraenkel (1910) proposed that the human uterus underwent proliferative changes due to the influence of the corpus luteum during the second half of the cycle in preparation for implantation of the embryo; he called this type of endometrium premenstrual. If fertilization and implantation did not occur, menstruation followed. The observations of Fraenkel (1910) supported those of Ancel and Villemin (1907) who suggested ovulation occurred twelve days before menstruation.

Shortly after this, two Vienna gynecologists, Hitschmann and Alder (1908) reported their histological findings on human uteri at well-defined stages of the menstrual cycle. Although they had no knowledge of the time of ovulation in women, they suggested the uterine proliferative changes that occurred the last seven to ten days before menstruation represented a preparation for the reception of an ovum released and fertilized at some earlier time. Hitschmann and Alder (1908) suggested implantation began in the late premenstrual phase. All of these results supported the concept that proliferation of the endometrium accompanied the formation and development of the corpus luteum in placentalial mammals.

Corner (1928) repeated the experiments of Fraenkel (1903) and Bouin and Ancel (1910) on the importance of corpora lutea to the proliferation of endometrium and implantation. Corner (1928) confirmed the existence of progestational proliferation of the rabbit uterus during early pregnancy and its dependence upon the presence of corpora lutea. Corner (1928) also confirmed Fraenkel's concept that corpora lutea were

necessary for implantation. Corner (1928) extended knowledge of this phase of reproduction by finding that uterine proliferation was a requirement not only for implantation but also for the nutrition of the free blastocysts during the three or four days between their arrival in the uterus and beginning implantation.

Identification of Progesterone

Corner and Allen (1929) found alcoholic extracts of swine corpora lutea induced progestational changes in the endometrium of spayed rabbits. This led to the development of a biological assay for the corpus luteum hormone (Corner and Allen, 1929). The minimal amount of extract (divided into five daily doses) which produced a proliferative endometrium throughout both cornua equivalent to that of a normal pregnancy was designated as one rabbit unit. Allen and Corner (1929) showed the survival of embryos of rabbits castrated after fertilization could be effected by treatment with corpus luteum extracts. This indicated the condition of the uterus produced by corpus luteum extracts was functionally as well as morphologically identical with normal early pregnancy.

The development of the bioassay technique by Corner and Allen (1929) initiated a series of investigations which culminated in the identification of the corpus luteum hormone (progesterone) several years later. In 1934, the hormone of the corpus luteum was isolated in pure form by the independent endeavor of four laboratories: Butenandt and associates in Danzig (Butenandt, Westphal and Hohlweg, 1934; Butenandt and Westphal, 1934a); Wintersteiner and Allen (1934) in New York and Rochester (also Allen and Wintersteiner, 1934); Hartmann and

Wettstein (1934a,b) in Basle; and Slotta, Ruchig and Fels (1934) in Breslau. The structure was determined in 1934 by Butenandt et al. (1934), Butenandt and Westphal (1934b), Fernholz (1934) and Butenandt and Schmidt (1934). The hormone was to become known as "progesterone" several years later (1937) through the melding of "progestin" suggested by Allen and Wintersteiner and "luteosterone" by Slotta and associates (Amoroso, 1968). Progress in research was slow, however, following the isolation and identification of progesterone because of two factors: (1) low levels of the hormone in biological materials precluded the use of biological assay (1 mg was required for a positive test) and (2) urinary metabolites were devoid of biological activity thus necessitating the development of chemical tests.

Isolation of Progesterone from Biological Materials

Extraction

A number of solvents have been used to extract progesterone from biological tissues. Boiling ethanol was used to extract lipids from the corpus luteum by several investigators (Allen, 1932; Loy, McShan and Casida, 1957; Oertel, Weiss and Eik-Nes, 1959). The plasma proteins were precipitated and the alcoholic residue was dissolved in ether for further purification procedures. Ethanol and ether (3:1, v/v), a classical solvent combination for lipid extraction, was used for progesterone extraction by Butt, Morris, Morris, and Williams (1951), Edgar (1953a), Zander and Simmer (1954) and Raeside and Turner (1955).

Sommerville and Deshpande (1958) used dichloromethane:ether (4:1, v/v) to extract progesterone from human plasma while Haskins (1950) used only ether. Short (1958a) found ether effective for extraction of

progesterone from the plasma of domestic animals. Hudson, Coghlan, Dulmanis, Wintour and Ekkel (1963) found dichloromethane an effective solvent for extraction of testosterone from human plasma.

Removal of Lipids

Allen (1932) while working on the isolation of purified "progestin" developed several techniques which were to become very important for the initial purification of progesterone. He used a partition system of 70% methanol-petroleum ether in which cholesterol and neutral lipids remained in the hydrocarbon phase while the progestins moved into the alcoholic phase. Allen (1932) also removed lipids by precipitation in cold methanol; this technique, likewise, found widespread usage. Allen (1932) also suggested the removal of fatty acids from ethereal solutions of the hormone by the use of alkali (sodium hydroxide). Hudson et al. (1963) saponified the solvent extract of plasma as a routine procedure for the isolation of testosterone; however, no comment was made as to its effectiveness. This procedure was to become an important part of the isolation technique used in the current dissertation.

Prelog and Meister (1949) developed an extraction technique in which alkali pretreatment of a mash of corpora lutea (from whales) was followed with ether extraction. This was done to reduce the amount of lipid removed during the extraction process. Noall, Salhanick, Neher, and Zarrow (1953) found purer extracts could be obtained if the sodium hydroxide treatment proceeded without ether overlay; ether extraction was started two days after the addition of sodium hydroxide. Based on these findings, Haskins (1954) developed a routine procedure using alkali pretreatment for the extraction of progesterone from the human

placenta. From these studies it became apparent that progesterone was not nearly as sensitive to alkali as was thought previously.

Sommerville and Deshpande (1958) and Short (1958a) used alkali treatment of human and domestic animal plasma before extraction. Zander (1962) conducted a series of extractions of pooled human umbilical cord plasma; ten samples were extracted after alkali treatment (Short, 1958a) while nine samples were extracted without pretreatment with alkali. Both methods gave the same average progesterone concentration but the precision was better with alkali treatment.

Countercurrent Distribution

Craig, Golumbic, Mighton and Titus (1945) presented the countercurrent distribution method for the separation of compounds. Loy et al. (1957) used this technique for purification of progesterone isolated from sow corpora lutea. The development of paper chromatography at this time, with its simplicity and speed, forestalled the use of the countercurrent distribution method.

Column Chromatography

Column chromatography has been widely used in the preliminary isolation procedure for progesterone. Samuels (1947) reported the use of an aluminum oxide column to isolate progesterone from liver preparations. Loy et al. (1957) used this procedure for purification of progesterone isolated from swine corpora lutea; the column was developed with hexane and hexane:chloroform (95:5, v/v). Progesterone was eluted with hexane: chloroform (80:20, v/v). The aluminum oxide column was also used by Hinsberg, Pelzer and Seuken (1956), Sommerville (1957), and Sommerville

and Deshpande (1958) following extraction of progesterone from plasma. Aluminum oxide column chromatography did not yield pure progesterone; further purification procedures were still necessary.

Sommerville, Pickett, Collins, and Denyer (1963) used a silica gel column to aid in the isolation of progesterone. The crude extract was applied in ethyl acetate:hexane (1:4, v/v) and progesterone was eluted with ethyl acetate:hexane (1:1, v/v).

Butt et al. (1951) isolated progesterone for routine analysis by use of a column of Hyflo Supercel (Johns Manville, celite); this was preceded by removal of lipids. Good separation of progesterone was found with the use of 70% methanol as the stationary phase and n-hexane as the mobile phase.

Paper Chromatography

Haskins, Sherman and Allen (1950) were the first to attempt to isolate progesterone by the use of paper chromatography; a "monophasic" 80% methanol system was used to separate progesterone from commercial oils. The use of paper chromatography was greatly refined by Zaffaroni, Burton and Keutmann (1950) and Bush (1952) who established it as the most important method for the isolation of progesterone from extracts of biological materials.

Edgar (1953a) and Raeside and Turner (1955) utilized the "A" system of Bush to isolate progesterone; methanol (80%) served as the stationary phase and light petroleum as the mobile phase. Zander and Simmer (1954), Hinsberg et al. (1956), Short (1958a) and Gorski, Erb, Dickson and Butler (1958) used variations of the Bush "A" system to isolate progesterone. Prior removal of lipids was necessary for adequate separation of steriods

on paper chromatography (Bush systems) in order that reliable identification and quantitation could be effected by infrared spectrophotometry and UV absorption, respectively.

Paper chromatography was the first method that effected sharp separation of weakly polar Δ4-3-ketosteroids such as progesterone, androst-4-ene-3,17-dione, testosterone and 17α-hydroxyprogesterone. In addition, Zander, Forbes, von Münstermann and Neher (1958) were able to isolate substances other than progesterone with progestational activity, namely, 20α-OH and 20β-OH. Thus paper chromatography became an important micro-analytical tool by which small amounts of steroid could be visualized and isolated.

Thin Layer Chromatography

Lisboa (1963) reported on the separation and characterization of a number of Δ4-3-ketosteroids of the pregnane series by thin layer chromatography (TLC) using silica gel G. Futterweit, McNiven and Dorfman (1963) isolated human plasma progesterone on TLC plates coated with the adsorbant silica gel G; development was in a benzene:ethyl acetate (3:2, v/v) system. Collins and Sommerville (1964) were likewise able to isolate progesterone from human plasma by two-dimensional TLC chromatography. van der Molen, Runnebaum, Nishizawa, Kristensen, Kirschbaum, Wiest and Eik-Nes (1965) used TLC (alumina) to isolate progesterone from human plasma; quantitation was by GLC with hydrogen flame detection. van der Molen and Groen (1965) used TLC to isolate progesterone from human plasma (the chloroacetate derivative was measured by GLC). van der Molen and Groen (1965) found that progesterone migrated further in a benzene:ethyl acetate (2:1, v/v) system as compared to other steroids

that might be present in plasma (the one exception was estrone); thus progesterone separation was effected in one single-dimension TLC system.

Luisi, Gambassi, Marescotti, Savi and Polvani (1965) used horizontal development of silica gel G plates to isolate progesterone obtained from human plasma. Progesterone did not move from the origin when the development was with 70% methanol saturated with heptane. Following this, the plate was left in place, the solvents were allowed to evaporate and a second development was performed using cyclohexane-ethyl acetate (1:1, v/v). Progesterone was effectively isolated in this system (Rf, 0.548).

Following celite column chromatography, Lurie, Villee and Reid (1966) used TLC (silica gel) to further purify progesterone from human plasma. Development was in chloroform:acetone (9:1, v/v) with elution of progesterone from silica gel accomplished by using chloroform:ether (1:1, v/v) as the eluant.

Neill, Johnsson, Datta and Knobil (1967) used silica gel sheets ("chromatogram", Eastman) previously washed in absolute methanol for the isolation of progesterone. The sheets were developed in ether:benzene (2:1, v/v) by ascending chromatography; appropriate areas were eluted with absolute methanol.

Methods for Quantitation of Progesterone

Bioassay

The first attempts at the quantitation of progesterone began with the work of Corner and Allen (1929) who measured the effects of crude extracts of swine corpora lutea on rabbit endometrium. They designated a rabbit unit as that amount of crude extract which would produce

proliferative changes in both cornua of a castrate rabbit equal to that produced by pregnancy. For a number of years the bioassay approach was the only method available for the quantitation of progesterone. Hooker and Forbes (1947) refined the bioassay technique in mice whereby 0.2 mμg of progesterone gave a positive uterine response. The inability of the method to differentiate progesterone from other progestational steroids limited its usefulness.

Spectrophotometry

A major step toward precise quantitation was contributed by Reynolds and Ginsburg (1942) who used the characteristic maximal UV absorption exhibited by Δ4-3-ketosteroids at 240 mμ to measure progesterone. This method was based on selective absorption by chromophores in the UV region of the light spectrum. The chromophores are resonating groups involving two or more atoms coupled by sharing four, rather than two electrons. The presence of a strong and characteristic chromophore grouping, namely, that of an α,β-unsaturated ketone (R-C(=O)-C=C<) allowed progesterone to strongly absorb UV light at 240 mμ. While the technique was sensitive, Reynolds and Ginsburg (1942) were not particularly successful in quantitating progesterone isolated from biological fluids; adequate techniques for the separation of Δ4-3-ketosteroids were not yet available. It was also found that materials of unknown composition and origin absorbed light at 240 mμ; this resulted in an overestimation of progesterone from biological materials. Allen (1950) devised a correction factor that minimized the error. Absorption was measured at 225 mμ, 240 mμ and 255 mμ; the values were calculated as follows, (O.D. = optical density):

$$O.D._{240\ \text{corrected}} = O.D._{240\ \text{observed}} - \frac{O.D._{225\ \text{observed}} + O.D._{255\ \text{observed}}}{2}$$

The elution of chromatographic paper revealed the presence of small amounts of material absorbing in the same UV region as progesterone. This was overcome by using eluates from paper blanks as the reference point for spectrophotometry.

Spectrophotometry became the main technique for progesterone quantitation during the 1950's and 1960's mainly because of its sensitivity; if preceded by adequate separation measures its specificity was adequate.

Progesterone derivatives

Evans and Gillam (1943) found thiosemicarbazone derivatives of α,β-unsaturated ketones exhibited a maximum absorption at 300 mμ. The technique had the advantage of increased intensity of absorption of progesterone thiosemicarbazone at this wave length (ε_{max} = 29,950-37,000) as well as the advantage that impurities usually absorbed at shorter wavelengths. Pearlman and Cerceo (1953) first used the technique for the estimation of progesterone isolated from human placentae. Zander and von Münstermann (1956) encountered difficulties in the quantitative estimation of progesterone if the extract was contaminated by trace amounts of ketonic impurities; the thiosemicarbazone method gave only an estimate of the total number of carbonyl groups present regardless of origin.

Umberger (1955) used the isonicotinic acid hydrazone derivative for the estimation of large amounts of progesterone in oily solutions. Maximum absorption was at 380 mμ. Sommerville and Deshpande (1958) adapted the technique on a microanalytical basis for progesterone determination

in human plasma. Unfortunately, the reaction was relatively insensitive as the absorption at 380 mμ was not intense (ε_{max} = 11,800).

Reich, Sanfilippo and Crane (1952) quantitatively estimated progesterone by measuring its dinitrophenyl-hydrazone derivative. Maximum absorption was at the same wavelength as the isonicotinic acid hydrazone derivative of progesterone, namely, 380 mμ; however, the intensity of absorption was much greater for the dinitrophenyl-hydrazone derivative (ε_{max} = 36,665). Hinsberg et al. (1956) used the dinitrophenyl-hydrazone derivative method for the quantitation of progesterone in human plasma. Preliminary removal of lipids was essential as derivatives of ether-soluble impurities (apparently lipids) interfered by absorbing at 366 mμ.

Butt et al. (1951) developed a quantitative method for estimating progesterone through the condensation of progesterone with trimethylacethydrazide ammonium chloride (Girard's reagent T). Polarographic waves produced by the condensate could be measured quantitatively since the wave span was proportional to the concentration of the steroid. This method was not widely used because of its lack of sensitivity; 10 μg of steroid were necessary for quantitation.

Zaffaroni (1950) showed that steroids (in ethanol) treated with sulfuric acid produced chromogens with distinctive spectra between 200 and 600 mμ. The chromogen produced from progesterone showed a single absorption peak at 290-295 mμ; unfortunately a number of other α,β-unsaturated ketosteroid chromogens, including 20α-OH and 20β-OH, exhibited an absorption peak at 290-295 mμ. Oertel et al. (1959) modified the method of Zander and Simmer (1954) by reducing the concentration of sulfuric acid (to 66%) and produced chromogens from progesterone isolated from human plasma. While the molar extinction coefficient at 290 mμ was

higher (19,000) than that of progesterone in ethanol at 240 mμ, the absorption peak around 290 mμ was much wider than at 240 mμ (Short, 1961). After application of the Allen correction, the sulfuric acid-ethanol reaction was less sensitive than direct UV measurement at 240 mμ.

Fluorescence

Abelson and Bondy (1955) reported an alkali fluorescence reaction for the estimation of α,β-unsaturated oxosteroids in solution. The reagent producing the fluorescence, potassium ter-butoxide was prepared by refluxing molten potassium metal with tertiary butanol. Short (1961) found the technique less sensitive when compared to UV absorption of progesterone in ethanol at 240 mμ.

Touchstone and Murawec (1960) pretreated progesterone with 2N potassium hydroxide at 60C for 30 min before dissolving it in sulfuric acid. A maximal fluorescence was produced at 490 mμ when prior excitation was carried out at 390 mμ. Touchstone and Murawec (1960) reported sensitivity was increased a hundredfold over treatment with sulfuric acid alone. Short and Levett (1962) used this technique to assay progesterone from human plasma obtained during pregnancy and the menstrual cycle. They suggested that while the technique was useful, it overestimated progesterone levels due to the presence of high and somewhat variable fluorescence of blanks.

Heap (1964) found 20β-OH produced a more intense fluorescence in sulfuric acid-ethanol as compared to progesterone. The fluorimeter absorption peak was at 520 mμ. Progesterone was converted to 20β-OH by the enzyme 20β-hydroxysteroid dehydrogenase. Major, Armstrong and Greep (1967) used the method to quantitate progesterone in corpora lutea

obtained from the rat.

Double Isotope Derivative Methods

Woolever and Goldfien (1963) developed a double-isotope derivative method for assaying plasma progesterone. Progesterone-^{14}C used as the indicator was added to the plasma. The reduction of progesterone by sodium borotritide followed; partial reoxidation by manganese dioxide increased the recovery rate and precision of the method. Following two paper chromatogram steps, quantitation was effected by the determination of the $^{3}H/^{14}C$ ratio.

Riondel, Tait, Tait, Gut and Little (1965) used a double isotope technique for the estimation of human plasma progesterone; the technique was developed originally to measure testosterone (Riondel, Tait, Gut, Tait, Joachim and Little, 1963). Progesterone-1,2-^{3}H was used as the indicator while thiosemicarbazide-^{35}S was used to form the thiosemicarbazone derivative of progesterone. Thin layer and paper chromatograms were used to isolate the derivative. Hydrolysis to the monothiosemicarbazone form with pyruvic acid was followed by a second TLC step. Acetylation was carried out with acetic anhydride to form the 3-thiosemicarbazone 2', 4'-diacetate. Thin layer and paper chromatograms preceded quantitation by determination of the $^{35}S/^{3}H$ ratio.

Wiest (1967a) reported a double isotope derivative assay for progesterone and 20β-OH. Steroids (^{14}C) were used as indicators while acetic-^{3}H anhydride was used as the derivatizing reagent to estimate steroid mass. The following flow chart emphasizes the difficulty as to the laboriousness of the technique:

1. Paper chromatogram for progesterone isolation.

2. Purification of progesterone by acetylation of residue.
3. Enzymatic conversion of progesterone to 20β-OH.
4. Acetylation of 20β-OH.
5. Paper chromatogram for purification of 20β-acetoxypregn-4-en-3-one.
6. Paper chromatogram for purification of 20β-acetoxypregn-4-en-3-one.
7. Removal of tritium contaminants with 0.4% chromium trioxide.
8. Formation of methyloxime derivative.
9. Paper chromatogram for purification of 20β-acetoxypregn-4-en-3-o-methyloxime.
10. Paper chromatogram for purification of 20β-acetoxypregn-4-en-3-o-methyloxime.

Gas-Liquid Chromatography

The introduction of commercial gas chromatographs in the mid 1950's opened new possibilities for the separation and quantitation of steroids, VandenHeuvel, Sweeley and Horning (1960) reported the first successful separation of steroids by GLC. They used a thermostable methyl-substituted silicone gum (SE-30, General Electric) as the liquid phase.

The first report of GLC quantitation of progesterone in human pregnancy plasma was by Futterweit et al. (1963). They were able to detect as little as 15 mμg of progesterone by the use of a SE-30 (6.8%) column coupled with hydrogen flame detection. Accurate quantitation was hindered, however, by the presence of a nonsteroidal contaminant. Collins and Sommerville (1964) reported the use of argon ionization detection for quantitation of progesterone from human plasma. Unfortunately, the

retention time on the liquid phase, cyclohexane-dimethanol succinate (1%), was very long (70 min).

van der Molen et al. (1965) reported the quantitation of progesterone from the plasma of normal women by GLC with radium ionization detection; a 1% XE-60 (cyanoethylmethyl and dimethyl substituted silicone gum, General Electric) column was used. GLC tracings were very poor even though prior partition and TLC steps were included.

Yannone, McComas and Goldfien (1964) were able to detect progesterone from human plasma by GLC with hydrogen flame detection. The key to their success was preliminary purification of progesterone by a 70% methanol:heptane partition system and paper chromatography.

Luisi et al. (1965) were able to obtain very good GLC chromatograms of progesterone isolated from the plasma of pregnant women. A partition system of 70% methanol and heptane plus TLC were used to purify the progesterone. Hydrogen flame detection was used in conjunction with 3% SE-30 liquid phase column. Lurie et al. (1966) also estimated progesterone from human plasma by GLC with hydrogen flame detection. Preliminary purification by celite column and thin layer chromatography resulted in good GLC chromatograms.

Clark and Wotiz (1963) reported the separation of nanogram (mμg) amounts of steroid in conjunction with the heptafluorobutyrate derivatives by GLC with electron capture detection. van der Molen and Groen (1965) used the chloroacetate derivative of 20β-OH to quantitate progesterone from human peripheral blood; progesterone was enzymatically converted to 20β-OH. Separation was by a 1% XE-60 column with electron capture detection. The pure 20β-OH chloroacetate could be detected in quantities as small as 1 mμg.

Competitive Protein-Binding Radioassay

Neill et al. (1967) used the competitive protein-binding radio-assay of Murphy (1964, 1967) to estimate progesterone in peripheral plasma during the menstrual cycle. The technique hinged on the displacement of corticosterone-1,2-^{3}H from canine corticosteroid-binding globulin (CBG) by progesterone molecules. Progesterone was quantitated by measuring the amount of corticosterone-1,2-^{3}H remaining bound to CBG. Displaced corticosterone-1,2-^{3}H was removed by the use of Florisil (activated magnesium silicate, Fisher Scientific Co.).

A comparison of the sensitivities of the important techniques for estimating progesterone are shown in Table I.

Estimation of Progesterone in Biological Fluids

The development of a refined progestin bioassay by Hooker and Forbes (1947) stimulated interest in the development of chemical means for the estimation of progesterone. Progestin as originally used by Hooker and Forbes (1947) was synonomous with progesterone. Haskins (1950), however, using spectrophotometry was unable to verify the progesterone levels found by Hooker & Forbes (1949); blood was examined from gravid, postmenopausal and postpartum women. Progesterone injected intravenously into intact rabbits could be isolated but had a short half-life (Haskins, 1950). The negative findings of Butt et al. (1951) who estimated progesterone polarographically supported the results of Haskins (1950). It thus became apparent that the bioassay technique of Hooker & Forbes (1947) was not specific for progesterone.

Human Peripheral Plasma Progesterone Levels

It was not until 1954 that quantitation of progesterone in

TABLE I

A SUMMARY OF THE SENSITIVITY OF TECHNIQUES USED TO QUANTITATE PROGESTERONE (SMALLEST AMOUNT OF PURE PROGESTERONE ACCORDING TO THE AUTHORS THAT COULD BE MEASURED)

Investigator	Method	Sensitivity (mμg)
Zander & Simmer (1954)	UV absorption at 240 mμ	500
Short (1958a)	UV absorption at 240 mμ	500
Oertel et al. (1959)	UV absorption of sulfuric acid chromogens at 290 mμ	500
Sommerville et al. (1963)	UV absorption of thiosemicarbazone derivative at 302 mμ	100
Luisi et al. (1965)	GLC with hydrogen flame detection (liquid injection)	$\geq$ 25
Woolever & Goldfien (1963)	Double isotope (sodium borotritide reduction of progesterone)	25
Touchstone & Murawec (1960)	Fluorescence in sulfuric acid at 490 mμ (alkali pretreatment)	10
Collins & Sommerville (1964)	GLC with argon ionization detection (solid injection)	< 10
Riondel et al. (1965)	Double isotope (progesterone 3-thiosemicarbazone 2',4'-diacetate)	2.5 to 5
Lurie et al. (1966)	GLC with hydrogen flame detection (solid injection)	3
Yannone et al. (1964)	GLC with hydrogen flame detection (solid injection)	3
Heap (1964)	Fluorescence of 20β-hydroxypregn-4-en-3-one in sulfuric acide at 520 mμ	3
van der Molen & Groen (1965)	GLC with electron capture detection (chloroacetate derivative)	1 to 2
Neill et al. (1967)	Competitive protein-binding radioassay	0.2

peripheral blood (human) was accomplished (UV spectrometry); an average of 142 mμg/ml plasma were found (Zander & Simmer, 1954; Zander, 1954). The quantitation was carried out on plasma from women in late pregnancy. The choice of plasma was fortunate because, as was later apparent, blood from pregnant women had much higher levels when compared to nonpregnant women as well as pregnant or nonpregnant domestic animals. Sommerville (1957) reported 98, 110 and 123 mμg of progesterone/ml of plasma from pregnant women. The determinations made by spectrophotometry of the isonicotinic acid hydrazone derivative of progesterone agreed with the data of Zander & Simmer (1954).

Oertel et al. (1959) used the absorption of progesterone in sulfuric acid-ethanol at 290 mμ to measure the hormone in nonpregnant and pregnant women. Levels ranged from 25 to 50 mμg/ml plasma in nonpregnant women with no correlation to the menstrual cycle; pregnancy levels ranged from 119 mμg/ml at the ninth week to 449 mμg/ml at the forty-first week. Short (1961) found progesterone increased in the peripheral plasma of pregnant women from about 30 mμg/ml (eighth to eighteenth week) to 160 mμg/ml at the fortieth week of gestation.

Short and Levett (1962) found a cyclic pattern of progesterone in peripheral plasma of women during the menstrual cycle; levels ranged from 12 mμg/ml during the follicular phase to 30 mμg/ml on the twenty-first and twenty-fifth day. These values were obtained by the microfluorescence technique of Touchstone & Murawec (1960).

Sommerville et al. (1963) reported progesterone levels in the plasma of nonpregnant and pregnant women. They found 15 to 39 mμg/ml during peak luteal function and 9 to 13 mμg/ml in the late premenstrual period.

Pregnancy levels of progesterone ranged from 19 mμg/ml at the ninth to eleventh week to 165 mμg/ml at the thirty-ninth week; levels during labor were similar to those of the last trimester of pregnancy. The thiosemicarbazone derivative of progesterone was measured spectrophotometrically.

Several investigators used double isotope derivative methods for the determination of plasma progesterone. Woolever (1963) using sodium borotritide reduction of progesterone reported an average of 1.7 mμg/ml for preovulatory plasma progesterone levels while levels in the second half of the cycle increased rapidly to 21 mμg/ml. Riondel et al. (1965) used a double isotope derivative method (thiosemicarbazide-^{35}S) and found 1.3 mμg/ml during the follicular phase and 10.6 mμg/ml during the luteal phase of the menstrual cycle of normal women. Wiest (1967b) found an average of 71 mμg/ml plasma during the first half of pregnancy which rose to an average of 173.5 mμg/ml during the second half. Acetic-^{3}H anhydride was used as the derivatizing reagent to estimate mass (Wiest, 1967a).

Still other workers used GLC to advantage to estimate progesterone in peripheral blood. Collins & Sommerville (1964) reported an average of 7.9 mμg/ml of plasma during the first half of the menstrual cycle and 15.0 mμg/ml the latter half. Determinations were made by GLC with argon ionization detection. van der Molen et al. (1965) estimated progesterone in the peripheral plasma of nonpregnant women. They reported less than 1.0 mμg/ml on days 1-10, 16.0 mμg/ml on days 11-15, 12.5 mμg/ml on days 16-20 and 4.4 mμg/ml from day 21 to the first day of the next cycle. These results were obtained from pooled blood samples by GLC with radium ionization detection. Luisi et al. (1965) found an average of

4.0 mμg/ml plasma during the preovulatory stage in women and 27.7 mμg/ml during the second half of the cycle. Determinations were made by GLC with hydrogen flame detection.

van der Molen & Groen (1965) used GLC of the chloroacetate derivative of progesterone to quantitate the hormone in the peripheral plasma of nonpregnant women. They found 0.5 mμg/ml at days 1-9, 1.3 mμg/ml on days 7-16, 15.2 mμg/ml on days 16-24 and 7.1 mμg/ml on days 22-30.

Neill et al. (1967) used the competitive protein-binding radioassay technique of Murphy (1967) to estimate progesterone in the peripheral plasma of nonpregnant women. An average of 0.4 mμg/ml plasma was found during the follicular phase with a peak average of 14 mμg/ml approximately one week after estimated time of ovulation. These results were in close agreement with those recorded by van der Molen and Groen (1965).

Domestic Animal Peripheral Plasma Progesterone Levels

Edgar (1953b) was the first to determine progesterone in the blood of domestic animals; this was accomplished by the use of UV spectrophotometry preceded by paper chromatography. Edgar (1953b) found progesterone in blood from the ovarian vein of the pregnant and nonpregnant ewe and in the nonpregnant sow; it was not found in the peripheral blood of the pregnant and nonpregnant ewe, nor was it found in the peripheral blood of the mare, cow, sow and doe as well as in the castrated ram. Raeside & Turner (1955) using a technique similar to Edgar (1953a) and Zander & Simmer (1954) were unable to find progesterone in the peripheral blood of several domestic species including the pregnant cow and goat; Raeside & Turner (1955) found progesterone in the venous effluent of the ovary of a pregnant goat.

Short (1957) demonstrated progesterone in peripheral blood of both pregnant and nonpregnant cows. In one cow he found 4.0 mμg/ml of plasma 24 hr before ovulation while another had 3.8 mμg/ml on the twelfth day following ovulation; levels in cows during the middle trimester of pregnancy ranged from 4.8 to 5.6 mμg/ml. This report was the first concerning progesterone in the peripheral blood of cows. Later Short (1958b) reported 7.4 to 9.8 mμg/ml plasma from the thirty-second to the 256th day of pregnancy in cows with a decrease noted a few days before parturition. Short (1957, 1958a) used UV absorption at 240 mμ to quantitate progesterone.

Melampy, Hearn & Rakes (1959) reported progesterone in pregnant cows ranging from 9 mμg/ml plasma during early pregnancy to 40 mμg/ml in the third trimester. These results were considerably higher than those reported earlier by Short (1958b). Bowerman & Melampy (1962) found no correlation of peripheral plasma progesterone with the stage of pregnancy in cows. Progesterone levels were lower than reported previously (Melampy et al., 1959) ranging from undetectable to 21.3 mμg/ml (ave., 10.3 mμg/ml). Melampy et al. (1959) and Bowerman and Melampy (1962) used UV spectrophotometry to quantitate progesterone.

McCracken (1963) used the technique of Short (1958a) and observed average progesterone values of 9.6 and 8.8 mμg/ml in the peripheral plasma of two cows in the luteal phase of the estrous cycle. Gomes, Estergreen, Frost & Erb (1963) were unable to correlate peripheral plasma progesterone with the stage of the estrous cycle. Levels, in fact, decreased from 18 mμg/ml on day 2 (estrus = 1) to 2.7 mμg/ml at day 7; this was followed by a rise to 22 mμg/ml at day 12, and thence a decline through the rest of the cycle. Gomes, Frost and Estergreen

(1962) had previously reported an average of 9.2 mμg/ml plasma in two cows at the 250th and 251st day of pregnancy.

Plotka, Erb, Callahan & Gomes (1967) reported progesterone levels in peripheral plasma of cyclic cows average 10 mμg/ml at estrus and 25 mμg/ml at the fifteenth day of the cycle. These determinations were made with the double isotope method of Woolever & Goldfien (1963).

CHAPTER III

MATERIALS AND METHODS

Reagents

A number of the solvents used including benzene, methanol, ethyl acetate and toluene were nanograde in quality (Mallinckrodt Chemical Works). Ether and dichloromethane (analytical reagent grade, Mallinckrodt) were glass distilled 2 times. Water used in the procedure was distilled 2 times followed by ether extraction and finally redistillation. Hydrochloric acid was analytical reagent grade (Mallinckrodt). Pyridine (spectrophotometric grade, Mallinckrodt) was stored over 1/16" molecular sieve pellets in a desiccator. Tetrahydrofuran (analytical reagent grade, Mallinckrodt) was refluxed for 3 hr over sodium hydroxide and distilled off fresh sodium hydroxide in the absence of air. It was stored over elemental sodium at 5C. Scintillation fluid contained 4 g 2,5-diphenyloxazole (PPO) and 300 mg 1,4-bis-[2-(5-phenyloxazolyl)]-benzene (POPOP) per 1 of toluene.

Silica Gel

Silica gel (Silicar TLC-7GF, Mallinckrodt) was washed 3 times with dilute acetic acid over a sintered glass filter followed by 3 rinses with boiling triple distilled water and finally washed 2 times with boiling nanograde methanol. It was dried 18-24 hr at 110C. Thin layer chromatography plates were 0.25 mm thick and had a ratio of 33 g

silica gel to 78 ml water.

Enzyme Preparation

In general, the technique of Henning and Zander (1962) as used by van der Molen and Groen (1965) was followed. Phosphate buffer (pH 5.2) used as the medium for enzymatic conversion of progesterone to 20β-OH was prepared by mixing 0.15M monobasic potassium phosphate (97.5 ml) and 0.15M dibasic sodium phosphate (2.5 ml); to this was added 100 mg (2.7×10^{-3}M) EDTA (Sigma). Enzymatic conversion was by 20β-hydroxysteroid dehydrogenase (Sigma) obtained in concentrated form in 2.2M ammonium sulfate; the enzyme was diluted with tris buffer (Sigma, pH 7.4) to a final concentration of 0.1 mg/ml buffer. The enzyme in this dilution was stable for at least 6 weeks. β-NADH (Sigma) kept in dry form was dissolved in the phosphate buffer just before enzymatic conversion.

Steroids

Progesterone and 20β-OH were obtained from Zori Ltd. (Tel Aviv, Israel). Progesterone-7-^{3}H (New England Nuclear) was purified 2 times by TLC systems of benzene:ethyl acetate (2:1, v/v and 6:1, v/v). Purity was checked by a radiochromatogram scanner (Packard, Model 7201) and identity by the use of standard preparations in adjacent lanes on the TLC plate. The isotopically labeled steroid was checked by TLC (radiochromatogram scanner) at 1 to 2 month intervals. It was stored in benzene at a dilution of about 250,000 cpm/ml at 5C.

Cleaning of Glassware

Glassware was rinsed immediately after use, soaked in detergent,

brushed and placed in chromic acid overnight. Following 7 to 8 tap water rinses the glassware was soaked in detergent, rinsed again with tap water and placed in dilute hydrochloric acid. After being rinsed 10 times with tap water, 10 times with distilled water and once with nanograde methanol, the glassware was air dried.

Animals and Collection of Blood

The animals used in this study were multiparous Holstein-Friesian cows (one animal was a Holstein-Friesian-shorthorn crossbreed). All animals had normal breeding histories including at least 2 lactations. Three of the cows (no. 07, 24 and 40) had not lactated for about 1 yr while the others (no. 11, 17 and 46) were lactating at the start of the experiment. The animals were housed in a large paddock and fed a ration of alfalfa and ground milo. Sexual behavior was used to assess the onset of pyschic estrus which was designated day 1 of the estrous cycle. Blood was obtained from five cows (no. 07, 11, 17, 24, 46) daily between 8 and 10 a.m. starting on April 3, 1967 regardless of the stage of cycle until data had been collected for 1 complete estrous cycle. Blood was obtained from cow no. 40 for 45 consecutive days or through 2 complete cycles. One hundred ml of jugular vein blood were obtained daily using 1 ml potassium oxalate (10% solution) per 9 ml blood. The bleeding regimen had no adverse effect on the cows as judged by hematocrit values (Appendix E), physical condition and occurrence of estrus.

Outline of Method

(1) Addition of progesterone-7-^{3}H to plasma followed by 3 extractions

with 2.5, 2.5 and 2 vol of dichloromethane.

(2) Saponification of the solvent extract (in ether) with sodium hydroxide.

(3) Isolation of progesterone by TLC using benzene:ethyl acetate (3:1, v/v).

(4) Enzymatic conversion of progesterone to 20β-OH.

(5) Acetylation of 20β-OH by chloroacetic anhydride.

(6) Isolation of the chloroacetate derivative of 20β-OH by TLC using benzene:ethyl acetate (6:1, v/v).

(7) Quantitation by GLC with electron capture detection and liquid scintillation spectrometry.

The Method in Detail

(1) Preparation of Plasma

The volume of blood was measured and duplicate hematocrit values were determined in order to estimate the dilution of plasma by the anticoagulant. Blood was centrifuged (International High-Speed Refrigerated Centrifuge, Model HR-1) for 20 min at 7200 g (0C) and plasma was removed and stored at 5C. Radioactive steroid (progesterone-7-^{3}H, 2500 cpm) was added to each extraction bottle in 10 µl benzene. A short equilibration period was allowed before extraction. Plasma samples were extracted and the residue saponified and washed the same day as collected; the residue was stored in benzene prior to thin layer chromatography (TLC). Of 150 samples examined, all were done in duplicate except 14 samples on which 1 determination was made for a total of 286 assays.

(2) Extraction and Saponification

Two 20 ml aliquots of plasma from each sample were extracted 3 times

using 2.5, 2.5 and 2 vol of dichloromethane in 250 ml polyethylene bottles (Fig. 1). Extractions were carried out on a vortex shaker for 30 sec. The separation of plasma and solvent was effected by spinning at 7200 g for 10 min (10C). A serum lifter was used to remove the solvent from the polyethylene bottles into 80 ml conical tubes (Fig. 1). The solvent was dried under nitrogen in a water bath (45C) between extractions. Following final drying of dichloromethane, the residue was dissolved in ether (15 ml) and saponification carried out by adding 5 ml 0.1N sodium hydroxide and shaking the tubes 100 times. The sodium hydroxide layer was removed by using Pasteur pipettes (230 mm). The ether was washed 2 times with 5 ml water. Tubes were centrifuged after saponification and each washing. After drying, the ether tubes were washed with 3, 2 and 1 ml dichloromethane to concentrate the residue; the final product was stored in 0.2 ml benzene prior to isolation of progesterone.

(3) Isolation of Progesterone

TLC plates were set up with 9 lanes (2 cm wide), 8 for samples and 1 for standard progesterone and 20β-OH. In addition to the 0.2 ml benzene, 3 and 2 drops of benzene were used to spot the sample within a 1 cm diameter. Development was in benzene:ethyl acetate (3:1, v/v). As unknown samples did not contain sufficient progesterone to fluoresce under UV light, areas corresponding to the standard progesterone were eluted. A fritted disc eluter (Dependable Scientific) was used to trap the silica gel on a sintered glass filter (medium pore size) prior to elution with 8 to 10 ml hot methanol. Following drying, the sample was concentrated by 2 and 1.5 ml rinses of benzene. Random checks were made

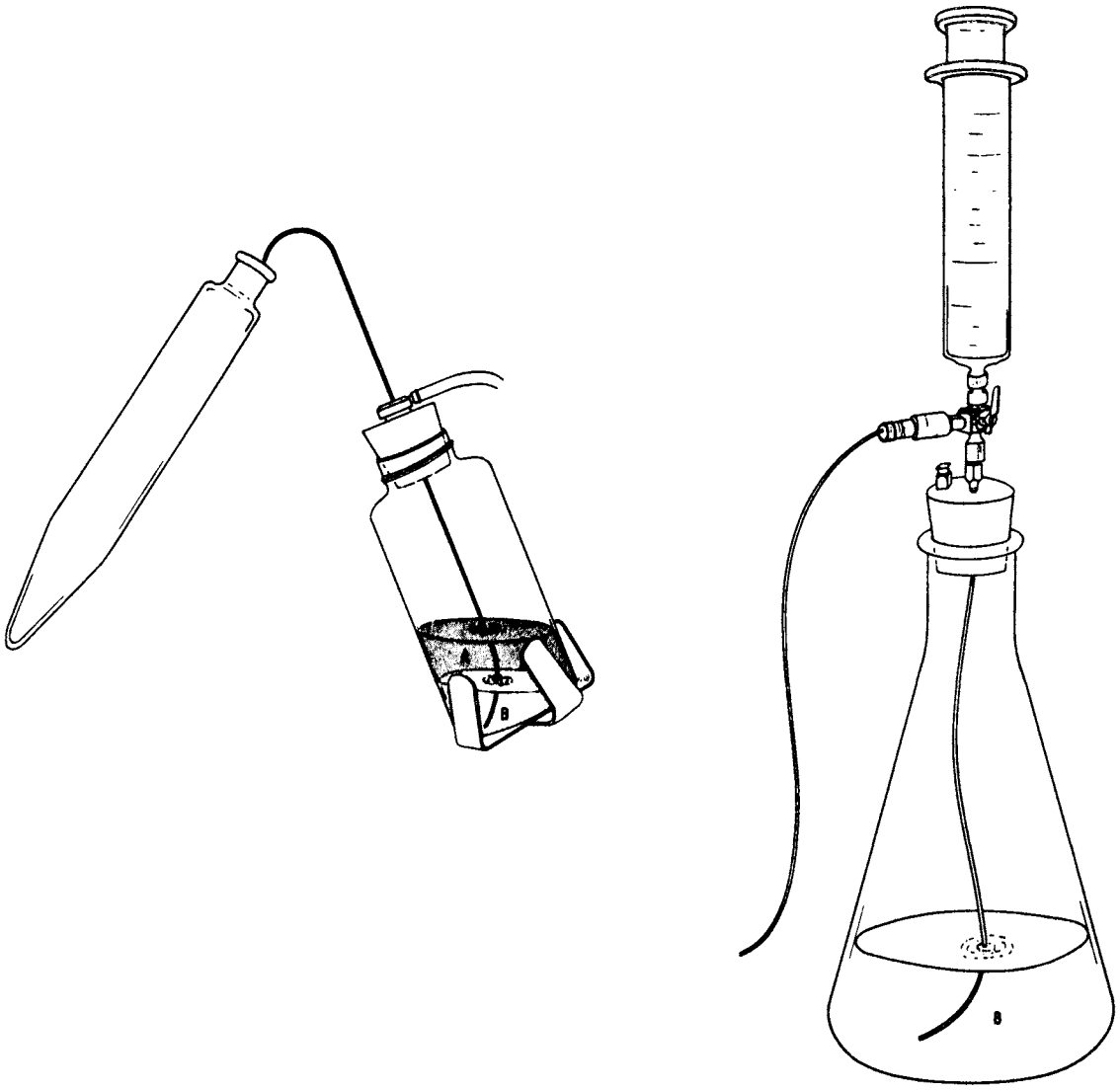

Figure 1. Apparatus for the Extraction of Progesterone from Bovine Plasma; Following Extraction, the Serum Lifter (Left) is Backwashed with Dichloromethane (Right). (A=Plasma; B= Dichloromethane).

on the efficiency of washing the eluter between samples with 10-15 ml hot methanol; 24 analyses showed an average of 169 counts/10 min as compared to the background values of 172 counts/10 min.

(4) Enzymatic Conversion

Progesterone was enzymatically converted to 20β-OH using 10 μg of 20β-hydroxysteroid dehydrogenase in 0.1 ml tris buffer (pH 7.4). Two ml phosphate buffer (pH 5.2) were added followed by incubation at 37C to 40C for 1½ to 2 hr. 20β-OH was extracted from the buffer with 2 ml of benzene; it was held in 0.5 ml benzene prior to acetylation.

(5) Acetylation

Tubes were placed in a desiccator for several hours before and also during acetylation. Monochloroacetic anhydride (Eastman), 5.0 mg/0.5 ml tetrahydrofuran and 0.1 ml pyridine were added to each tube; the reaction was allowed to proceed for approximately 16 hr. Following the addition of 0.5 ml water the acetylation reagents were extracted 3 times with 2 ml benzene. The benzene extract was washed with 1 ml 6N hydrochloric acid to remove acid soluble products formed during the acetylation reaction; this was followed by 2-1 ml water washes. Following concentration by 2, 1 and 0.5 ml benzene, the product was spotted on a TLC plate using 3, 2 and 2 drops of benzene; development was in benzene: ethyl acetate (6:1, v/v). The area corresponding to the 20β-OH chloroacetate standard was placed in a 35 ml tube and eluted with a benzene-water partition system; 3 extractions using 1 ml benzene with 0.5 ml water were carried out. The benzene was pooled in a 15 ml tube and dried under nitrogen. Following the addition of 1 ml of benzene, a 20%

aliquot was taken for scintillation counting and 40% to 70% placed in a 2 ml conical centrifuge tube for GLC.

(6) Gas-Liquid Chromatography

A Barber-Colman gas chromatograph (Selecta series 5000) equipped with an electron capture detector (Model 5120) was used to quantitate the chloroacetate derivative of progesterone. Samples were applied in 5 to 10 µl of toluene. Operating conditions included flash heater, 240C; column bath, 218C; and detector, 210C. A U-shaped glass column (3 ft long, i.d. 4 mm) silanized with a 5% solution of dimethyldichlorosilane in toluene and packed with Gas-Chrom Q (80-100 mesh) coated with 1% XE-60 (Applied Science Laboratories, Inc.) was used to effect separation of the steroids. Pure nitrogen was used as a carrier gas with an outlet velocity of approximately 200 ml/min; a purge gas was not used. The electron capture detector was operated in the D.C. mode and voltage was adjusted to give a standing current 60% of that obtained at the plateau of the voltage input curve. Sensitivity and linearity of detector response to standard chloroacetate derivatives of 20β-OH and testosterone were ascertained each day of data collection on GLC; samples were not run unless the response was linear over the range of expected progesterone concentration. The requirement that the standard curve must pass through zero of the abscissa and ordinate was adhered to very rigidly. The size of peaks was recorded by multiplying one-half the height (measured by a perpendicular line to the base) by the base.

The importance of air drying glassware became apparent during the early phases of the experiment. The inadvertent heat drying of pipettes

and tubes resulted in the appearance of extraneous peaks on the gas chromatograms; these peaks obscured the internal standard (testosterone chloroacetate) as well as the 20β-OH chloroacetate.

(7) Monitoring of Progesterone Loss Through Method

Progesterone loss prior to GLC was determined by measuring the loss of progesterone-7-^{3}H (New England Nuclear) added to the plasma sample before extraction. The average recovery rate prior to GLC for all samples was 50.8%. Adjustments for losses incurred during quantitation by GLC were made by the addition of an internal standard (testosterone chloroacetate) as previously outlined (Horning, Maddock, Anthony and VandenHeuvel, 1963).

(8) Calculation of Progesterone Concentration

The amount of progesterone in 1 ml of plasma was calculated by a formula modified from van der Molen and Groen (1965), to which were added adjustments for (1) anticoagulant dilution of plasma, (2) gas-liquid chromatography aliquot, and (3) plasma volume. The formula used is as follows:

$$\text{Progesterone (m}\mu\text{g/ml plasma)} = R \times C \times U \times A \times .8 \times DF \times X \times P$$

$$R = \frac{\text{counts/10 min progesterone-7-}^{3}\text{H added to plasma sample}}{5 \times \text{counts/10 min in aliquot (20\%) obtained prior to GLC}}$$

$$C = \frac{\text{peak area (cm}^2\text{) of 10 m}\mu\text{g testosterone chloroacetate standard}}{\text{peak area (cm}^2\text{) of 10 m}\mu\text{g 20}\beta\text{-OH chloroacetate standard}}$$

$$U = \frac{\text{peak area (cm}^2\text{) of 20}\beta\text{-OH chloroacetate in sample}}{\text{peak area (cm}^2\text{) of testosterone chloroacetate in sample}}$$

$$A = \text{m}\mu\text{g testosterone chloroacetate added as internal standard}$$

$$0.8 = \frac{\text{molecular weight of progesterone}}{\text{molecular weight of 20}\beta\text{-OH chloroacetate}}$$

$$DF = \frac{(100\text{-hematocrit})\ (\text{blood volume})}{[(100\text{-hematocrit})\ (\text{blood volume})]\text{-}(\text{no. of ml anticoagulant})}$$

$$X = \frac{1}{\text{GLC aliquot}}$$

$$P = \frac{1}{\text{no. of ml of plasma extracted}}$$

A Fortran IV computer program was developed to simplify the handling of data (Appendix F); IBM cards were punched from data sheets as shown in Appendix F. The Fortran IV computer program was set up to handle duplicate samples and calculate both individual and mean values.

(9) Identification of Progesterone

The chemical purity of progesterone isolated from plasma was shown by establishing a constant specific activity of the chloroacetate derivative of 20β-OH following TLC in a number of different solvent systems (Table II). Rf values of unknown and standard 20β-OH chloroacetate were identical in all TLC solvent systems. The nature of the method, i.e., enzymatic conversion of progesterone to 20β-OH and its subsequent acetylation and reisolation on TLC tends to support the identity of the hormone.

The mass spectrum of the unknown compound was determined by a mass spectrometer-gas chromatograph (LKB-9000, prototype). Details of its operation have been reported by Waller (1967) and Ryhage (1967). The finding of a molecular weight of 392 with the presence of an atom of chlorine supports the identity of the compound as 20β-OH chloroacetate. Fig. 2 presents the mass spectrum of the standard 20β-OH chloroacetate and Fig. 3 presents the mass spectrum of the unknown compound.

TABLE II

SPECIFIC RADIOACTIVITY (cpm/mμg) OF 20β-HYDROXYPREGN-4-EN-3-ONE CHLOROACETATE (ISOLATED AS PROGESTERONE FROM PERIPHERAL COW PLASMA) FOLLOWING DEVELOPMENT IN DIFFERENT TLC SYSTEMS

	Purification Step		Rf Value	cpm/mμg
1.	After TLC, benzene:ethyl acetate, 3:1	(1)	0.47	232
2.	After TLC, benzene:ethyl acetate, 2:1 and TLC, benzene:ethyl acetate, 6:1	(2) (3)	0.51 0.36	215
3.	After TLC, toluene:chloroform:methanol:water, 120:60:20:1	(4)	0.64	202
4.	After TLC, benzene:ethyl acetate, 4:1	(5)	0.42	220

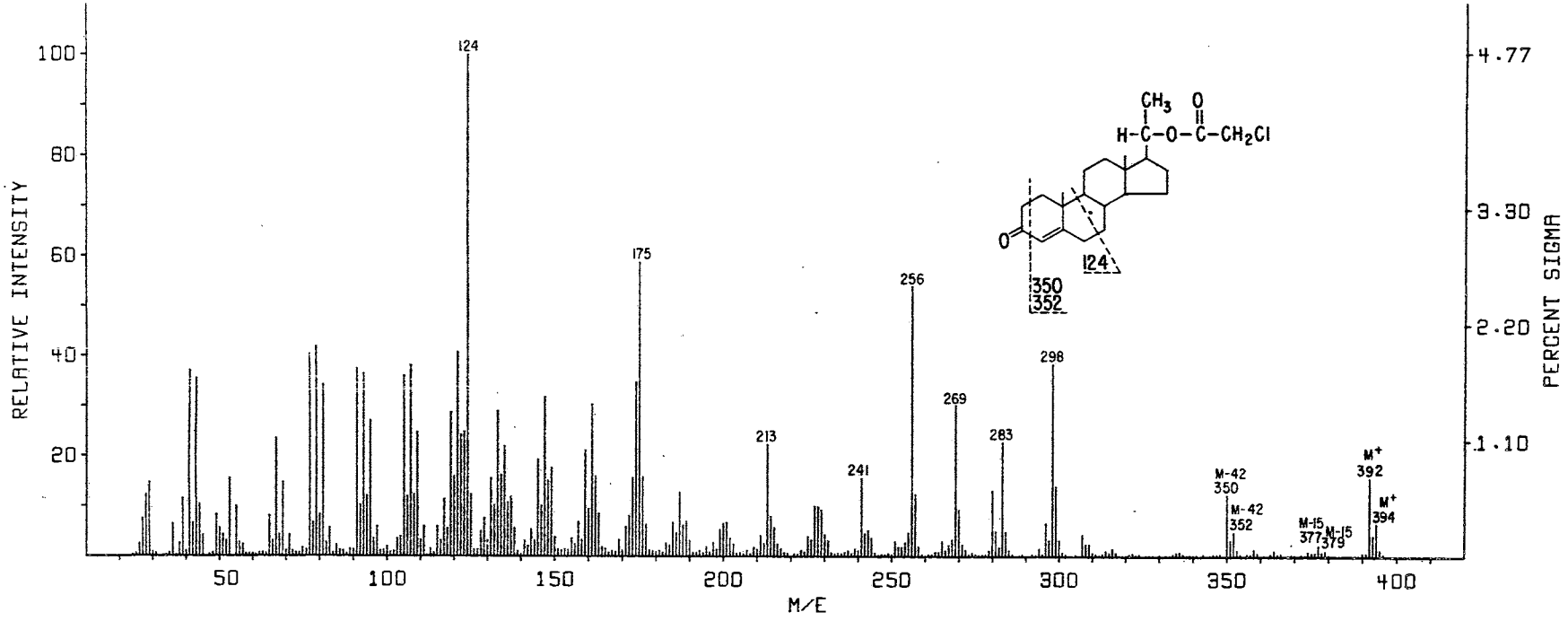

Figure 2. Mass Spectrum of Standard 20β-hydroxypregn-4-en-3-one Monochloroacetate.

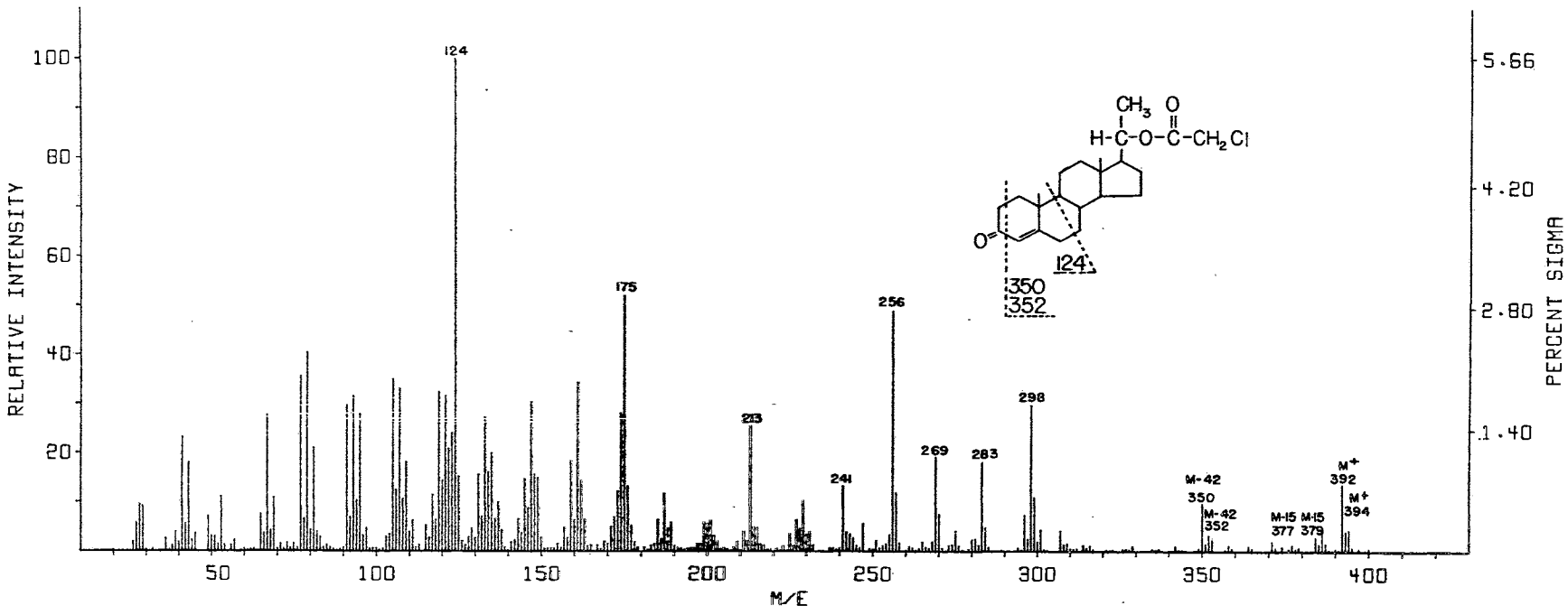

Figure 3. Mass Spectrum of 20β-hydroxypregn-4-en-3-one Monochloroacetate Isolated as Progesterone from the Peripheral Blood of Cows.

CHAPTER IV

RESULTS

Evaluation of Method

(1) Isolation Technique

Dichloromethane was selected as the extraction solvent of choice because it removed a minimal amount of pigment from bovine plasma. Subsequently, dichloromethane proved to be effective regardless of the source of plasma. The problem of emulsion formation between dichloromethane and plasma was partially solved by centrifugation at 7200 g for 10 min at 10C; recovery rates, however, were still minimal. The increase in recovery rate (% $\pm$ S. D.) (77.0 $\pm$ 7.6 to 96.5 $\pm$ 6.4) (Table III) observed when the plasma extraction residue was saponified rather than the plasma was due mainly to decreased emulsion formation and thus greater recovery of solvent. Most of the yellow pigments found in bovine plasma moved readily from the solvent into the sodium hydroxide phase upon shaking.

Saponification of the solvent residue resulted in a much cleaner sample; any plasma carried over during extraction could be removed in the sodium hydroxide phase. The isolation procedure was also aided by saponification in ether rather than dichloromethane; it was more efficient to remove the lower layer (sodium hydroxide) from a conical tube when ether was used than the upper layer (sodium hydroxide) when dichloromethane was used (the densities of ether and dichloromethane being .708 and 1.32

respectively). Emulsion formation was less during saponification with ether as the solvent as opposed to dichloromethane.

TABLE III

INFLUENCE OF SAPONIFICATION UPON EFFICIENCY OF EXTRACTION OF PROGESTERONE FROM BOVINE PLASMA

Addition of NaOH BEFORE Extraction			Addition of NaOH AFTER Extraction		
1.	[a]2X (160 mls) (4)	[b]69.5 ± 1.3	1.	2X (160 mls) (4)	92.5 ± 8.5
2.	3X (180 mls) (2)	77.6 ± 5.1	2.	3X (180 mls) (4)	99.5 ± 4.5
3.	4X (240 mls) (2)	86.6 ± 4.8	3.	4X (240 mls) (3)	100.4 ± 1.7
4.	5X (200 mls) (4)	75.8 ± 6.6	4.	5X (200 mls) (4)	94.4 ± 4.4
5.	6X (240 mls) (4)	80.6 ± 8.4	5.	6X (240 mls) (5)	96.7 ± 4.3
	$\bar{x}$	77.0 ± 7.6		$\bar{x}$	96.5 ± 6.4

[a]Number of extractions and total amount of solvent.

[b]Percent recovery based on recovery of progesterone-7-^{3}H added to the plasma prior to extraction.

The effect of solvent volume on steroid recovery from plasma was determined (Table IV). Seven volumes of solvent distributed among three extractions resulted in about 96% of the steroid being recovered as measured by liquid scintillation counting of progesterone-7-^{3}H. These data support a partition coefficient of about 0.67. The final extraction scheme used was 2.5, 2.5 and 2 vol of solvent.

TABLE IV

EFFECT OF SOLVENT VOLUME (DICHLOROMETHANE) ON RECOVERY RATE OF PROGESTERONE-7-^{3}H ADDED TO 20 ml BOVINE PLASMA (PROCEDURE INCLUDED SAPONIFICATION AND TWO WATER WASHINGS BEFORE ALIQUOTS FOR RECOVERY WERE OBTAINED)

	Amt. of Solvent (ml) & No. of Volumes per Extraction	Total No. of Volumes	Percent Recovery ± S. D.
Series #1	60 : 60 : 60 3 : 3 : 3	9	99.7 ± 2.2 (12)
Series #2	60 : 60 : 40 3 : 3 : 2	8	96.7 ± 5.3 (60)
Series #3	50 : 50 : 50 2½ : 2½ ; 2½	7½	95.2 ± 2.9 (15)
Series #4	50 : 50 : 40 2½ : 2½ : 2½	7	96.1 ± 3.5 (9)
Series #5	40 : 40 : 40 2 : 2 : 2	6	92.6 ± 2.4 (12)
Series #6	60 : 60 3 : 3	6	89.2 ± 5.1 (66)

As progesterone was converted to 20β-OH later in the method, it was important that any endogenous 20β-OH be removed at the first TLC step. Several solvent systems were employed to find one that would give maximum TLC separation of progesterone from 20β-OH as well as from plasma pigments found in the cow. The benzene:ethyl acetate system (2:1, v/v) used by van der Molen and Groen (1965) did not give adequate separation of progesterone from pigments although progesterone and 20β-OH were well separated; benzene:ethyl acetate (3:1, v/v) gave better separation of

progesterone and pigments. The Rf values for progesterone, 20β-OH and pigments in various solvent systems are shown in Table V. It was found the distance between pigments and progesterone increased with an increasing benzene:ethyl acetate ratio while separation of progesterone and 20β-OH remained quite constant.

TABLE V

EFFECT OF BENZENE:ETHYL ACETATE RATIO ON SEPARATION OF PROGESTERONE, 20β-HYDROXYPREGN-4-EN-3-ONE (20β-OH) AND PLASMA PIGMENTS BY TLC

	Benzene:ethyl Acetate (v/v)							
	2:1		2.5:1		3:1		3.5:1	
Substance	Rf	ΔRf	Rf	ΔRf	Rf	ΔRf	Rf	ΔRf
20β-OH	0.29		0.25		0.22		0.19	
		0.19		0.17		0.17		0.16
Progesterone	0.48		0.42		0.39		0.35	
		0.21		0.22		0.30		0.32
Pigments	0.69				0.69		0.67	
			0.64					

The lower limit of elution of silica gel for progesterone was half the distance between the progesterone and 20β-OH standards; an area equal to this was also eluted above the progesterone standard. On analysis of the distribution of progesterone-7-^{3}H on TLC plates following development,

it was found about 10% of the sample was immediately above the area normally eluted for progesterone (Table VI). By making a 0.5 cm asymmetrical cut toward the solvent front, progesterone recovery (% ± S.D.) through extraction and saponification increased from 77.4 ± 5.9 (n=27) to 87.7 ± 6.2 (n=18).

TABLE VI

INFLUENCE OF SOLVENT, TUBE SIZE AND AREA OF ELUTION ON PERCENT RECOVERY OF PROGESTERONE-7-^{3}H BY TLC (BENZENE:ETHYL ACETATE, 3:1, V/V)

	35 ml Tube			12 ml Tube		
Area	[a]Water	[b]Benzene	[c]Methanol	Water	Benzene	Methanol
A	[d]11.0	8.8	10.1	10.1	9.8	9.3
B	81.4	81.0	91.1	73.1	85.7	94.5
C	[e]	6.9	[e]	[e]	[e]	[e]
D	[e]	[e]	[e]	[e]	[e]	[e]
Total Recovery	92.4	96.7	101.2	83.2	95.5	103.8

[a]Water added to silica gel before benzene prior to extraction.

[b]Benzene added to silica gel before water prior to extraction.

[c]Methanol (boiling) elution of silica gel only.

[d]Percent recovery of progesterone-7-^{3}H spotted at origin.

[e]Percent recovery of progesterone-7-^{3}H was less than 1%.

A=3 cm area immediately above progesterone (B).

B=3 cm area centered on progesterone standard.

C=3 cm area centered on 20β-OH (immediately beneath progesterone).

D=3 cm area immediately beneath C including the origin.

A check was made of duplicate isotope standards routinely set up at the same time isotope was added to plasma. That the isotope pipetting contribution to experimental error was neglible was shown by a comparison of 43 duplicates with an average difference of 0.8%.

(2) Enzymatic Conversion and Acetylation

The efficiency of enzymatic conversion of progesterone-7-^{3}H to 20β-OH was determined by isolating both steroids on TLC following conversion (Table VII). The 20β-OH spot on TLC contained 81.5 ± 4.7% (n=8) of the isotope originally added as progesterone-7-^{3}H whereas the progesterone area contained 2.8 ± 0.9% (n=8). This is a conservative estimate of the amount of 20β-OH present following enzymatic conversion as the steroid is normally acetylated without an intervening TLC step. The percent distribution of the isotope recovered in this experiment was 96.6 ± 1.0% (n=8) in the 20β-OH area as compared to 3.4 ± 1.1% (n=8) in the progesterone area on TLC. This suggests enzymatic conversion was quite efficient.

An attempt was made to determine the efficiency of the acetylation step whereby 20β-OH-4-^{14}C was acetylated by the usual procedure; 88.8 ± 3.4% (n=8) was found in the 20β-OH chloroacetate spot on TLC (Table VIII). The recovery rate of 69.2 ± 5.6% (n=51) found for the combined steps of enzymatic conversion, acetylation and TLC agrees well with the two combined individual experiments. Data on the effect of time on enzymatic conversion and acetylation suggested the use of a 1½ to 2 hr (vs 1 hr) enzymatic conversion period followed by a minimum of 12 hr of acetylation (Table IX).

TABLE VII

EFFICIENCY OF ENZYMATIC CONVERSION OF [a]PROGESTERONE TO 20β-HYDROXYPREGN-4-EN-3-ONE

	Percent of Isotope Originally Added		Percent of Isotope Recovered	
Tube No.	Progesterone	20β-OH	Progesterone	20β-OH
1	[b]2.3	84.9	[c]2.6	97.4
2	3.5	79.1	4.3	95.7
3	2.3	75.3	3.0	97.0
4	3.1	87.5	3.4	96.6
5	4.7	77.7	5.7	94.3
6	2.2	87.0	2.5	97.5
7	2.1	77.6	2.7	97.3
8	2.5	82.5	3.0	97.0
$\bar{x}$	2.8 ± 0.9	81.5 ± 4.7	3.4 ± 1.1	96.6 ± 1.0

[a]200 mμg of unlabeled progesterone added prior to enzymatic conversion.

[b]Percent recovery based on progesterone-7-^{3}H originally added.

[c]Percent of total isotope recovered.

TABLE VIII

EFFICIENCY OF ACETYLATION OF [a]20β-HYDROXYPREGN-4-EN-3-ONE BY CHLOROACETIC ANHYDRIDE

Tube No.	1	2	3	4	5	6	7	8	$\bar{x} \pm$ S.D.
Percent Recovery	[b]81.2	89.7	88.9	92.1	90.7	91.1	86.9	90.0	88.8 ± 3.4

[a]200 mμg of unlabeled 20β-hydroxypregn-4-en-3-one added prior to acetylation.

[b]Percent recovery based on 20β-hydroxypregn-4-en-3-one-^{14}C chloroacetate (originally added as 20β-hydroxypregn-4-en-3-one-^{14}C).

TABLE IX

EFFECT OF TIME ON EFFICIENCY (% ± S.D.) OF ENZYMATIC CONVERSION ([a]PROGESTERONE-7-^{3}H TO 20β-HYDROXYPREGN-4-EN-3-ONE-7-^{3}H) AND ACETYLATION (20β-HYDROXYPREGN-4-EN-3-ONE-7-^{3}H TO ITS CHLOROACETATE DERIVATIVE)

	Enzymatic Conversion (hr)		
Acetylation (hr)	2	1½	1
16	[b]69.2 ± 5.6 (51)	70.7 ± 4.1 (5)	66.9 ± 9.4 (4)
12	69.3 ± 6.8 (10)	70.2 ± 6.8 (6)	61.1 ± 3.8 (4)

[a]200 mμg unlabeled progesterone added prior to enzymatic conversion.

[b]Percent recovery based on 20β-hydroxypregn-4-en-3-one-7-^{3}H chloroacetate (originally added as progesterone-7-^{3}H).

The addition of EDTA to the buffer during enzymatic conversion increased the recovery rate of progesterone for the combined steps of

enzymatic conversion, acetylation and TLC from 62.4 ± 6.2 (n=7) to 73.3 ± 5.1 (n=5). The data previously mentioned for conversion and acetylation (69.2 ± 5.6%, n=51) included the use of EDTA. These data probably reflect the ability of EDTA to chelate enzyme inhibiting metal ions present in the incubating medium.

The importance of water as a deterent to effective acetylation became apparent during this experiment which was conducted over several months. Recovery rates of 50% to 60% through the method would drop at times to 30% to 40% when proper attention was not given to the desiccant used to dry the chloroacetic anhydride crystals. Replacement of desiccant usually was followed in several days by a definite increase in recovery percentages.

As reported by van der Molen and Groen (1965), the recovery (% ± S.D.) of 20β-OH chloroacetate was found dependent upon whether benzene (68.3 ± 7.9, n=26) or water (60.4 ± 4.0, n=12) was added first when extracting the chloroacetate derivative from silica gel (these recoveries included enzymatic conversion, acetylation and TLC). It was found the extraction tube size also had an effect on recovery; rates were higher when 35 ml tubes were used when compared to 12 ml tubes (Table X). It appeared the greater surface area of the 35 ml tube allowed more effective contact between steroid and solvent.

(3) Gas-Liquid Chromatography

Figs. 4 and 5 presents typical GLC tracings of progesterone (in the form of 20β-OH chloroacetate) obtained from bovine plasma during the time just prior to and during anticipated luteal regression. As can be seen, the addition of progesterone-7-^{3}H to the water blank did not

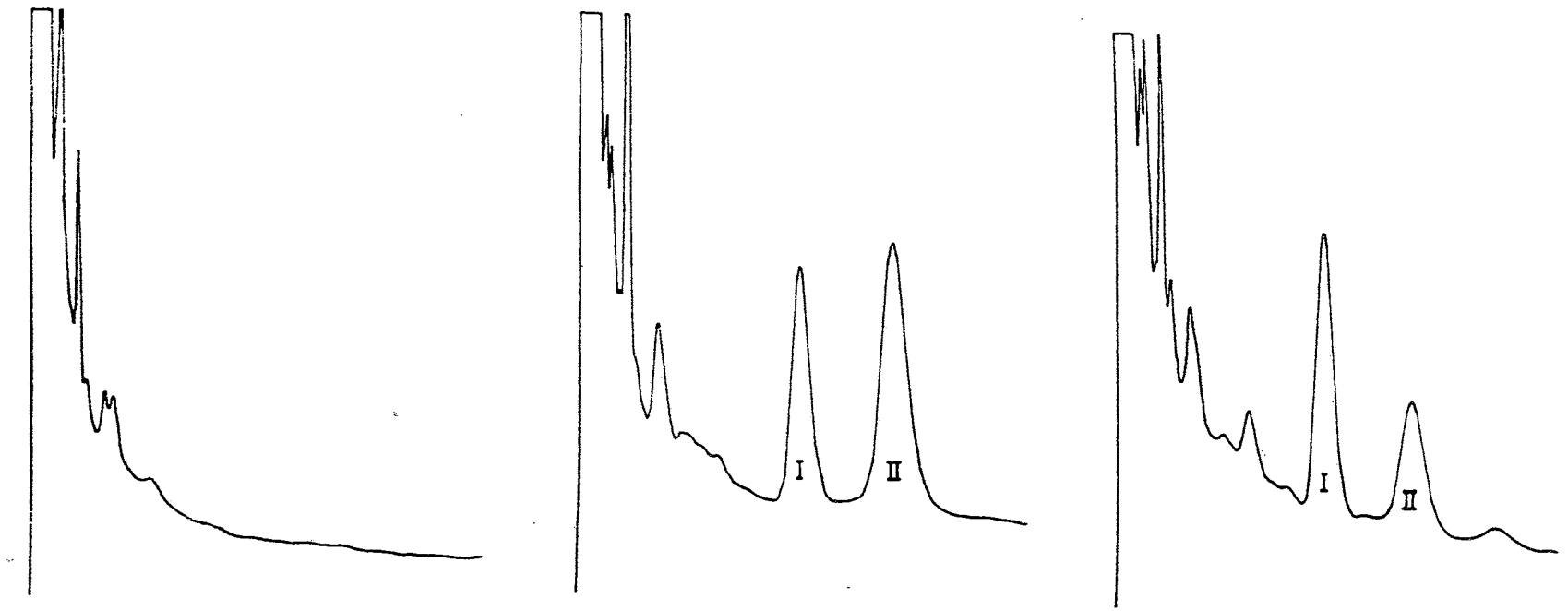

Figure 4. (Left) GLC Tracing of a Water Blank (Plus Progesterone-^{3}H) Carried Through the Method.

(Middle) GLC Tracing of Progesterone from 2.4 ml Plasma, Day 17 of the Estrous Cycle (Cow No. 07). I = Testosterone Chloroacetate; II = 20β-hydroxypregn-4-en-3-one Chloroacetate (Progesterone).

(Right) GLC Tracing of Progesterone from 3.2 ml Plasma, Day 18 of the Estrous Cycle (Cow No. 07). I = Testosterone Chloroacetate; II = 20β-hydroxypregn-4-en-3-one Chloroacetate (Progesterone).

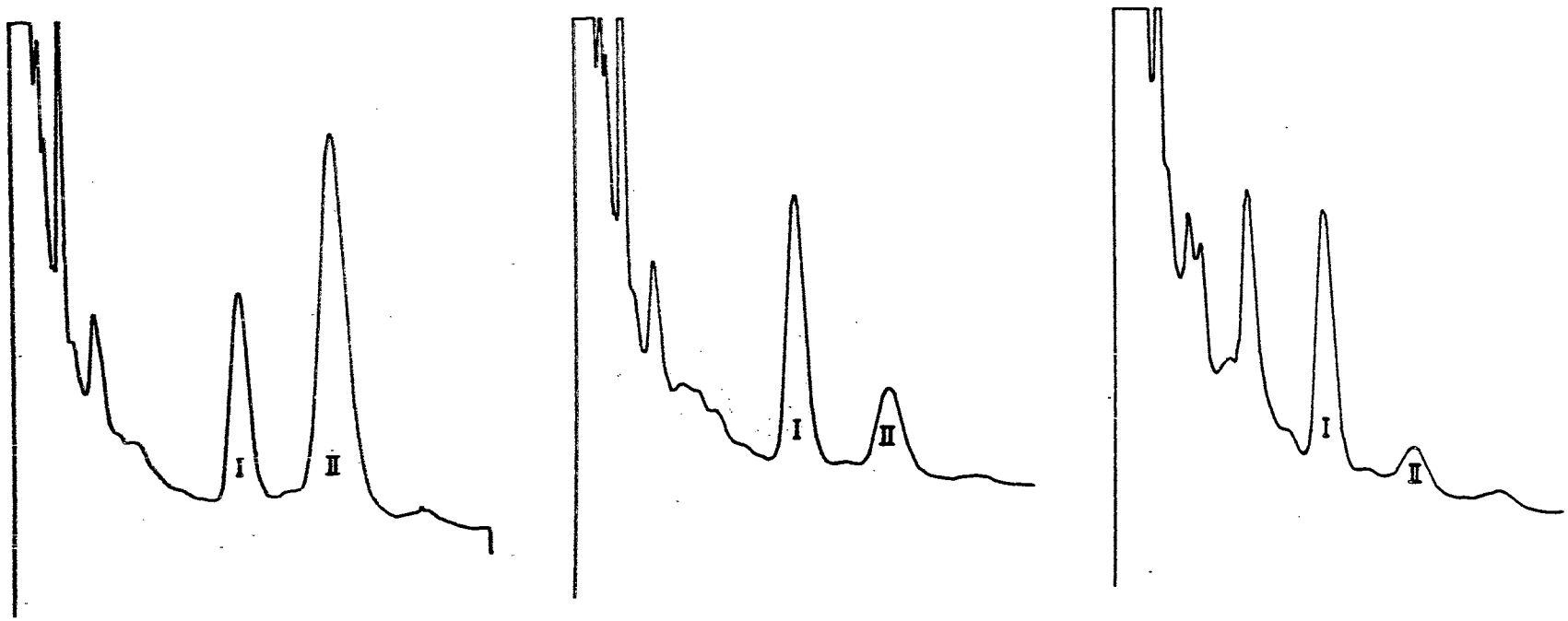

Figure 5. (Left) GLC Tracing of Progesterone from 2.5 ml Plasma, Day 17 of the Estrous Cycle (Cow No. 24). I = Testosterone Chloroacetate; II = 20β-hydroxypregn-4-en-3-one Chloroacetate (Progesterone).

(Middle) GLC Tracing of Progesterone from 3.2 ml Plasma, Day 18 of the Estrous Cycle (Cow No. 24). I = Testosterone Chloroacetate; II = 20β-hydroxypregn-4-en-3-one Chloroacetate (Progesterone).

(Right) GLC Tracing of Progesterone from 3.0 ml Plasma, Day 19 of the Estrous Cycle (Cow No. 24). I = Testosterone Chloroacetate; II = 20β-hydroxypregn-4-en-3-one Chloroacetate (Progesterone).

produce measurable peaks (Fig. 4); the mass of progesterone-7-^{3}H added was approximately 0.2 mμg.

TABLE X

EFFECT OF SOLVENT ADDITION AND TUBE SIZE ON RECOVERY RATE (% ± S.D.) OF [a]20β-HYDROXYPREGN-4-EN-3-ONE-7-^{3}H CHLOROACETATE FROM SILICA GEL (TLC)

Tube Size	Solvent [b]Benzene (first)	[c]Water (first)
35 ml	[d]71.5 ± 6.2 (3)	67.8 ± 2.3 (4)
12 ml	68.9 ± 4.9 (4)	59.2 ± 1.5 (4)

[a]200 mμg unlabeled progesterone added prior to enzymatic conversion and acetylation.

[b]Benzene added to silica gel before water prior to extraction.

[c]Water added to silica gel before benzene prior to extraction.

[d]Percent recovery based on 20β-hydroxypregn-4-en-3-one-7-^{3}H chloroacetate (originally added as progesterone-7-^{3}H) with isolation by TLC.

While detector sensitivity varied to some extent from day to day, the ratio between standard 20β-OH chloroacetate and standard testosterone chloroacetate (cm^2) peaks remained quite constant during the experiment conducted over several months. The ratio of 20β-OH chloroacetate/testosterone chloroacetate was 1.23 ± .06 (S.D.) (n=69) which compares favorably to the ratio of 1.15 found by van der Molen and Groen (1965).

The type of GLC column used has considerable influence on this ratio; a 1% XE-60 column was used in the current study. The sensitivity (cm^2 $\pm$ S.D.) averaged 8.8 $\pm$ 1.0 for 10 mμg 20β-OH chloroacetate and 7.1 $\pm$ 1.1 for 10 mμg testosterone chloroacetate (n=69) over several months. The level of sensitivity of the electron capture detector was kept considerably below the maximal limit.

(4) Recoveries

About 96% of the progesterone-7-^{3}H was recovered following extraction of plasma and saponification and water washing of the extract. After isolation of the steroid by TLC, 87.7% of the hormone was still present as indicated by isotope recovery. Recovery of progesterone through the steps of enzymatic conversion, acetylation and TLC averaged 69.2% thus enabling 60% recovery through the method to GLC.

A few duplicate samples had widely varying recovery rates. However, after adjustment for isotope loss was made, agreement between duplicates was very good. This emphasizes that a high recovery rate through the procedure is not imperative as long as an adequate isotope count/background count ratio is maintained. It is important if more than one GLC determination is desired.

(5) Precision and Accuracy

From Table XI it can be seen that precision of determination of quantities of progesterone ranging from 10 mμg to 250 mμg is very good, i.e., about 3%. The accuracy results indicate a tendency to overestimate the amount of progesterone present by approximately 5% with the exception of the 10 mμg level where an overestimation of 13.5% was made.

Difficulty in pipetting the progesterone-7-^{3}H may have resulted in a smaller amount of labeled steroid in the samples when compared to the standards. This would tend to cause an over-adjustment to be made for loss of progesterone-7-^{3}H through the method.

TABLE XI

DETERMINATION OF PROGESTERONE ADDED TO WATER
(CONTAINING 0.75% BOVINE SERUM ALBUMIN)

Amt. added	10 mμg	25 mμg	100 mμg	250 mμg
Recovered				
1	11.0	26.3	105.2	267.5
2	11.2	26.0	108.4	249.9
3	11.6	27.1	102.6	253.3
4	11.6	25.4	106.8	264.5
$\bar{x}$	11.35	26.2	105.75	258.8
S.D.	.3	.7	2.5	8.5
Precision %	2.6%	2.7%	2.4%	3.3%

The greater discrepancy at the 10 mμg level when compared to the other levels was probably due to the fact that larger amounts of the sample were injected into the gas chromatograph. The larger solvent fronts resulted in a sloping baseline from which the steroids were measured. The sloping baseline tends to distort the accuracy of quantitation

when compared to that obtained with a flat baseline.

Progesterone Levels in Plasma

The data for the seven estrous cycles examined in six cows are shown in Table XII. Five cycles were of twenty-one days duration while the other two were twenty-two and twenty-three days in length. The data were combined in several ways to show: (1) all seven cycles regardless of cycle length, (2) cycles of twenty-one days duration and (3) cycles of twenty-one days duration arranged to illustrate the sharp decline in progesterone that occurred toward the end of the cycle.

TABLE XII

SUMMARY OF DATA ON COWS USED IN PROGESTERONE EXPERIMENT

Cow (No.)	Cycle Length (Days)	Peak Plasma Progesterone (mμg/ml plasma)/day of cycle	Day of Plasma Progesterone Decline
07	21	6.7/11	18
11	21	9.8/20	21
17	21	8.9/15	19
24	21	10.2/16	18
40	21	6.1/13	21
	22	7.6/17	21
46	23	10.2/14	20

[a]All animals used in this study were Holstein Friesian cows except 07 which was a Holstein-shorthorn crossbreed.

The composite data for all cycles (Fig. 6) show progesterone levels varied from less than 0.5 mμg/ml plasma at estrus to approximately 7 mμg/ml plasma (range of 6.1-10.2 mμg) at peak luteal function. The decline noted on day 18 continued until the next estrous cycle; the first significant increase in the next cycle occurred on day 4. The large standard error of the mean (SEM) of progesterone levels seen on days 18, 19 and 20 of the cycle is indicative of the variation that existed among the cows during this period of the cycle.

Data were combined from four cows with twenty-one day cycles in an attempt to present a more accurate description of the typical cow by eliminating some of the variation seen toward the end of the cycle (Fig. 7). Progesterone levels increased rapidly from day 3 to day 8 with a much slower rate of increase from day 8 through day 17. The large SEM recorded at day 5 is indicative of the large variation of progesterone levels observed in individual cows and may be a reflection of individual variation of initial CL development following ovulation. It is again evident from the SEM that days 18 through 20 were extremely variable. This can be seen in that progesterone decreased more than 50% from the peak levels at day 18 in two cows, day 19 in one cow and day 21 in the remaining cow. This is interpreted to be the result of individual variation among cows as to time of cessation of CL function.

Because of the wide variation in the decline of CL function that existed among cows another method for presenting the composite data was used (Fig. 8). The data from each cow (21 day cycles only) were coordinated by designating the first day progesterone fell 50% as day 1. The decrease in progesterone in the peripheral plasma that occurred toward the end of the cycle is evident and is representative of what

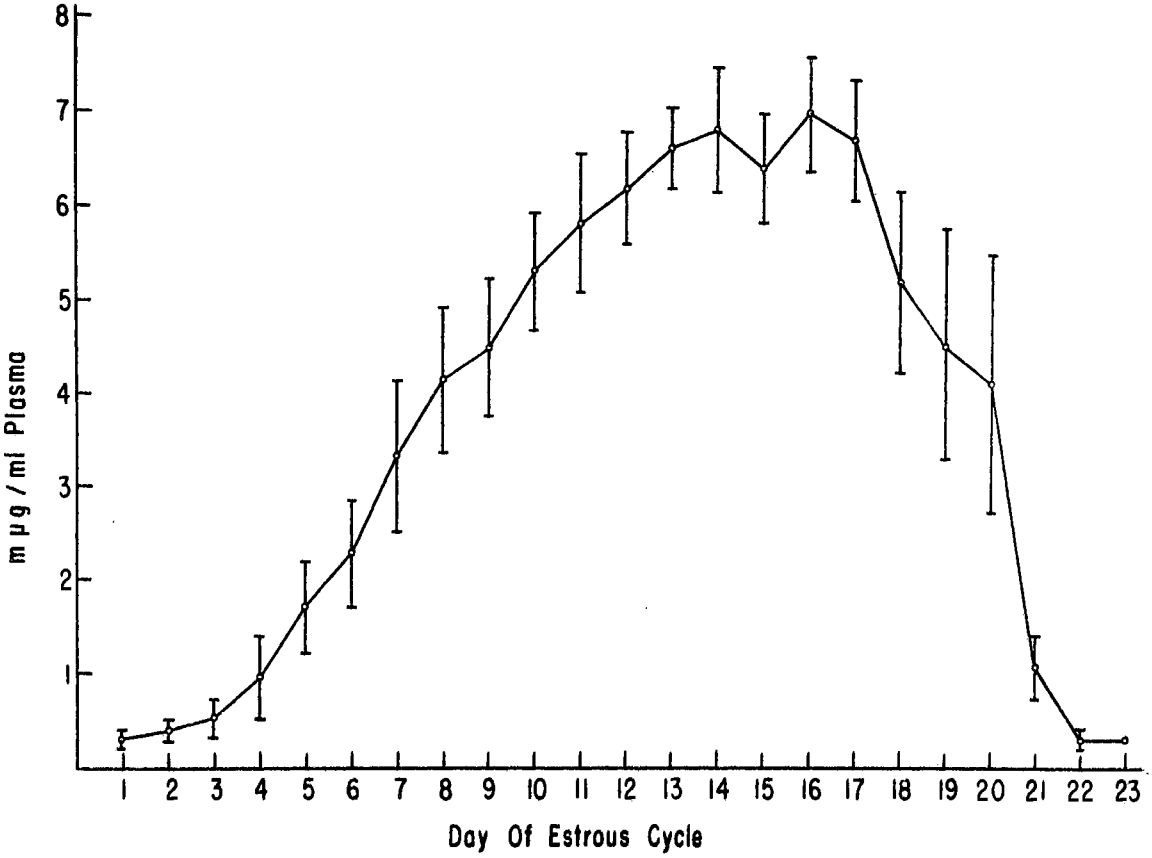

Figure 6. Progesterone Concentration in Jugular Venous Plasma During the Bovine Estrous Cycle (Estrus=Day 1). The Data Represents the Composite of Seven Cycles (Six Cows) with a Duration of Twenty-one Days (Five), Twenty-two Days (One) and Twenty-three Days (One). Vertical Bars Represent the Standard Error of the Mean.

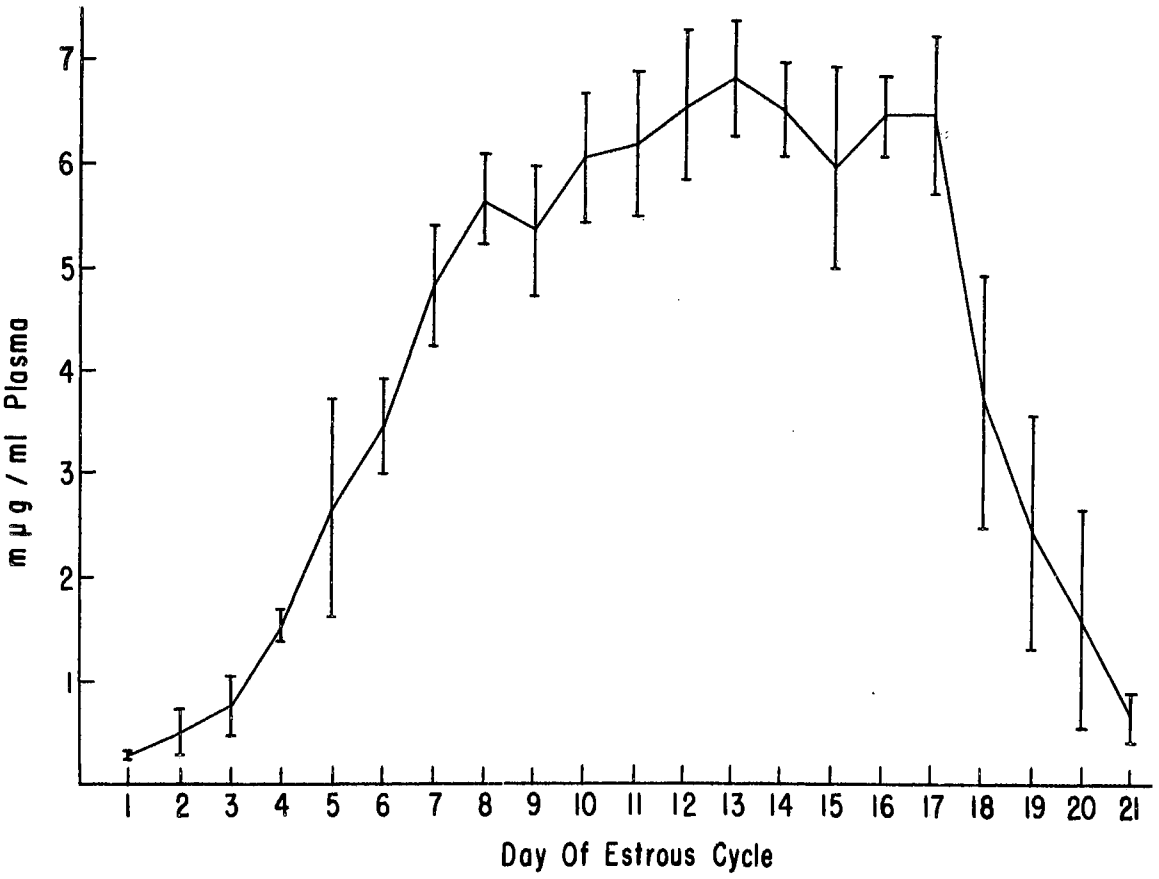

Figure 7. Progesterone Concentration in Jugular Venous Plasma from Four Cows with Twenty-one Day Estrous Cycles (Estrus=Day 1). Vertical Bars Represent the Standard Error of the Mean.

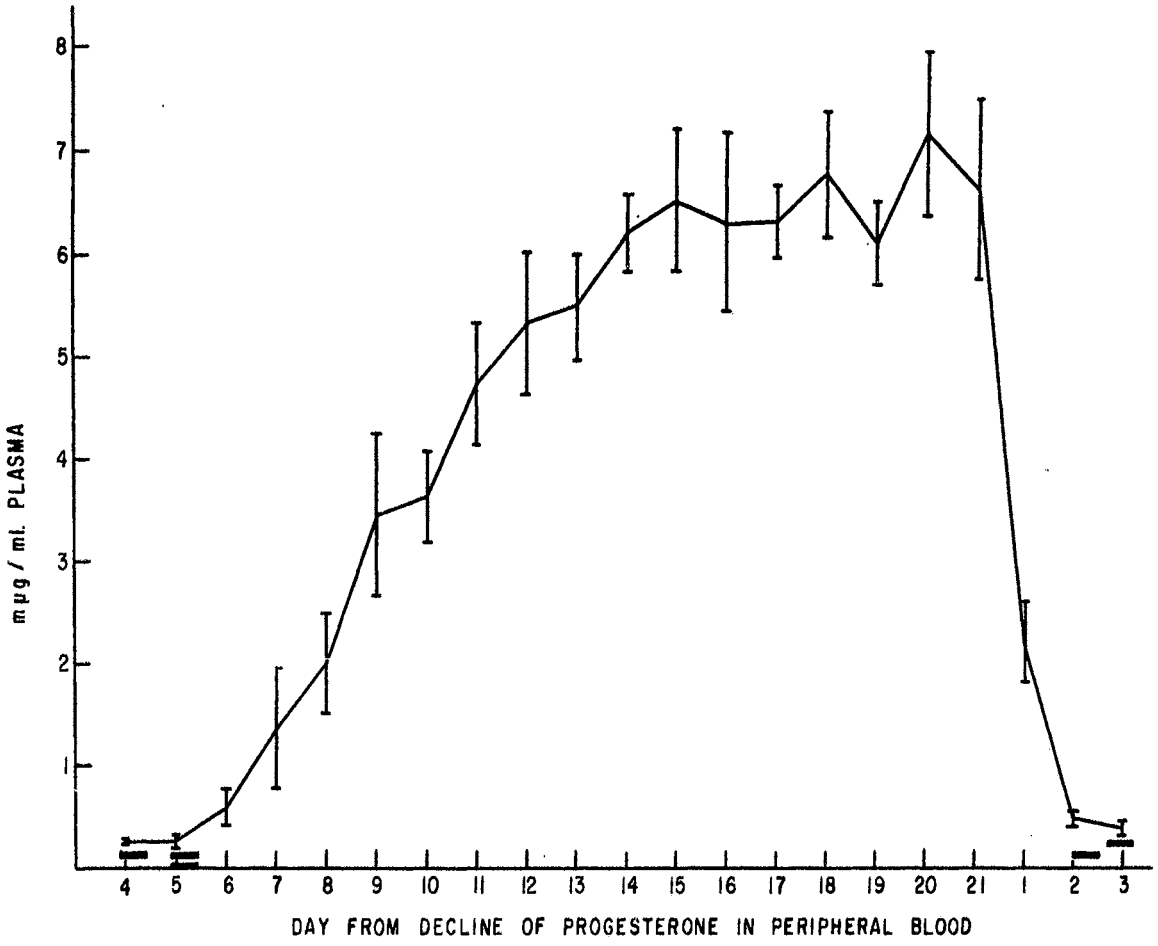

Figure 8. Progesterone Concentration in Jugular Venous Plasma from Five Cows with Twenty-one Day Estrous Cycles. Data from Cows Were Adjusted So That Day 1 Represented the First Day at the End of Luteal Function in Which a Decrease in Progesterone Was Greater than 50%. Vertical Bars Represent the Standard Error of the Mean; Horizontal Bars Indicate the Time of Estrus for Individual Cows.

happened in a typical cow. Another pertinent point is the timing of estrus following the fall in progesterone; animals varied in the occurrence of estrus from two to five days following the decline. That Fig. 8 more closely approximates conditions in a typical cow can be seen by inspecting data for individual cows seen in Figs. 9 and 10. Curves for individual cows in general all showed the same cyclic pattern with a precipitous fall in progesterone one to several days preceding estrus.

Typical gas chromatograms are presented in Figs. 4 and 5. The two main points of interest are (1) progesterone-7-^{3}H added to water blanks did not produce a measurable peak (the added mass was calculated to be 0.2 mμg) and (2) the decline in progesterone concentration in plasma was precipitous toward the end of the estrous cycle.

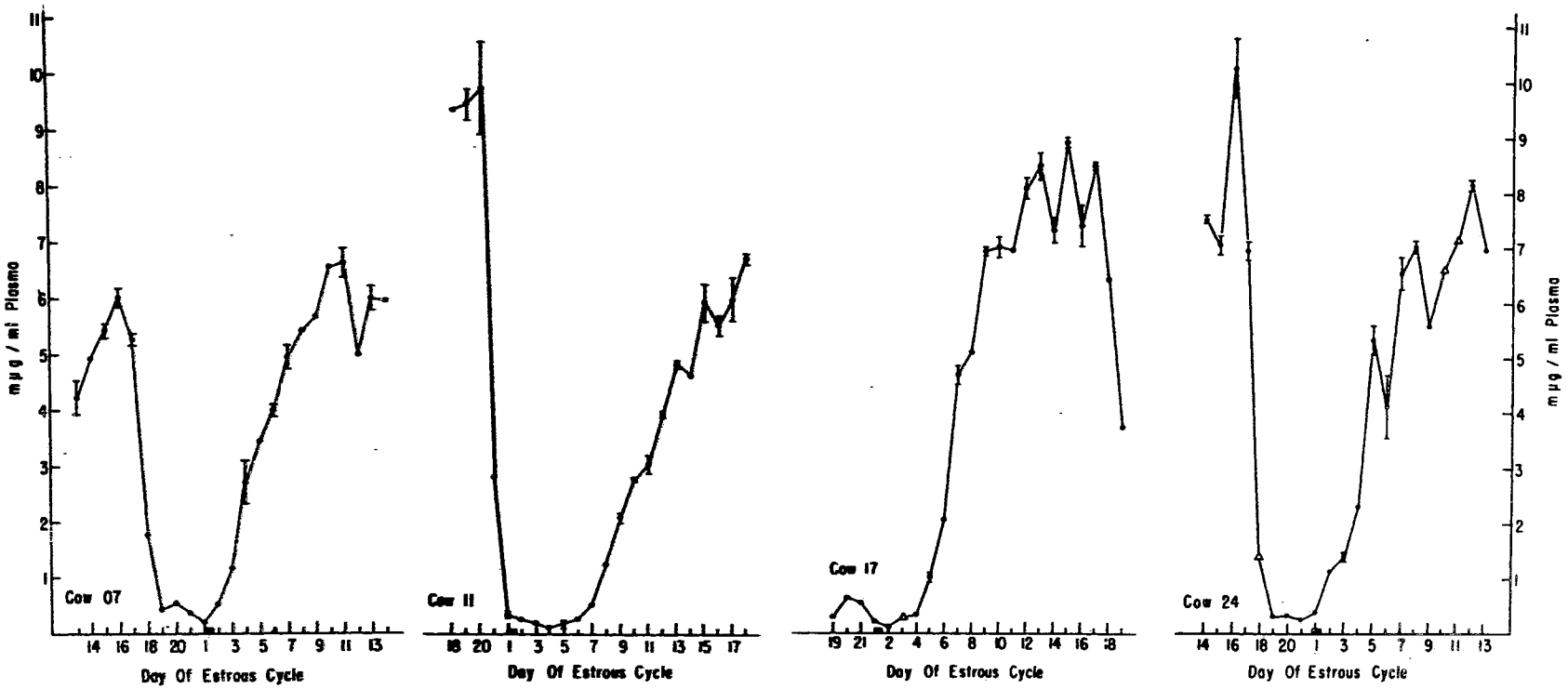

Figure 9. Progesterone Concentration in Jugular Venous Plasma of Individual Cows During the Bovine Estrous Cycle (Estrus=Day 1). Vertical Bars Indicate the Range of Duplicate Determinations.

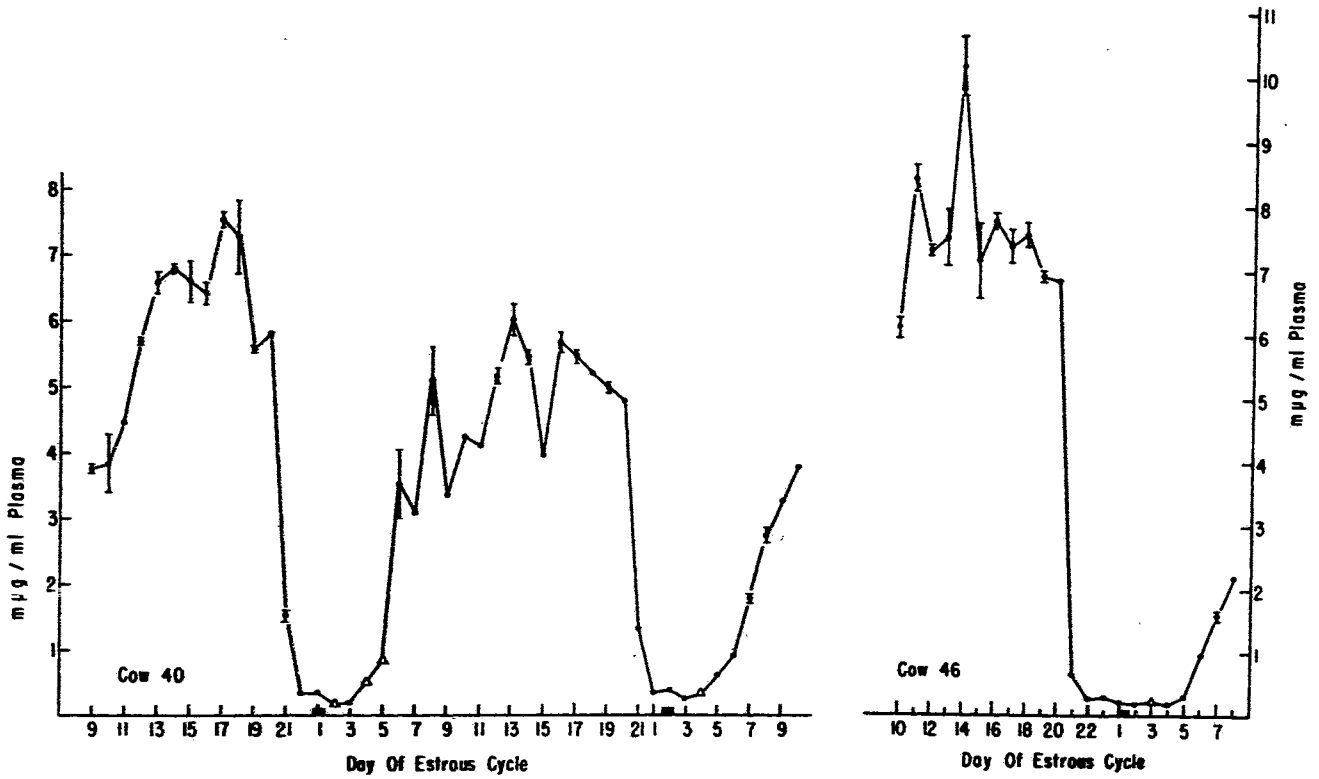

Figure 10. Progesterone Concentration in Jugular Venous Plasma of Individual Cows During the Bovine Estrous Cycle (Estrus=Day 1). Vertical Bars Indicate the Range of Duplicate Determinations.

CHAPTER V

DISCUSSION

Method

Initial progesterone purification procedures are complicated in both the cow and the horse by the presence of yellow pigments especially when animals have access to fresh green feeds (Palmer, 1916). These pigments, mainly carotenes, are not present in other species such as man, dog, pig and ewe. The partition of the plasma extract in a 70% methanol:light petroleum or petroleum ether system has been a particularly effective method for removing lipids and pigments from plasma extraction residues. Other preliminary isolation procedures such as column and paper chromatography have been effective in removing materials interfering in the assays; however, both the partition and the chromatography methods have proven laborious to perform. Consequently, an attempt was made to by-pass the use of the partition system and/or column and paper chromatography by the use of TLC. It was found, however, that the amount of pigment extracted from alkalinized plasma by ether was such that samples could not easily be spotted on TLC plates. This was resolved, in part, by using dichloromethane which removed less pigment from plasma when compared to ether.

In the current study, progesterone-7-^{3}H addition to the plasma was followed by a 30 minute equilibration period before extraction; the amount of progesterone added was approximately 0.2 mμg. The assumption was

made that the labeled progesterone was bound in the same manner to plasma proteins as was the endogenous hormone. The recovery of about 96% of the labeled progesterone by the extraction procedure used herein, suggests that protein-binding of progesterone in plasma did not interfere with the recovery of progesterone.

Slaunwhite and Sandberg (1959) concluded albumin was responsible for binding most of the non-corticosteroids. Sandberg, Slaunwhite and Antoniades (1957) found the binding of progesterone to plasma proteins was relatively weak and release could be effected easily with organic solvents. Eik-Nes, Schellman, Lumry and Samuels (1955) studied the protein-binding of the non-aromatic steroid hormones and found the binding was strongest with estrogens and weakest with corticosteroids while progesterone and testosterone were intermediate.

Seven volumes of dichloromethane distributed over three extractions were used to remove progesterone from plasma. As shown in Table IV, the use of three volumes for each of two extractions is sufficient to remove approximately 90% of the isotope; this approach should be sufficient for most experiments. Brownie et al. (1964) found ether removed testosterone from alkaline plasma much more effectively than did dichloromethane; this was due, at least in part, to emulsion formation. This problem was resolved in this study by centrifugation at 7200 g for 10 minutes at 10C.

The saponification of the solvent rather than the plasma was very effective in removing lipids and pigments from the sample with a minimal loss of isotope. Allen (1932) originally suggested the removal of fatty acids from ethereal extracts of swine corpora lutea by the use of alkali (sodium hydroxide). Hudson et al. (1963) saponified the solvent extract

of plasma as a routine procedure for the isolation of testosterone; however, no comment was made as to its effectiveness. Removal of the methanol:petroleum ether partition system and/or column and paper chromatography from the isolation procedure greatly shortened the time required for isolating progesterone from plasma. The alkalinization of the extraction solvent residue is a useful technique regardless of the species involved and enables one-step isolation of progesterone via TLC without prior purification. The technique is particularly useful for samples containing large amounts of pigments and/or lipids.

The saponification of the extraction residue in ether rather than dichloromethane was an important adaptation of the technique. It was very difficult to remove the aqueous saponifying layer in its entirety from the top of the dichloromethane; as a consequence, a small portion of the aqueous layer was usually left to be dried with the solvent. This allowed substances interfering with GLC to be carried through the process. With ether as the solvent (upper layer) and the aqueous layer on the bottom, a very precise removal of the saponified lipid and pigment was possible. This, coupled with an efficient TLC system for the isolation of progesterone, is essential for producing GLC tracings without interfering peaks.

Several experimental approaches reported in recent years have lowered the amount of progesterone necessary for quantitation. Short and Levett (1962) applied the fluorescence technique of Touchstone and Murawec (1960) for the estimation of progesterone in human plasma. They found approximately 10 mμg of progesterone/ml of plasma during the follicular phase of the menstrual cycle; this was much higher than the 0.49 mμg/ml for days 1 to 9 of the cycle reported by van der Molen and

Groen (1965) and 0.4 mμg/ml found by Neill et al. (1967) during the first twelve days of the cycle. Heap (1964) utilized the increased fluorescent sensitivity of 20β-hydroxypregn-4-en-3-one (enzymatically converted from progesterone) in sulfuric acid and ethanol to measure progesterone. The finding of high blank values (Short & Levett, 1962) points up the major problem associated with the fluorescent techniques, namely, lack of specificity. This would appear to be of particular consequence if the determinations were made at the lower limit of the sensitivity of the technique.

Several double isotope derivative techniques have been reported for the estimation of progesterone in human plasma (Woolever and Goldfien, 1963; Riondel et al., 1965; Wiest, 1967a). These techniques also have the disadvantage of some lack of specificity due to the presence of high blank values (Woolever, 1965). The preovulatory values of 1.7 mμg/ml plasma (Woolever, 1963) and 1.32 mμg/ml plasma (Riondel et al., 1965) are higher than those reported by van der Molen and Groen (1965) and Neill et al. (1967). The laboriousness of the double isotope technique is illustrated by the use of a minimum of three paper chromatograms by Woolever and Goldfien (1963), two paper chromatograms and three thin layer chromatograms by Riondel et al. (1965) and five paper chromatograms by Wiest (1967a) in order to attain theoretical $^{3}H/^{14}C$ ratios.

The competitive protein-binding radioassay system for progesterone analysis developed by Murphy (1964, 1967) has been used to measure human plasma levels (Neill et al., 1967). A high level of sensitivity was attained; 0.5 mμg progesterone could be quantitated with good accuracy and precision. The procedure appears to be concise and should prove to be

an important method for routine progesterone analyses.

Besides the need for sensitivity for the determination of progesterone in the peripheral plasma of domestic animals, it was deemed important to be able to establish the identity of progesterone by other than chromatographic techniques. The 20β-OH chloroacetate derivative method of van der Molen and Groen (1965) allowed supplemental identification by such methods as the establishment of a constant specific activity and by mass spectrometry. Identification of progesterone was precluded with the use of the fluorescence, double isotope derivative and competitive protein-binding radioassay methods.

Progesterone Levels

The cyclic pattern of progesterone concentration in jugular plasma found in this study is in agreement with known changes in corpus luteum (CL) function in the cow that occur during the cycle. The rapid decline of progesterone in peripheral plasma toward the end of the cycle as well as the marked rise in concentration during the time of CL development is strong evidence for suggesting CL function can be monitored in peripheral plasma by progesterone determination. A cyclic pattern of progesterone has been reported in the guinea pig by Feder, Resko & Goy (1966). That the plasma progesterone levels would decrease rapidly with declining CL function is suggested by the short biological half-life of progesterone (36.3 minutes) reported by Miller, Williams, Pipes & Turner (1963) for the nonpregnant cow. Imori (1967) reported the half-life of progesterone in nonpregnant cows during the regressing luteal phase of the cycle was 22.4 minutes.

A few reports, although fragmentary, support the present data as

to progesterone concentration during the luteal phase of the cycle. Short (1961) reported 9.4 mμg/ml plasma in one cow in the luteal phase while McCracken (1963) found an average of 9.6 and 8.8 mμg/ml plasma on day 12 in two cows for two consecutive cycles. Stabenfeldt, Ewing and McDonald (1966) reported 7.3 mμg/ml plasma in a cow at the 167th day of gestation. The peak progesterone levels found in the study reported herein, ranged from 6.1 to 10.2 mμg/ml plasma (Table XII).

In a recent study, Plotka et al. (1967) estimated progesterone in the peripheral plasma of twelve cows on alternate days during the estrous cycle using the double isotope derivative technique of Woolever & Goldfien (1963). While the data showed a cyclic pattern of progesterone concentration, the shape of the curve as well as the amount of hormone present differed considerably from this study. The sharp decline observed at day 7 by Plotka et al. (1967) was not observed in the present study; as seen in Fig. 6, the rise in progesterone is continuous from day 3 until about day 8 with a slower rate of increase until about day 17.

The progesterone levels reported by Plotka et al. (1967) declined gradually from day 15 until the next estrus. In the study reported herein, peak progesterone levels were high until at least day 17; the subsequent decline in concentration was rapid. The levels of progesterone observed by Plotka et al. (1967) were at least twenty times higher than those found in the current study during the follicular phase (10 mμg vs 0.5 mμg/ml plasma); peak luteal levels were three to four times higher (25 mμg vs 7 mμg/ml plasma). One of the principal problems of the double isotope technique revolves around removal of excess tritiated products. The presence of tritium, other than that associated with

progesterone, would result in a quantitative overestimation of progesterone. While the luteal phase progesterone levels in human plasma as estimated by the double isotope technique (Woolever, 1963) were only slightly higher than those determined by the 20β-OH chloroacetate derivative method (van der Molen & Groen, 1965) and the competitive protein-binding radioassay method (Neill et al., 1967), the follicular phase levels of Woolever (1963) were four times higher. These follicular phase levels were later acknowledged to be due to unrecognized high blank values (Woolever, 1965). High blank values may partially explain the discrepancy between the progesterone data reported herein and those of Plotka et al. (1967).

Earlier attempts to correlate peripheral plasma progesterone with the stage of the estrous cycle were unsuccessful. Gomes, Estergreen, Frost and Erb (1963) were unable to relate CL progesterone and ovarian venous progesterone concentrations with the estrous cycle. That these workers did not find a correlation of jugular plasma progesterone with the stage of the estrous cycle is probably attributable to the fact that the pooling of samples from individual animals with considerable inherent variability obscured meaningful relationships. Also, Gomes and Erb (1965) pointed out that the readings (Gomes et al., 1963) were made near the limit of the sensitivity of the method which increased the possibility of error. However, Shemesh, Ayalon and Lindner (1968) reported a three-fold progesterone drop in peripheral plasma from day 19 to day 20 of the cycle of nonpregnant cows; pregnant cows did not show this decrease.

Mares, Zimbelman and Casida (1962) found peak luteal function at day 16 (estrus=1) of the bovine cycle as indicated by peak progesterone

concentration in CL. Likewise, Gomes et al. (1963) obtained bovine CL at various times of the cycle with peak luteal progesterone concentration recorded at day 15; the decrease in progesterone concentration observed on days 16 and 17 was interpreted as evidence for failing CL function. Stormshak, Inskeep, Lynn, Pope and Casida (1963) found a correlation of luteal progesterone and ovarian venous progesterone in sheep with the day of the estrous cycle. Luteal progesterone was suggested as being an appropriate indicator of the amount of progesterone released into the general circulation. In the present study, cows had elevated progesterone levels in the peripheral blood at days 17 to 20 of the cycle; because of the short half-life of progesterone the assumption may be made that these animals had functional CL during this time. If peak luteal progesterone concentration does occur at either day 15 or 16 as reported by Gomes et al. (1963) and Mares et al. (1962), it appears from the data recorded in this study that CL may be able to maintain high levels of progesterone in the blood even though the concentration of hormone in the organ is declining. These observations raise the question as to the validity of using hormone concentration in endocrine glands as an absolute indicator of physiological activity. Nalbandov (1966), as well as others, has strongly emphasized the inadequacy of pituitary assays without examination of peripheral or subdural plasma hormone levels. It may be that high CL progesterone content is not an absolute requirement for maintaining plasma levels. Staples & Hansel (1961) reported data that show a base level of 100 µg of luteal progesterone may be sufficient for embryo maintenance in heifers although levels two to three times greater have been observed more commonly in normal animals.

That the CL may be functional beyond day 15 was shown in studies conducted in vitro on the bovine CL by Armstrong and Black (1966). These workers found CL could respond to reduced nicotinamide-adenine dinucleotide (NADH) or indirectly to luteinizing hormone (LH) by progesterone biosynthesis through day 18; LH stimulated NADH production by CL. The failure of the CL to synthesize progesterone toward the end of the cycle was due to an inability of the CL to respond to LH by NADH production and not an inability to utilize NADH.

The time that estrus occurs following progesterone decline in the cow differs from data observed by the author for both the gilt and the ewe. The variation in the appearance of estrus in the cow following decline of CL function as indicated by progesterone levels ranged from two to five days. The time interval between CL regression and estrus in the ewe appears to be one to two days (Stabenfeldt, Ewing, Holt & McDonald, 1968a). Swine appear to represent the other extreme; in four gilts studied through one estrous cycle each, the average interval from time of decline of peripheral plasma progesterone to estrus was six days (Stabenfeldt, Akins, Ewing, McDonald & Morrissette, 1968b). These three species apparently differ in the time interval requirements for follicular growth and maturation following the regression of corpora lutea. This may be related to differences of circulating progesterone levels with follicular growth in the gilt suppressed by the high plasma progesterone levels. Follicular development in the ewe, on the other hand, may be continuous throughout the luteal phase because of the much lower plasma progesterone levels. This could allow early maturation of the follicle in the ewe allowing sexual receptivity to be manifested soon after CL regression.

The considerable variation noted for cows as to the length of time required for follicle development may be one reason for the lack of precise grouping of estrus encountered following estrus synchronization. Likewise, the low conception rate often observed for cows at the first estrus after synchronization may be a result of the production of psychic estrus by certain critical levels of estrogens and synthetic progestins without sufficient time lapse for follicle development and maturation.

van der Molen & Groen (1965) reported 0.5 mμg progesterone/ml plasma in women during the first part of the follicular phase of the menstrual cycle (days 1-9). Likewise, Neill et al. (1967) found 0.4 mμg/ml plasma in women during the twelve days of the menstrual cycle which preceded LH release (follicular phase). These levels are very similar to those reported here for the cow (0.4 mμg/ml plasma, Table XIII), gilt (0.5 mμg/ml plasma, Table XIV) and ewe (0.2 mμg/ml plasma, Table XV). This indicates the base levels of progesterone in peripheral plasma during the follicular phase of the cycle are very similar in cows, gilts, ewes and women.

That progesterone may originate, at least in part, from sources other than the ovary is suggested by the finding of 0.9 mμg progesterone/ml of peripheral plasma obtained from nine castrated male pigs (barrows) (Stabenfeldt et al., 1968b). Identification of the hormone was by the Rf value on GLC only and is therefore presumptive; the amount of progesterone available was insufficient for other identification procedures. The adrenal cortex with its steroid synthesizing capability would appear to be one source of the hormone in the barrow as well as a source of progesterone in females during the follicular stage of the cycle. Balfour, Comline and Short (1957) isolated progesterone

from the adrenal venous blood of bulls, cows and calves as well as from young boars; progesterone was also isolated in the adrenal venous effluent of rams and ewes, both intact and castrate.

Fig. 11 presents a composite of the peripheral plasma levels found during the estrous cycle of the cow, ewe (Stabenfeldt et al., 1968a) and gilt (Stabenfeldt et al., 1968b) as well as during the human menstrual cycle (Neill et al., 1967). The most striking feature is that all four species have decidely different peripheral plasma levels of progesterone during the luteal phase of the cycle. One implication could be that each species requires different progesterone levels for the progestational proliferation of the uterine endometrium in preparation for implantation of the zygote(s) (see Tables XIII, XIV and XV). Also, it could be that the uterine epithelium and musculature of these species varies as to progesterone-trapping capabilities. As mentioned previously, the biological half-life of progesterone in the nonpregnant cow was 36.3 minutes (Miller et al., 1963) and 22.4 minutes (Imori, 1967). The lower levels of plasma progesterone in the ewe may be explained by the short biological half-life of progesterone (4 minutes) found by Short (1961).

The decline of CL function as indicated by peripheral plasma progesterone levels appears to be somewhat different among the four species (Fig. 11). Cows and gilts appear to have a very precipitous regression of the CL while ewes and women appear to have a more gradual decline of CL function. These data (Fig. 11) suggest several possibilities: (1) differences may exist among species as to steroidogenic capabilities of CL; (2) differences may exist among species as to the metabolic clearance rate of progesterone; and (3) differences may exist as to mechanisms of regression of the CL.

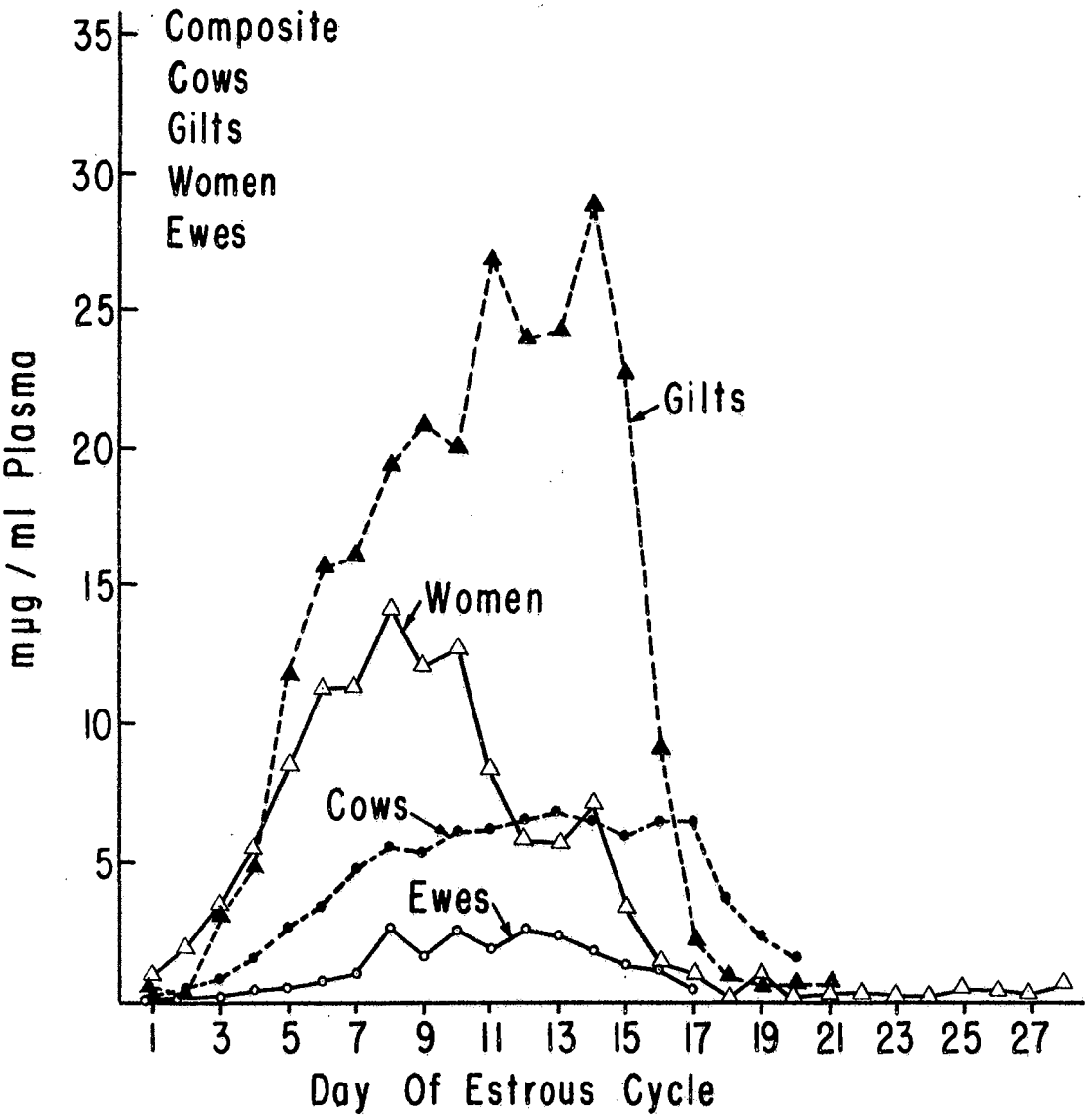

Figure 11. Progesterone Concentration in the Peripheral Plasma of Cows, Gilts and Ewes During the Estrous Cycle and in Women During the Menstrual Cycle (Taken from Neill et al., 1967). Day 1 Represents the First Day of Sexual Receptivity in Cows, Gilts and Ewes While Day 1 in Women Represents the Day of Luteinizing Hormone Release at Mid-cycle.

During this investigation, the importance of studying individual animals on a daily basis became apparent because of the variation seen in individual cows as to (1) the day of beginning CL function following estrus; (2) the day of peak luteal function as shown by peripheral plasma progesterone (Table XIV); (3) the day of decline of progesterone in plasma at the end of the estrous cycle; and (4) the interval between progesterone decline and manifestations of estrus. It was particularly important to study cows on a daily basis at the end of the luteal function where a two to ten-fold reduction in progesterone occurred in the peripheral plasma within a twenty-four hour sampling period. In the past, interpretation of data on the CL has been hampered because of the necessity of pooling data from animals which had considerable variation in CL function. It is apparent no precise limitation can be placed on the cessation of CL function because of this variation.

Methods that allow monitoring of hormone levels in peripheral plasma on a daily or even more frequent basis should enable the collection of data that will reflect more accurately the physiological status of the animal than has been possible to date with various *in vitro* and morphological approaches.

TABLE XIII

AVERAGE PERIPHERAL PLASMA PROGESTERONE LEVELS IN FOLLICULAR PHASE AND PEAK LUTEAL PHASE OF THE BOVINE ESTROUS CYCLE

Cow (No.)	[a]Follicular Phase (mμg/ml Plasma)	Duration (Days)	[b]Luteal Phase (mμg/ml Plasma)	Duration (Days)
07	0.4	5	5.2	5
11	0.3	7	5.7	6
17	0.3	8	7.8	6
24	0.4	4	7.9	5
40	0.4	6	5.3	8
40	0.5	6	6.6	8
46	0.4	9	7.7	8

[a]The days of the cycle including estrus in which levels of progesterone were less than 1.0 mμg/ml plasma.

[b]Day 13 of the estrous cycle (estrus=day 1) through the last day of the cycle with elevated progesterone levels.

TABLE XIV

AVERAGE PERIPHERAL PLASMA PROGESTERONE LEVELS IN FOLLICULAR PHASE AND PEAK LUTEAL PHASE OF THE PORCINE ESTROUS CYCLE

Gilt (No.)	[a]Follicular Phase (mμg/ml Plasma)	Duration (Days)	[b]Luteal Phase (mμg/ml Plasma)	Duration (Days)
241	0.5	6	---	-
241	0.5	5	25.2	5
244	0.5	5	26.8	6
245	0.6	5	31.5	5
245	---	-	24.7	5

[a]The days of the cycle including estrus in which levels of progesterone were less than 1.0 mμg/ml plasma.

[b]Day 10 of the estrous cycle (estrus=1) through the last day of the cycle with elevated progesterone levels.

TABLE XV

AVERAGE PERIPHERAL PLASMA PROGESTERONE LEVELS IN FOLLICULAR PHASE AND PEAK LUTEAL PHASE OF THE OVINE ESTROUS CYCLE

Sheep (No.)	[a]Follicular Phase (mμg/ml Plasma)	Duration (Days)	[b]Luteal Phase (mμg/ml Plasma)	Duration (Days)
10	0.3	3	2.6	5
10	0.1	3	---	-
12	0.1	3	1.5	5
12	0.2	3	---	-
13	---	-	2.3	5
16	0.7	3	1.8	5
16	0.2	3	---	-
18	0.1	3	3.1	5
21	0.1	3	1.5	5
28	0.1	3	1.8	4
28	0.2	3	---	-

[a]Days 1, 2 and 3 of the estrous cycle (estrus=day 1).

[b]Days 10, 11, 12, 13 and 14 of the estrous cycle (estrus=day 1).

CHAPTER VI

SUMMARY AND CONCLUSIONS

In the present study, progesterone concentration was determined daily in the peripheral venous plasma of six cows during the estrous cycle. The technique of van der Molen and Groen (1965) was adapted whereby progesterone was converted enzymatically to 20β-hydroxypregn-4-en-3-one and thence acetylated with monochloroacetic anhydride.

The presence of large amounts of substances in plasma that interfere with the quantitation of progesterone have necessitated the use of involved isolation procedures. Initial progesterone purification procedures have been complicated in the cow by the presence of yellow pigments in the plasma. These pigments, mainly carotenes, while present in the plasma of horses, are not found in the plasma of other species such as man, dog, pig and sheep. The use of methanol:petroleum ether partition systems as well as column and/or paper chromatography have allowed the isolation of relatively pure progesterone from plasma; unfortunately, both the partition and the chromatography methods have been laborious. In the present study, dichloromethane was used as the extraction solvent because it removed fewer pigments from plasma when compared to other solvents. Dichloromethane was effective in the extraction of progesterone from plasma.

It was found that saponification of the extraction residue rather than the plasma was a much more effective method for separation of the

plasma pigments from progesterone. The resultant product could be placed easily on thin layer chromatography plates. Thus progesterone isolation was accomplished without intervening partition or chromatography steps. The alkalinization of the extraction solvent residue is a useful technique regardless of the species involved and enables one-step isolation of progesterone by thin layer chromatography.

Progesterone levels determined daily in the peripheral blood of cows ranged from less than 0.5 mμg/ml plasma during the follicular phase to approximately 7 mμg/ml plasma (6.1 mμg to 10.2 mμg) at peak luteal phase. Progesterone levels in cows with twenty-one day cycles increased rapidly from day 3 to day 8 (estrus=day 1) with a much slower rate of increase from day 8 through day 17. These cows showed a decrease of more than 50% from the peak luteal progesterone levels on days 18 (two cows), 19 (one cow) and 21 (two cows). Two other cows with cycles of twenty-two days duration and twenty-three days duration both had similar progesterone decreases on day 20.

If peak luteal progesterone concentration occurs at either day 15 or 16 as reported in the literature, it appears from the data recorded in this study that corpora lutea may be able to maintain high levels of progesterone in the blood even though the concentration of the hormone in the organ is declining. These observations raise the question as to the validity of using hormone concentration in endocrine glands as an absolute indicator of physiological activity. It may be that high corpora lutea progesterone content is not an absolute requirement for maintaining plasma levels.

The levels of progesterone found in the cow during the follicular stage of the estrous cycle (0.4 mμg/ml plasma) are very similar to those

found in women (0.4 mμg/ml plasma), gilts (0.5 mμg/ml plasma) and ewes (0.2 mμg/ml plasma). This suggests sources other than corpora lutea (such as the adrenal cortex) contribute to a base level of progesterone in the peripheral plasma.

Peak luteal progesterone levels for the cow are generally lower than those reported for women and gilts but higher than those found for ewes. This suggest differences may exist among species as to progesterone synthesizing and/or releasing capabilities of corpora lutea; differences also may exist as to the metabolic clearance rate of progesterone.

The variation in the appearance of estrus in the cows following the decline of corpus luteum function as indicated by peripheral plasma progesterone levels ranged from two to five days. The cow thus appears to be quite variable as to the length of time required for follicle development and maturation. This is in contradistinction to the ewe and the gilt; the ewe shows signs of estrus the day following corpus luteum regression while the gilt exhibits signs about six days after luteal regression.

The considerable variation noted for cows as to length of time required for follicle development may be one reason for the lack of precise grouping of estrus encountered following estrus synchronization. Likewise, the low conception rates often observed for cows at the first estrus after synchronization may be a result of the production of psychic estrus by certain critical levels of estrogens and synthetic progestins without sufficient time lapse for follicle development and maturation.

During this investigation, the importance of studying individual animals on a daily basis became apparent because of the variation

observed in individual cows. It was particularly important to study the cows on a daily basis at the end of luteal function where a two to ten-fold reduction in progesterone occurred in the peripheral plasma within a twenty-four hour sampling period. It is suggested that corpus luteum function can be monitored by studying progesterone concentration in the peripheral blood.

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A P P E N D I C E S

A P P E N D I X A

TABLE XVI

EFFECT OF METHOD OF EXTRACTION ON RECOVERY OF PROGESTERONE FROM 20 ml BOVINE PLASMA

[a]Stirring	[b]Shaking
[c]83.5	83.7
90.8	89.3
89.4	99.2
81.8	99.1
90.3	91.8
99.1	----

[a]Extraction was accomplished by stirring plasma and solvent manually with a glass rod for 1 min/each of 3 extractions.

[b]Extraction was accomplished by shaking plasma and solvent on a vortex mixer for 1 min/each of 3 extractions.

[c]Percent recovery based on recovery of progesterone-7-^{3}H added to plasma before extraction.

TABLE XVII

DATA FOR INFLUENCE OF SAPONIFICATION UPON EFFICIENCY OF EXTRACTION OF PROGESTERONE FROM BOVINE PLASMA

Extraction	[a]Before	[b]After
1. 2X (160 ml)	[c]83.7	83.2
	78.1	101.9
	73.3	94.1
	68.2	89.8
2. 3X (180 ml)	72.1	100.1
	75.1	93.0
	----	101.6
	----	103.1
3. 4X (240 ml)	90.1	101.5
	84.9	101.3
	----	98.3
4. 5X (200 ml)	81.2	89.3
	73.9	92.3
	90.0	98.5
	83.2	97.6
5. 6X (240 ml)	69.0	99.1
	71.1	91.8
	68.0	80.4
	70.0	102.3

[a]Sodium hydroxide added to plasma before extraction.

[b]Sodium hydroxide added to plasma extraction residue.

[c]Percent recovery based on recovery of progesterone-7-^{3}H added to plasma prior to extraction.

TABLE XVIII

EFFECT OF SOLVENT VOLUME (ml OF DICHLOROMETHANE) AND NUMBER OF EXTRACTIONS ON RECOVERY OF PROGESTERONE FROM BOVINE PLASMA (BASED ON RECOVERY OF PROGESTERONE-7-^{3}H)

Date	Extraction Procedure	Percent Recovery	Date	Extraction Procedure	Percent Recovery	Date	Extraction Procedure	Percent Recovery
0808	60 : 60 : 60	99.2	0925	60 : 60 : 40	87.3	0203	60 : 60 : 40	98.1
0808	60 : 60 : 60	100.7	0925	60 : 60 : 40	86.7	0203	60 : 60 : 40	100.9
0812	60 : 60 : 60	101.9	0925	60 : 60 : 40	83.4	0203	60 : 60 : 40	99.4
0812	60 : 60 : 60	100.6	0925	60 : 60 : 40	83.3	0203	60 : 60 : 40	100.5
0815	60 : 60 : 60	97.0	0925	60 : 60 : 40	95.1	0203	60 : 60 : 40	102.3
0815	60 : 60 : 60	102.0	0925	60 : 60 : 40	78.2	0203	60 : 60 : 40	96.5
0815	60 : 60 : 60	98.2	0925	60 : 60 : 40	97.0	0203	60 : 60 : 40	94.4
0815	60 : 60 : 60	101.3	0928	60 : 60 : 40	91.2	0203	60 : 60 : 40	101.3
0815	60 : 60 : 60	99.7	0928	60 : 60 : 40	91.7	0207	60 : 60 : 40	97.9
0815	60 : 60 : 60	97.8	0928	60 : 60 : 40	96.1	0207	60 : 60 : 40	97.5
0815	60 : 60 : 60	95.5	0928	60 : 60 : 40	94.4	0207	60 : 60 : 40	98.9
0815	60 : 60 : 60	102.1	0928	60 : 60 : 40	100.1	0207	60 : 60 : 40	98.0
			0928	60 : 60 : 40	93.6	0207	60 : 60 : 40	93.8
0816	60 : 60 : 40	100.5	0928	60 : 60 : 40	93.7	0207	60 : 60 : 40	99.2
0816	60 : 60 : 40	96.5	0928	60 : 60 : 40	97.7	0207	60 : 60 : 40	94.3
0816	60 : 60 : 40	97.9	0930	60 : 60 : 40	102.3			
0816	60 : 60 : 40	98.9	0930	60 : 60 : 40	97.2	0831	50 : 50 : 50	92.1
0819	60 : 60 : 40	101.5	0930	60 : 60 : 40	95.5	0831	50 : 50 : 50	92.0
0819	60 : 60 : 40	104.8	0930	60 : 60 : 40	106.8	0831	50 : 50 : 50	88.0
0819	60 : 60 : 40	101.5	0930	60 : 60 : 40	97.6	0831	50 : 50 : 50	96.8
0819	60 : 60 : 40	98.4	0930	60 : 60 : 40	104.1	0831	50 : 50 : 50	95.6
0822	60 : 60 : 40	97.3	0930	60 : 60 : 40	100.3	0831	50 : 50 : 50	94.6
0822	60 : 60 : 40	98.1	0930	60 : 60 : 40	108.4	0831	50 : 50 : 50	97.8
0822	60 : 60 : 40	95.7	1005	60 : 60 : 40	89.9	0831	50 : 50 : 50	97.0
0822	60 : 60 : 40	96.2	1005	60 : 60 : 40	98.2	0901	50 : 50 : 50	97.2
0822	60 : 60 : 40	94.8	1005	60 : 60 : 40	97.7	0901	50 : 50 : 50	94.2
0822	60 : 60 : 40	93.5	1005	60 : 60 : 40	92.5	0901	50 : 50 : 50	93.5
0822	60 : 60 : 40	95.3	1005	60 : 60 : 40	97.7	0901	50 : 50 : 50	94.9
0822	60 : 60 : 40	98.4	1005	60 : 60 : 40	99.6	0901	50 : 50 : 50	98.7
						0901	50 : 50 : 50	97.9
						0901	50 : 50 : 50	97.7

TABLE XVIII (Continued)

Date	Extraction Procedure	Percent Recovery	Date	Extraction Procedure	Percent Recovery	Date	Extraction Procedure	Percent Recovery
0308	50 : 50 : 40	96.7	1013	60 : 60	89.8	1022	60 : 60	91.7
0308	50 : 50 : 40	96.7	1013	60 : 60	90.0	1022	60 : 60	86.4
0308	50 : 50 : 40	99.0	1013	60 : 60	83.8	1022	60 : 60	88.8
0308	50 : 50 : 40	92.0	1013	60 : 60	91.5	1023	60 : 60	77.6
0311	50 : 50 : 40	95.5	1013	60 : 60	90.8	1023	60 : 60	95.6
0311	50 : 50 : 40	95.9	1013	60 : 60	94.6	1023	60 : 60	93.2
0311	50 : 50 : 40	93.6	1013	60 : 60	79.4	1023	60 : 60	96.6
0311	50 : 50 : 40	92.3	1016	60 : 60	89.6	1023	60 : 60	92.8
0319	50 : 50 : 40	103.3	1016	60 : 60	89.0	1023	60 : 60	96.1
			1016	60 : 60	89.5	1029	60 : 60	91.1
0823	40 : 40 : 40	90.3	1016	60 : 60	87.1	1029	60 : 60	91.2
0823	40 : 40 : 40	93.7	1016	60 : 60	81.5	1029	60 : 60	92.3
0823	40 : 40 : 40	93.4	1016	60 : 60	85.8	1029	60 : 60	91.7
0823	40 : 40 : 40	87.7	1016	60 : 60	88.0	1029	60 : 60	84.0
0308	40 : 40 : 40	92.8	1017	60 : 60	86.4	1029	60 : 60	93.3
0308	40 : 40 : 40	95.4	1017	60 : 60	86.4	1029	60 : 60	92.0
0308	40 : 40 : 40	94.4	1017	60 : 60	86.8	1101	60 : 60	94.9
0308	40 : 40 : 40	95.6	1017	60 : 60	79.3	1101	60 : 60	93.6
0311	40 : 40 : 40	94.0	1017	60 : 60	82.5	1101	60 : 60	89.6
0311	40 : 40 : 40	91.6	1017	60 : 60	84.5	1101	60 : 60	84.5
0311	40 : 40 : 40	89.8	1020	60 : 60	91.0	1101	60 : 60	91.6
0311	40 : 40 : 40	92.3	1020	60 : 60	86.9	1101	60 : 60	88.9
			1020	60 : 60	87.6	1101	60 : 60	91.0
1009	60 : 60	92.7	1020	60 : 60	90.5	1101	60 : 60	96.6
1009	60 : 60	87.7	1020	60 : 60	93.7	1102	60 : 60	89.9
1009	60 : 60	78.1	1020	60 : 60	71.6	1102	60 : 60	93.2
1009	60 : 60	88.1	1022	60 : 60	91.9	1102	60 : 60	87.6
1009	60 : 60	84.4	1022	60 : 60	90.7	1102	60 : 60	95.5
1009	60 : 60	93.6	1022	60 : 60	89.9	1102	60 : 60	89.0
1009	60 : 60	98.2	1022	60 : 60	97.0			

TABLE XIX

EFFECT OF ELUTION AREA (TLC) ON PERCENT RECOVERY OF PROGESTERONE-7-^{3}H

[a]Symmetrical Cut (Percent Recovery)	[b] Asymmetrical Cut (Percent Recovery)
[c]79.5	82.4
84.8	81.2
73.4	80.7
78.9	91.0
88.0	80.0
81.8	81.5
71.7	80.0
84.3	82.0
76.2	94.3
82.4	94.2
80.8	91.9
80.6	81.2
85.6	94.3
87.5	94.2
81.0	94.3
71.4	94.5
72.1	88.0
74.0	92.2
74.0	----
66.7	----
68.4	----
69.3	----
71.1	----
74.9	----
76.2	----
79.0	----
77.5	----

[a]Symmetrical cut refers to a 3 cm area of silica gel removed for elution which was centered on the adjacent progesterone standard.

[b]Asymmetrical cut refers to a 3 cm area of silica gel removed for elution which was centered on the adjacent progesterone standard plus an additional 0.5 cm area toward the solvent front.

[c]Percent recovery based on amount of progesterone-7-^{3}H recovered following extraction, saponification, two water washes and TLC.

TABLE XX

CHECK OF EFFICIENCY OF BACKWASHING THE ELUTER USED TO REMOVE PROGESTERONE FROM SILICA GEL FOLLOWING ISOLATION BY TLC

Date	[a,b]Counts/10 min
0421	211
0426	186
0427	170
0428	157
0429	157
0430	179
0501	178
0502	161
0503	158
0504	153
0505	157
0506	167
0507	164
0508	175
0509	160
0510	169
0511	168
0512	176
0513	156
0514	175
0515	178
0516	174
0517	169

[a]Hot methanol used to backwash eluter was trapped in a scintillation vial, dried and the residue counted in a liquid scintillation spectrometer.

[b]Background count/10 min averaged 180.

A P P E N D I X B

TABLE XXI

DATA FOR EFFECT OF TIME ON EFFICIENCY OF ENZYMATIC CONVERSION AND ACETYLATION

[a]EC (1 hr) [b]A (12 hr)	EC (2 hr) A (12 hr)	EC (2 hr) A (16 hr)
[c]61.4	67.4	67.8
60.9	71.1	63.3
65.7	73.1	67.3
56.4	60.4	69.8
	69.4	64.4
	71.1	60.0
EC (1 hr)	71.3	69.3
A (16 hr)	83.4	71.4
	66.7	71.9
54.8	59.2	70.7
66.2		70.1
77.4		74.4
69.1	EC (2 hr)	63.8
	A (16 hr)	74.1
EC (1½ hr)		69.7
A (12 hr)	73.0	67.2
	69.8	64.8
61.2	75.0	67.4
69.7	82.1	69.6
65.2	72.5	72.0
71.1	74.4	76.9
81.0	58.4	65.5
72.8	65.7	69.8
	70.5	68.0
	53.3	77.3
EC (1½ hr)	76.2	62.8
A (16 hr)	71.0	74.3
	71.0	67.8
73.1	69.9	70.5
76.0	69.1	78.8
70.1	62.1	65.2
69.2	57.0	69.1
65.1	69.7	72.4

[a]Enzymatic conversion (progesterone to 20β-hydroxypregn-4-en-3-one).

[b]Acetylation (20β-hydroxypregn-4-en-3-one to its chloroacetate derivative).

[c]Percent recovery of 20β-hydroxypregn-4-en-3-one-7-^{3}H chloroacetate (originally added as progesterone-7-^{3}H).

TABLE XXII

DATA ON EFFECT OF TUBE SIZE AND SOLVENT ON EXTRACTION OF 20β-HYDROXY-PREGN-4-EN-3-ONE CHLOROACETATE FROM SILICA GEL G (PROCEDURE INCLUDED ENZYMATIC CONVERSION, ACETYLATION AND ISOLATION BY TLC)

Tube Size	[a]Benzene	[b]Water
35 ml	[c]73.6	67.9
	76.4	70.6
	64.6	66.5
	----	66.1
12 ml	70.7	60.7
	69.3	60.4
	67.4	57.6
	69.1	58.2

[a]Benzene added to silica gel G before water.

[b]Water added to silica gel G before benzene.

[c]Percent recovery based on recovery of [^{3}H] associated with 20β-hydroxypregn-4-en-3-one chloroacetate.

TABLE XXIII

DATA ON EFFECT OF EDTA ON ENZYMATIC CONVERSION OF PROGESTERONE TO 20β-HYDROXYPREGN-4-EN-3-ONE (PROCEDURE INCLUDED ACETYLATION OF 20β-HYDROXYPREGN-4-EN-3-ONE WITH CHLOROACETIC ANHYDRIDE AND ISOLATION BY TLC)

[a]Buffer w/o EDTA	Buffer w/ EDTA
[b]63.8	73.6
65.8	73.1
71.4	77.4
66.4	77.4
58.3	64.8
58.4	----
53.0	----

[a]Phosphate buffer used for enzymatic conversion.

[b]Percent recovery based on recovery of [^{3}H] associated with 20β-hydroxypregn-4-en-3-one chloroacetate.

TABLE XXIV

DATA ON EFFECT OF SOLVENT ON EXTRACTION OF 20β-HYDROXYPREGN-4-EN-3-ONE CHLOROACETATE FROM SILICA GEL G (PROCEDURE INCLUDED ENZYMATIC CONVERSION, ACETYLATION AND ISOLATION BY TLC)

[a]Benzene	[b]Water
[c]73.2	60.9
69.8	65.1
75.0	65.0
82.1	60.3
72.5	57.6
74.4	66.2
58.4	58.4
65.7	60.9
62.2	59.4
59.8	60.1
82.4	50.9
71.7	60.1
56.0	----
62.6	----
70.5	----
53.3	----
76.2	----
71.0	----
54.8	----
66.2	----
77.4	----
67.1	----
70.9	----
72.5	----
60.6	----
69.1	----

[a]Benzene added to silica gel G before water.

[b]Water added to silica gel G before benzene.

[c]Percent recovery based on recovery of [^{3}H] associated with 20β-hydroxypregn-4-en-3-one chloroacetate.

A P P E N D I X C

TABLE XXV

VARIATION IN DETECTOR SENSITIVITY OF 20β-HYDROXYPREGN-4-EN-3-ONE CHLOROACETATE (20β-OH MCA) AND TESTOSTERONE CHLOROACETATE (TEST MCA) OVER A TWO MONTH INTERVAL

Peak Area (cm^2) (10 mμg) 20β-OH MCA	Peak Area (cm^2) (10 mμg) Test MCA	Ratio 20β-OH MCA/Test MCA	Peak Area (cm^2) (10 mμg) 20β-OH MCA	Peak Area (cm^2) (10 mμg) Test MCA	Ratio 20β-OH MCA/Test MCA
5.2	4.3	1.21	9.0	7.4	1.22
5.3	4.6	1.15	9.2	7.5	1.23
7.0	5.8	1.21	10.6	8.0	1.33
7.0	5.8	1.21	8.6	7.6	1.13
7.4	5.8	1.23	9.0	8.0	1.13
8.1	6.2	1.31	10.2	8.2	1.24
7.5	6.0	1.25	8.9	7.2	1.24
7.2	6.0	1.20	9.8	7.8	1.26
7.5	6.0	1.25	8.4	7.1	1.18
7.4	6.5	1.14	7.8	6.6	1.18
6.2	4.9	1.27	8.9	7.8	1.14
6.8	5.5	1.24	8.4	7.5	1.13
6.8	5.2	1.31	10.0	8.6	1.16
6.4	5.4	1.19	9.6	8.6	1.12
6.6	5.4	1.22	8.6	7.3	1.18
6.9	5.5	1.25	9.7	7.4	1.31
8.0	6.0	1.33	10.0	7.3	1.37
8.7	6.8	1.28	11.0	8.3	1.33
8.7	7.0	1.24	9.4	7.0	1.34
10.4	8.6	1.21	9.8	7.6	1.29
10.4	8.7	1.20	10.5	8.3	1.27
10.0	8.4	1.19	9.4	7.1	1.32
10.8	8.2	1.32	10.2	8.2	1.24
12.0	9.3	1.29	9.9	7.9	1.25
10.2	8.6	1.19	8.4	7.1	1.18
9.1	7.8	1.17	8.4	7.0	1.20
9.0	7.3	1.23	8.1	6.6	1.23
9.3	8.0	1.16	9.8	7.6	1.29
9.4	7.3	1.29	8.3	6.4	1.30
9.8	8.3	1.18	7.9	6.2	1.27
10.0	8.4	1.19	8.5	6.6	1.29
11.1	9.8	1.13	9.0	7.2	1.25
9.1	7.7	1.19	9.0	7.3	1.23
8.8	7.7	1.21	8.1	6.3	1.29
8.6	7.3	1.18	---	---	----

TABLE XXVI

COMPARISON OF PROGESTERONE-7-^{3}H STANDARDS USED TO ESTIMATE PROGESTERONE RECOVERY

Date	$\bar{x}$ [a]Std.No.1	$\bar{x}$ Std.No.2	$\bar{x}$ Stds.	Percent Deviation (±) from Mean
0403	24,393	24,570	24,482	0.4
0404	24,005	24,791	24,390	1.6
0405	25,110	25,557	25,247	0.5
0406	25,643	25,713	25,678	0.1
0407	25,840	26,454	26,058	0.8
0408	26,556	25,638	26,097	1.8
0409	26,681	26,651	26,667	0.1
0410	26,047	26,585	26,226	0.7
0411	27,303	27,060	27,181	0.5
0412	27,597	27,337	27,467	0.5
0414	28,180	26,826	27,503	2.5
0415	27,590	26,483	27,037	2.1
0416	28,057	28,108	28,083	0.1
0417	27,071	27,685	27,378	1.1
0418	26,671	26,606	26,638	0.1
0419	27,565	28,842	28,204	2.3
0420	24,486	24,572	24,529	0.2
0422	29,839	29,349	29,594	0.8
0423	29,935	29,558	29,746	0.6
0424	25,901	25,836	25,869	0.1
0425	26,567	25,692	26,129	1.7
0426	26,269	25,385	25,836	1.7
0427	26,016	26,304	26,160	0.6
0428	25,762	27,015	26,388	2.4
0429	27,253	27,894	27,574	1.2
0430	26,853	26,327	26,576	0.9
0501	27,661	27,925	27,793	0.5
0502	28,374	28,341	28,358	0.1
0503	24,738	24,957	24,847	0.4
0504	24,664	24,993	24,829	0.7
0505	23,546	24,121	23,834	1.2
0506	25,255	25,125	25,190	0.3
0507	25,162	25,277	25,219	0.2
0508	25,380	25,109	25,244	0.5
0509	24,305	24,721	24,513	0.9
0510	24,366	24,866	24,616	1.0
0511	25,012	25,042	25,027	0.1
0512	24,884	25,511	25,198	1.2
0513	25,277	25,362	25,319	0.2
0514	25,049	25,032	25,040	0.0
0515	24,828	25,979	25,404	2.3
0516	25,187	25,891	25,539	1.4
0517	25,691	25,694	25,693	0.0

[a]Net counts/10 min of progesterone-7-^{3}H (average of three 10 min counts per standard).

A P P E N D I X D

TABLE XXVII

PROGESTERONE DATA FOR COW NO. 07 (mμg/ml PLASMA)

Date	Day of Cycle	Percent Recovery	mμg/ml	$\bar{x}$	Percent Deviation from Mean (±)	Date	Day of Cycle	Percent Recovery	mμg/ml	$\bar{x}$	Percent Deviation from Mean (±)
0403	13	41.9	4.5	4.3	7.0	0415	4	44.2	2.3	2.7	14.1
0403	13	26.3	3.9			0415	4	33.4	3.1		
0404	14	58.8	4.9	4.9	0.1	0416	5	37.1	3.5	3.5	1.8
0404	14	57.1	4.9			0416	5	39.3	3.4		
0405	15	45.0	5.3	5.4	2.4	0417	6	46.2	3.9	4.0	2.5
0405	15	56.9	5.6			0417	6	25.5	4.1		
0406	16	80.0	5.9	6.0	2.8	0418	7	38.6	4.8	5.0	4.3
0406	16	48.0	6.2			0418	7	32.5	5.2		
0407	17	48.0	5.2	5.3	1.9	0419	8	56.4	5.5	5.5	0.2
0407	17	51.8	5.4			0419	8	49.9	5.5		
0408	18	47.0	1.7	1.8	2.9	0420	9	48.3	5.7	5.7	---
0408	18	48.3	1.8			0420	9	----	---		
0409	19	48.0	0.4	0.4	3.5	0421	10	55.9	6.6	6.6	---
0409	19	52.6	0.4			0421	10	----	---		
0410	20	50.1	0.6	0.6	7.9	0422	11	41.3	7.0	6.7	3.9
0410	20	58.4	0.5			0422	11	45.4	6.4		
0411	21	63.6	0.4	0.4	0.8	0423	12	58.0	5.0	5.0	0.7
0411	21	54.4	0.4			0423	12	64.0	5.1		
0412	1	51.1	0.2	0.2	2.5	0424	13	49.0	5.9	6.1	3.4
0412	1	59.9	0.2			0424	13	50.9	6.3		
0413	2	61.9	0.5	0.5	6.2	0425	14	60.1	6.0	6.0	0.5
0413	2	56.2	0.6			0425	14	56.0	6.1		
0414	3	47.6	1.1	1.2	5.1						
0414	3	43.9	1.2								

TABLE XXVIII

PROGESTERONE DATA FOR COW NO. 11 (mμg/ml PLASMA)

Date	Day of Cycle	Percent Recovery	mμg/ml	$\bar{x}$	Percent Deviation from Mean (±)	Date	Day of Cycle	Percent Recovery	mμg/ml	$\bar{x}$	Percent Deviation from Mean (±)
0426	18	48.7	9.4	9.4	---	0507	8	58.6	1.3	1.3	0.3
0426	18	----	---			0507	8	57.9	1.3		
0427	19	56.8	9.8	9.6	2.7	0508	9	53.9	2.2	2.1	4.5
0427	19	57.7	9.3			0508	9	59.2	2.0		
0428	20	44.9	9.0	9.8	8.4	0509	10	55.4	2.8	2.8	1.8
0428	20	36.0	10.6			0509	10	59.5	2.7		
0429	21	37.8	2.9	2.9	1.3	0510	11	60.3	2.9	3.1	5.3
0429	21	42.8	2.8			0510	11	56.5	3.2		
0430	1	31.6	0.3	0.4	18.0	0511	12	44.3	4.1	4.0	1.4
0430	1	48.5	0.4			0511	12	50.2	4.0		
0501	2	68.9	0.3	0.3	---	0512	13	47.4	5.0	4.9	1.4
0501	2	61.7	0.3			0512	13	47.5	4.8		
0502	3	63.3	0.2	0.2	4.4	0513	14	59.0	4.7	4.7	0.6
0502	3	63.9	0.2			0513	14	63.6	4.7		
0503	4	53.8	0.1	0.1	---	0514	15	54.2	6.4	6.0	5.6
0503	4	----	---			0514	15	43.9	5.7		
0504	5	51.0	0.3	0.2	30.7	0515	16	56.0	5.8	5.6	3.4
0504	5	64.3	0.1			0515	16	62.8	5.4		
0505	6	30.0	0.3	0.3	---	0516	17	38.1	6.5	6.1	6.5
0505	6	----	---			0516	17	47.0	5.7		
0506	7	54.3	0.5	0.5	0.9	0517	18	39.9	6.9	6.8	1.5
0506	7	43.7	0.5			0517	18	45.6	6.7		

TABLE XXIX

PROGESTERONE DATA FOR COW NO. 17 (mμg/ml PLASMA)

Date	Day of Cycle	Percent Recovery	mμg/ml	$\bar{x}$	Percent Deviation from Mean (±)	Date	Day of Cycle	Percent Recovery	mμg/ml	$\bar{x}$	Percent Deviation from Mean (±)
0426	18	57.7	0.1	0.1	57.9	0508	9	63.3	6.9	6.9	1.0
0426	18	60.1	0.2			0508	9	54.2	7.0		
0427	19	57.7	0.3	0.3	4.3	0509	10	65.2	6.8	7.0	2.7
0427	19	60.6	0.3			0509	10	59.0	7.2		
0428	20	35.2	0.7	0.7	4.9	0510	11	62.9	7.0	7.0	0.2
0428	20	38.3	0.6			0510	11	56.6	7.0		
0429	21	43.6	0.5	0.6	8.7	0511	12	52.9	7.9	8.1	2.4
0429	21	39.5	0.6			0511	12	42.5	8.3		
0430	1	49.7	0.2	0.2	6.5	0512	13	50.6	8.7	8.5	2.9
0430	1	45.5	0.2			0512	13	55.2	8.2		
0501	2	69.7	0.1	0.1	4.3	0513	14	67.4	7.1	7.3	3.0
0501	2	61.5	0.1			0513	14	65.9	7.6		
0502	3	62.3	0.3	0.3	---	0514	15	58.9	8.8	8.9	1.1
0502	3	----	---			0514	15	57.7	9.0		
0503	4	64.3	0.3	0.3	3.2	0515	16	63.5	7.8	7.4	5.1
0503	4	61.5	0.4			0515	16	44.0	7.0		
0504	5	63.9	1.1	1.0	7.4	0516	17	44.4	8.6	8.5	0.9
0504	5	63.6	1.0			0516	17	50.0	8.4		
0505	6	63.8	2.1	2.1	0.1	0517	18	41.8	6.4	6.4	0.3
0505	6	57.7	2.1			0517	18	40.0	6.5		
0506	7	53.4	4.6	4.7	3.4	0518	19	48.6	3.7	3.8	1.6
0506	7	59.6	4.9			0518	19	51.6	3.8		
0507	8	58.0	5.1	5.1	0.9						
0507	8	58.7	5.2								

TABLE XXX

PROGESTERONE DATA FOR COW NO. 24 (mμg/ml PLASMA)

Date	Day of Cycle	Percent Recovery	mμg/ml	$\bar{x}$	Percent Deviation from Mean (±)	Date	Day of Cycle	Percent Recovery	mμg/ml	$\bar{x}$	Percent Deviation from Mean (±)
0403	14	41.2	7.4	7.5	0.8	0415	5	10.5	5.1	5.3	4.8
0403	14	44.4	7.6			0415	5	5.4	5.6		
0404	15	58.0	7.2	7.0	2.5	0416	6	37.0	4.7	4.1	14.0
0404	15	61.9	6.8			0416	6	45.1	3.5		
0405	16	47.1	10.7	10.2	5.4	0417	7	24.9	6.8	6.5	4.6
0405	16	52.6	9.8			0417	7	30.0	6.2		
0406	17	56.6	6.8	6.9	2.5	0418	8	41.6	6.9	7.0	1.6
0406	17	59.5	7.1			0418	8	36.9	7.1		
0407	18	57.5	1.4	1.4	---	0419	9	53.9	5.6	5.6	0.4
0407	18	----	---			0419	9	52.3	5.5		
0408	19	41.7	0.6	0.6	0.5	0420	10	47.2	6.6	6.6	---
0408	19	28.6	0.6			0420	10	----	---		
0409	20	21.7	0.3	0.3	2.3	0421	11	53.9	7.1	7.1	---
0409	20	33.0	0.4			0421	11	----	---		
0410	21	48.7	0.3	0.3	0.8	0422	12	35.3	8.0	8.1	1.2
0410	21	62.0	0.3			0422	12	42.1	8.2		
0411	1	59.3	0.4	0.4	8.0	0423	13	62.3	6.9	7.0	0.2
0411	1	58.3	0.4			0423	13	57.8	7.0		
0412	2	63.3	1.1	1.1	4.1	0424	14	48.5	3.9	3.8	2.4
0412	2	62.1	1.2			0424	14	51.4	3.7		
0413	3	53.6	1.3	1.4	4.6	0425	15	58.8	5.6	5.7	1.7
0413	3	58.4	1.5			0425	15	54.8	5.8		
0414	4	39.8	2.5	2.6	1.7						
0414	4	40.0	2.6								

TABLE XXXI

PROGESTERONE DATA FOR COW NO. 40 (mμg/ml PLASMA)

Date	Day of Cycle	Percent Recovery	mμg/ml	$\bar{x}$	Percent Deviation from Mean (±)	Date	Day of Cycle	Percent Recovery	mμg/ml	$\bar{x}$	Percent Deviation from Mean (±)
0403	9	36.7	3.8	3.8	1.9	0415	21	----	1.6	1.5	4.7
0403	9	34.4	3.7			0415	21	36.6	1.5		
0404	10	59.1	3.4	3.8	11.5	0416	22	31.2	0.3	0.3	1.8
0404	10	61.0	4.3			0416	22	35.4	0.3		
0405	11	64.2	4.5	4.5	0.2	0417	1	38.9	0.3	0.3	1.5
0405	11	56.9	4.5			0417	1	42.4	0.3		
0406	12	52.6	5.8	5.7	0.9	0418	2	39.7	0.2	0.2	---
0406	12	50.0	5.7			0418	2	----	---		
0407	13	54.1	6.4	6.6	2.4	0419	3	52.7	0.2	0.2	5.7
0407	13	52.0	6.8			0419	3	46.8	0.2		
0408	14	28.0	6.7	6.8	1.1	0420	4	45.8	0.5	0.5	---
0408	14	33.1	6.9			0420	4	----	---		
0409	15	34.1	6.3	6.6	4.7	0421	5	56.4	0.8	0.8	---
0409	15	21.4	6.9			0421	5	----	---		
0410	16	31.8	6.6	6.4	2.6	0422	6	48.0	3.1	3.6	14.6
0410	16	32.1	6.3			0422	6	40.8	4.1		
0411	17	57.5	7.4	7.6	1.6	0423	7	52.6	3.1	3.1	0.1
0411	17	41.1	7.7			0423	7	52.5	3.1		
0412	18	56.5	6.7	7.3	7.8	0424	8	49.3	5.7	5.1	10.1
0412	18	43.7	7.9			0424	8	50.4	4.6		
0413	19	57.3	5.6	5.6	0.8	0425	9	55.0	3.4	3.4	0.1
0413	19	51.3	5.6			0425	9	52.8	3.4		
0414	20	28.8	5.9	5.8	0.2	0426	10	58.7	4.3	4.3	0.1
0414	20	36.9	5.8			0426	10	60.0	4.3		

TABLE XXXI (Continued)

Date	Day of Cycle	Percent Recovery	mμg/ml	$\bar{x}$	Percent Deviation from Mean (±)	Date	Day of Cycle	Percent Recovery	mμg/ml	$\bar{x}$	Percent Deviation from Mean (±)
0427	11	61.3	4.2	4.1	0.7	0508	1	57.2	0.3	0.3	1.8
0427	11	57.1	4.1			0508	1	65.1	0.3		
0428	12	39.7	5.1	5.2	2.2	0509	2	64.9	0.4	0.4	5.8
0428	12	34.7	5.3			0509	2	60.5	0.4		
0429	13	41.1	6.3	6.1	4.1	0510	3	57.0	0.3	0.2	15.6
0429	13	39.2	5.8			0510	3	61.5	0.2		
0430	14	60.0	5.4	5.5	2.1	0511	4	56.6	0.3	0.3	---
0430	14	40.2	5.6			0511	4	----	---		
0501	15	63.9	4.0	4.0	0.9	0512	5	33.2	0.6	0.6	0.5
0501	15	63.1	4.0			0512	5	38.0	0.6		
0502	16	66.8	5.6	5.7	2.8	0513	6	68.0	0.9	0.9	0.2
0502	16	61.9	5.9			0513	6	43.5	0.9		
0503	17	55.3	5.6	5.5	2.2	0514	7	59.9	1.9	1.8	3.3
0503	17	47.6	5.4			0514	7	62.4	1.7		
0504	18	54.0	5.3	5.3	0.2	0515	8	50.8	2.7	2.8	4.3
0504	18	62.1	5.3			0515	8	50.8	2.9		
0505	19	57.7	5.1	5.1	1.4	0516	9	45.7	3.3	3.3	1.1
0505	19	65.2	5.0			0516	9	51.5	3.3		
0506	20	55.1	4.8	4.9	0.6	0517	10	40.7	3.9	3.9	0.3
0506	20	57.4	4.9			0517	10	39.1	3.8		
0507	21	50.2	1.3	1.3	0.8						
0507	21	47.6	1.3								

TABLE XXXII

PROGESTERONE DATA FOR COW NO. 46 (mμg/ml PLASMA)

Date	Day of Cycle	Percent Recovery	mμg/ml	$\bar{x}$	Percent Deviation from Mean (±)	Date	Day of Cycle	Percent Recovery	mμg/ml	$\bar{x}$	Percent Deviation from Mean (±)
0426	10	52.7	6.0	6.1	2.7	0507	21	56.3	0.7	0.7	0.0
0426	10	55.4	6.3			0507	21	54.9	0.7		
0427	11	54.5	8.2	8.5	2.5	0508	22	61.5	0.3	0.3	2.3
0427	11	57.5	8.7			0508	22	61.5	0.3		
0428	12	31.5	7.2	7.3	1.2	0509	23	61.2	0.3	0.3	9.0
0428	12	32.2	7.4			0509	23	55.3	0.3		
0429	13	40.5	8.0	7.5	5.9	0510	1	61.1	0.2	0.2	23.7
0429	13	28.9	7.1			0510	1	43.0	0.3		
0430	14	40.2	10.6	10.2	4.3	0511	2	53.3	0.1	0.2	28.4
0430	14	25.7	9.8			0511	2	45.2	0.2		
0501	15	72.5	7.8	7.2	8.2	0512	3	56.9	0.2	0.2	---
0501	15	50.6	6.6			0512	3	----	---		
0502	16	68.0	7.9	7.8	1.7	0513	4	67.5	0.1	0.2	19.3
0502	16	65.1	7.6			0513	4	62.1	0.2		
0503	17	34.0	7.7	7.4	3.6	0514	5	55.2	0.5	0.5	5.9
0503	17	49.7	7.1			0514	5	48.8	0.6		
0504	18	55.8	7.8	7.6	2.5	0515	6	53.5	0.9	1.0	2.2
0504	18	64.2	7.4			0515	6	55.6	1.0		
0505	19	64.1	7.0	6.9	1.1	0516	7	50.2	1.7	1.6	5.3
0505	19	32.3	6.9			0516	7	56.7	1.5		
0506	20	55.1	6.8	6.9	0.9	0517	8	31.3	2.1	2.2	1.8
0506	20	55.8	6.9			0517	8	27.1	2.2		

A P P E N D I X E

TABLE XXXIII

HEMATOCRIT VALUES FOR COWS (ADJUSTED FOR ANTICOAGULANT)

Cow 07		Cow 11		Cow 17		Cow 24		Cow 46	
Date	Hematocrit	Date	Hematocrit	Date	Hematocrit	Date	Hematocrit	Date	Hematocrit
0325	38.5	0426	31.0	0426	30.5	0325	34.9	0426	24.8
0326	35.2	0427	30.5	0427	34.3	0326	31.1	0427	25.8
0327	35.3	0428	30.2	0428	34.9	0327	30.9	0428	24.2
0328	37.3	0429	29.9	0429	33.8	0328	30.8	0429	25.8
0329	32.0	0430	29.6	0430	33.3	0329	28.3	0430	25.9
0330	37.1	0501	28.9	0501	33.3	0330	33.9	0501	25.7
0331	31.5	0502	27.4	0502	34.0	0331	34.1	0502	23.8
0401	31.4	0503	27.2	0503	31.1	0401	31.1	0503	23.9
0402	33.3	0504	27.8	0504	31.1	0402	28.7	0504	23.7
0403	29.0	0505	27.3	0505	27.2	0403	29.2	0505	24.2
0404	30.4	0506	26.3	0506	32.5	0404	29.7	0506	24.5
0405	31.4	0507	27.2	0507	33.5	0405	32.2	0507	24.8
0406	31.8	0508	25.7	0508	32.4	0406	30.1	0508	25.8
0407	31.0	0509	24.7	0509	30.8	0407	29.9	0509	26.5
0408	31.7	0510	25.1	0510	31.3	0408	32.0	0510	24.9
0409	29.5	0511	24.6	0511	30.9	0409	30.8	0511	25.1
0410	30.8	0512	27.8	0512	31.9	0410	30.9	0512	27.3
0411	32.7	0513	26.5	0513	32.1	0411	30.1	0513	26.9
0412	34.0	0514	25.4	0514	32.8	0412	29.6	0514	27.1
0413	29.8	9515	28.0	0515	32.3	0413	28.1	0515	27.7
0414	30.4	0516	25.3	0516	31.3	0414	29.0	0516	27.2
0415	30.4	0517	25.9	0517	30.5	0415	29.6	0517	25.6
0416	31.4					0416	28.8		
0417	31.4					0417	29.2		
0418	31.5					0418	28.9		
0419	32.0					0419	30.7		
0420	31.3					0420	28.9		
0421	31.0					0421	28.3		
0422	31.9					0422	28.7		
0423	30.3					0423	27.6		
0424	30.3					0424	30.1		
0425	31.7					0425	28.8		

TABLE XXXIV

HEMATOCRIT VALUES FOR COWS (ADJUSTED FOR ANTICOAGULANT)

Cow 40			
Date	Hematocrit	Date	Hematocrit
0325	34.5	0421	29.7
0326	30.5	0422	30.1
0327	30.5	0423	28.0
0328	30.3	0424	29.0
0329	29.3	0425	28.5
0330	34.1	0426	28.9
0331	32.4	0427	28.3
0401	30.0	0428	29.0
0402	29.2	0429	28.9
0403	28.3	0430	28.9
0404	31.7	0501	28.9
0405	30.6	0502	26.9
0406	30.1	0503	27.5
0407	30.8	0504	26.8
0408	32.3	0505	28.6
0409	30.9	0506	27.8
0410	30.2	0507	28.1
0411	30.6	0508	28.3
0412	33.5	0509	26.3
0413	32.0	0510	25.5
0414	31.7	0511	25.0
0415	31.5	0512	29.9
0416	31.7	0513	28.8
0417	30.4	0514	28.2
0418	31.9	0515	29.4
0419	32.1	0516	26.9
0420	31.3	0517	29.5

A P P E N D I X F

TABLE XXXV

DATA SHEET FOR IBM CARD

Experiment name	1. XXX
Date	2. XXXX
Tube number	3. XX
Animal number	4. XXX
Day of cycle	5. XX
Koxalate volume (ml)	6. XX.
Blood volume (ml)	7. XXX.X
Hematocrit (%)	8. XX.X
Plasma volume (ml)	9. XX.X
G. L. C. aliquot	10. .X
Scint. aliquot	11. .X
20β-MCA peak base (cm)	12. X.X
20β-MCA peak height (cm)	13. XX.X
TMCA peak base (cm)	14. X.X
TMCA peak height (cm)	15. XX.X
20β-MCA std. peak area (cm^2)	16. XX.X
TMCA std. peak area (cm^2)	17. XX.X
TMCA internal std. (mμg)	18. XX.
Scint. count 1 (counts/10 min)	19. XXXX.
Scint. count 2 (counts/10 min)	20. XXXX.
Scint. count 3 (counts/10 min)	21. XXXX.
Scint. std. count (counts/10 min)	22. XXXXX.

Date

Isotope

Extraction vol

First TLC

Acetylation

Second TLC

GLC

COMMENTS

% recovery

mμg/ml plasma

TABLE XXXVI

FORTRAN IV COMPUTER PROGRAM FOR CALCULATION OF PLASMA PROGESTERONE CONCENTRATION

```
$JOB   WATFOR      G H STABENFELDT                2739-41701
C      G. H. STABENFELDT, DEPT. OF PHYSIOLOGY
C      CYCLING COW
C      ADJUSTMENT FACTORS IN PROGESTERONE LEVEL DETERMINATIONS
C      WITH MEANS
       DATA IZERO/3H000/
   20 FORMAT(A3,I4,I2,A3,I2,F3.0,F5.1,2F4.1,2F2.1,F3.1,F4.1,F3.1,3F4.1,
      1F3.0,3F5.0,F6.0)
  100 FORMAT(1H ,12X,A3,8X,I4,7X,I2,12X,A3,9X,I2,15X,F8.1,15X,F9.3)
   99 FORMAT(1H1,9X,8HEXP.NAME,5X,4HDATE,5X,8HTUBE NO.,5X,10HANIMAL NO.,
      25X,12HDAY OF CYCLE,5X,16HPERCENT RECOVERY,5X,16HNG.PER ML.PLASMA)
   98 FORMAT(105X,7HMEAN = , F7.3//)
       IDAOLD=1
       ALPHA=0.
       TLPROG = 0.
    5 WRITE(6,99)
       KNT=0
    6 READ(5,20)NAME,IDATE,ITUBE,IANML,IDACYC,OXYVOL,BLDVOL,HMCRT,PLSVOL
      3,GLCALQ,SNTALQ,PB20B,PH20B,PBTMCA,PHTMCA,A20BS,ATMCAS,TMCAIS,SNTCT
      41,SNTCT2,SNTCT3,SNTSTD
       IF(IDAOLD.NE.IDACYC)GO TO 200
  300 IF(IANML.EQ.IZERO)GO TO 7
       VOLLQ=(1.-HMCRT/100.)*BLDVOL
       DILFKR=VOLLQ/(VOLLQ-OXYVOL)
       RKYPC=(((SNTCT1+SNTCT2+SNTCT3)/3.-180.)/((SNTSTD-180.)*SNTALQ))*10
      7J.
       R=1./(RKYPC/100.)
       U=(PH20B*PB20B*.5)/(PHTMCA*PBTMCA*.5)
       C=ATMCAS/A20BS
       A=TMCAIS
       P=1./PLSVOL
       X=1./GLCALQ
       PROG=R*C*U*A*.8*DILFKR*X*P
       KNT=KNT+1
       WRITE(6,100)NAME,IDATE,ITUBE,IANML,IDACYC,RKYPC,PROG
       TLPROG=PROG + TLPROG
       ALPHA=ALPHA+1.
       GO TO 8
  200 AMEAN=TLPROG/ALPHA
       WRITE (6,98)AMEAN
       TLPROG=0.
       ALPHA=0.
       GO TO 300
    8 IDAOLD=IDACYC
       IF(KNT-23)6,5,5
    7 CALL EXIT
       END
$ENTRY
(INSERT DATA CARDS HERE)
00000 0
$IBSYS
```

VITA

George Harold Stabenfeldt

Candidate for the Degree of

Doctor of Philosophy

Thesis: STUDIES ON PROGESTERONE LEVELS IN THE PERIPHERAL BLOOD OF COWS DURING THE ESTROUS CYCLE

Major Field: Physiology

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