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ESTROGEN AND PROGESTIN CONTENT IN THE PERIPHERAL PLASMA AND FOLLICULAR FLUID OF CYCLING BEEF CATTLE

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee

James Joseph Ireland

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ABSTRACT

The objectives of this experiment are as follows: (1) to compare estrogen and progestin levels in the peripheral circulation to steroid levels in the follicular fluid, (2) to compare estrogen and progestin levels within small, medium, and large follicles, and (3) to obtain estrous dates along with morphological ovarian descriptions to use as a basis for the aforementioned comparisons.

There were 180 Hereford heifers (6 per pen) used in this experiment. The animals were slaughtered when the mean thickness of backfat for the animals in a pen (6 per pen) reached 12 mm as measured by the somascope. Blood and follicular fluid were sampled and assayed simultaneously for estrogen and progestin content using radioimmunoassay. Ovarian weights, corpus luteum weights, and follicle numbers were also recorded. Prior to slaughter, all heifers had been observed for estrous acitivities; and following slaughter, each pair of ovaries were classified as to stage of growth (MGU) and inspected for abnormalities.

Progestins and estrogens varied inversely throughout the MGU growth phases, and their patterns, levels, and ranges compared favorably with those previously described in the literature. Only progestin from medium follicles had secretion patterns similar to patterns in the plasma.

There were significant differences ($P<.001$) in estrogen and progestin levels and patterns from different size follicles. Progestins varied significantly among MGU; however, estrogen was quite variable and

showed no statistically significant relationship to MGU. Within size categories, steroid concentrations in the follicular fluid varied independently of volume changes. Follicle numbers per pair of ovaries remained constant for large follicles (one follicle per pair of ovaries) but varied within the small (12-100) and medium (1-6) classification.

Morphological ovarian descriptions (MGU) were used as a basis for comparisons of estrogen and progestin levels in the peripheral circulation with steroid levels in follicular fluid and for comparisons between follicle sizes. Behavioral estrous cycles were extremely variable with 27 percent having short cycles (<15 days) and 37 percent having long cycles (>26 days). Approximately 50 percent of the animals were observed in standing estrus while the other half were not observed standing, but did display a color change in the KaMar Heatmount Detector.

Within the MGU classifications, there was a disproportionality in numbers of heifers in MGU V and VI. This suggests that prior to and at the beginning of regression are the most variable stages in the corpus luteum morphology, thus emphasizing an explanation for variability in the cycle lengths that was observed in the heifer experiment.

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CHAPTER I

INTRODUCTION

Extensive research involving ovarian follicles of various species has been reported toward the elucidation of phenomenon such as luteinization, growth, atresia, and steroid production. Sensitive assays for enzymes and steroids, and *in vitro* techniques compounded with transmission and scanning electron microscopy have been utilized by recent investigators interested in these problems.

Controversy exists over the capacity of follicles to secrete steroids in significant amounts to alter plasma steroid levels, over which cells in the follicle secrete steroids, and recently, over the actual significance of quantitating plasma hormones. The significance of quantitating plasma steroid levels as an indication of target organ function is confounded by the possibility of a blood vasculature system that bypasses the general peripheral system and provides for an independent endocrine-target organ relationship. The ovary-uterus vascular complex may be a good example of this problem.

The role of gonadotrophins in the control of ovarian steroid production, follicular growth, atresia, and luteinization throughout the estrous cycle is unclear. Gonadotrophins may: (1) stimulate the steroid secreting cells of the follicle to produce and release steroids into the follicular fluid and/or plasma, (2) affect peak synthesis by granulosa and thecal cells, or (3) initiate the biochemical events necessary for follicular growth, luteinization, and atresia.

These are unanswered questions which require basic research and when explained, will provide scientists further clarification of the relationship between protein and steroid hormones as well as endocrine organs and target cells.

The purpose of these experiments was to establish the inter-relationship between progestins and estogens in the peripheral blood and in the follicular fluid of normal cycling beef heifers.

CHAPTER II

REVIEW OF LITERATURE

This review includes the following parameters of follicular function: growth, development, atresia, ultrastructural function and endocrine capacity. In addition, a brief review of sex steroids in the plasma of bovine and follicular fluid of various species is included. The review does not include RIA techniques for steroid quantitation since previous work in this area has been well documented (Batson, 1972; Diczfalusy, 1970; Odell and Daughaday, 1971; Peron and Caldwell, 1970).

I. GROWTH AND DEVELOPMENT

Mammalian follicular growth and development have been reviewed extensively (Asdell, 1960; Bjersing, 1967; Brambell, 1956; Hisaw, 1947; Lane and Davis, 1939; Lobel and Levy, 1968; Priedkalns et al., 1968; Rajakoski, 1960). This review will be concerned with mammalian follicular development, with special emphasis on the granulosa cells of vesicular follicles.

Primary Follicular Development

Follicular development has been classified into three stages: primary, growing, and graafian (or vesicular) (Brambell, 1956; Rajakoski, 1960). Primary follicles were characterized as containing an oocyte surrounded by a single layer of granulosa cells. Primary follicles showed no signs of growth. In most mammals oogenesis was generally

considered completed during or immediately after parturition (Brambell, 1956; Erickson, 1966; Hisaw, 1947; Lobel and Levy, 1968; Rajakoski, 1960).

Growing Follicular Development

The growing follicle displayed an increased mitotic rate in the bovine (Rajakoski, 1960); however, neither an antrum nor a thecal layer of tissue was developed (Hisaw, 1947; Rajakoski, 1960).

The primary and growing follicles have been associated with high levels of succinic dehydrogenase, diaphorases, ATPase, alkaline phosphatase and B-glucuronidase (Lobel and Levy, 1968). Steroid dehydrogenases were not found in small ovarian follicles from bovine (Lobel and Levy, 1968). This supports recent reports by Erickson et al., (1974) that granulosa cells from primary follicles of rabbits were endocrinologically inactive and unable to respond to LH-FSH stimuli.

These investigators concluded that prior to follicular antrum formation, the activities of granulosa cells were involved in growth and development of the ovum, not steroidogenesis.

Vesicular Follicle Development

The vesicular follicle was described in most mammals as a structure in which an antrum had formed (Brambell, 1956; Hisaw, 1947; Rajakoski, 1960) from coalescence of small fluid filled cavities among epithelial cells (Brambell, 1956). At that point, the ovum had reached its maximum size (Brambell, 1956). The antrum was lined with several layers of granulosa cells (Brambell, 1956), and thecal cell development had commenced (Brambell, 1956; Hisaw, 1947; Rajakoski, 1960). The granulosa in normal mammalian follicles possessed 6 to 10 layers of cells

and displayed mitotic activity dependent upon the stage of follicle development (Brambell, 1956; Hisaw, 1947; Lane and Davis, 1939; Rajakoski, 1960).

Cell Division

Formation of the theca interna and externa in vesicular follicles of the rat has been characterized (Brambell, 1956). Mitotic activity was greatest during the early stages of follicle development and decreased until ovulation (Rajakoski, 1960; Schmidt, 1942). In defining mitotic activity of the rat granulosa cells, significant differences between follicles of various sizes and stages of development has been found (Lane and Davis, 1939). Follicles of less than 200 micrometers (um) in diameter in the rat were mitotically inactive (Lane and Davis, 1939). Granulosa cells of small follicles in the guinea pig were inactive regardless of the cycle stage (Schmidt, 1942). Colchicine has been used to arrest mitosis, and the most mitotically active granulosa was observed in metestrus rat follicles (300 to 400 um in diameter) (Bullough, 1942; Lane and Davis, 1939). The mitotic index of the granulosa cells demonstrated its greatest depression during proestrus (Lane and Davis, 1939). Several investigators (Land and Davis, 1939; Rajakoski, 1960; Schmidt, 1942) agreed that mitosis was not directly related to follicular size but was more dependent upon cycle stage and hormonal influence. Mitotic activity in bovine granulosa cells was reported to have undergone extreme individual tissue variations as well as a diurnal rhythm (Lobel and Levy, 1968). Mitoses have been reported to be absent in proestrus, to have increased from Day three

to four, and maximized or disappeared by Day eight in bovine graafian follicles (Asdell, 1960).

Mitotic activity was highest when bovine follicles reached three to four mm with a continuous decrease in mitotic rate until ovulation (Rajakoski, 1960; Priedkalns et al., 1968). Most researchers agreed that follicular development proceeded to antrum formation in hypophysectomized animals (Simpson, 1959) or to the vesicular stage in intact mammals (Brambell, 1956; Dempsey, 1937; Hisaw, 1947; Rajakoski, 1960), independent of hormonal control. Recent evidence in rabbits, pigs, and monkeys (Channing and Kammerman, 1973; Erickson et al., 1974; Kammerman et al., 1972) suggested that the granulosa cell layer of primary and developing follicles was unable to respond to hormonal stimulation due to an absence of receptors and obligatory enzymes.

Growth Rate

The follicular growth rate of mouse follicles has been investigated (Pedersen, 1969). Autoradiographs were utilized to investigate granulosa cells prepared after pulse-labeling with tritiated thymidine (Pedersen, 1970a, b). Follicular development was not constant from birth until maturity, and follicles grew faster prior to puberty. Small follicles of mice were surrounded by single layers of granulosa cells and required 16 days to develop into large follicles characterized by several layers of granulosa cells and by cavity formation (Pedersen, 1970a, b).

In the guinea pig, follicular growth was constant for the first 12 days; increased for the next three to four days; and, at the beginning

of estrus, underwent extreme proliferation culminated by ovulation (Dempsey, 1937). In 116 cows studied, the number of primary follicles averaged 133,000 until the fourth year of life and reached the zero point in cows at 15 to 20 years of age (Erickson, 1966). Numbers of growing follicles increased between 50 and 80 days after parturition up to 120 days, then declined simultaneously with primary follicles during the fourth year. Vesicular follicles increased in number concurrently with growing follicles with no noticeable decline in numbers until the cows were 10 to 14 years of age (Erickson, 1966).

The growth rate of bovine follicles has been described as continuous and constant from one to 12 mm in diameter (Marion et al., 1968). Approximately 24 hours prior to ovulation in the cow, the ovulatory follicle increased from 10 mm in width to a diameter of 16 to 18 mm (Marion et al., 1968).

According to Dufour et al., (1972), the probability of the largest follicle ovulating in the bovine increased significantly beginning at Day 18 of the cycle. However, prior to Day 18 no growth pattern could be discerned (Dufour et al., 1972). This contrasted with Priedkalns' et al., (1968) suggestion that the ovulatory follicle began its development at midcycle and that 10 days were needed for development from a primary to a graafian follicle. Hammond (1927) suggested two cycles were required. Choudary et al. (1968) utilizing 21 cows could not identify a cyclical growth pattern in follicles greater than five mm in diameter as previously reported by Rajakoski (1960) during the bovine cycle. The contention of completely randomized follicular growth (Choudary et al., 1968) might be critized because

it was based on the utilization of a single cow per day of the cycle.

No distinct seasonal variation has been found among follicles five mm or larger in diameter during the winter or spring (Rajakoski, 1960). However, there was a significant increase in follicle numbers from autumn to winter. Individual variations within seasons supplied the greatest amount of variation (Rajakoski, 1960; 1961). He concluded from two separate studies (Rajakoski, 1960; 1961) that follicles underwent two growth waves: at three to four days and at 12 to 14 days. Follicular growth to one mm in diameter was rapid but variable, and growth from one to 12 mm appeared continuous and without growth waves (Rajakoski, 1960; 1961) in 700 dairy cows examined (Marion et al., 1968). Continuous growth in follicles has been observed in guinea pigs during the first 12 days of the cycle (Dempsey, 1937) and in bovine throughout the cycle (Priedkalns et al., 1968). In direct contrast to research reported by Brambell (1956) and Hisaw (1947) in which hypophysectomy had no effect on the rat follicle development, Dempsey (1937) reported that hypophysectomy stopped all follicular growth in the guinea pig.

II. ATRESIA

Follicular atresia has been reviewed extensively (Allen et al., 1930; Asami, 1920; Choudary et al., 1968; Erickson, 1966; Hisaw, 1947; Marion et al., 1968; Rajakoski, 1960; Schmidt, 1942; Wiltbank et al., 1953) and has been described as an intriguing physiological process that appeared necessary for normal ovarian function (Rajakoski, 1960). Waldeyer (1870), according to Asami (1920), described the process as a retrogression by which the ova and follicles degenerated and disappeared. Follicular degeneration has been studied in the rabbit

(Asami, 1920), cow (Asdell, 1960; Erickson, 1966; Lobel and Levy, 1968; Marion et al., 1968; Priedkalns et al., 1968; Wiltbank et al., 1953), human (Allen et al., 1930; Sturgis, 1950), snakes and birds (Brambell, 1956), and pigs (Allen et al., 1925).

Rate of Atresia

Ovaries from 50 swine possessed at least 45 follicles that averaged three mm in diameter (Allen et al., 1925). Ten to 14 days prior to ovulation, atresia eventually eliminated 85 percent of these follicles. Variations in numbers of atretic follicles corresponded to various cycle stages in mice (Engle, 1927). The maximum atresia correlated to the first day of the cycle and the minimum with the second day of diestrus (Engle, 1927). The ovulatory follicle of the rat and guinea pig was the one that survived extensive atresia. It became identifiable in the rat by the third day of the cycle (Lane and Davis, 1939) and in the guinea pig by the twelfth day (Schmidt, 1942). In primates, the majority of follicles seven mm in diameter showed definite signs of atresia regardless of menstrual cycle stage (Allen et al., 1930). Sheep and cattle had atretic and normal follicles that occurred in the same ratio during all phases of the estrous cycle (Cole, 1930). Follicular growth and atresia were considered independent of the stage of estrous cycle in 71 cows examined (Cole, 1930; Priedkalns, et al., 1968). According to Rajakoski (1960), there was only one aspect of atresia in sheep and cattle on which all researchers agreed. After ovulation, the large follicles degenerated, and a new wave of follicular growth commenced.

Primary Follicle Atresia

Follicular atresia has been described in the bovine during the primary, growing, and vesicular stages (Rajakoski, 1960). Fatty alteration in the oocyte, differential cytoplasmic and nuclear staining characteristics, chromatolysis and pyknosis were criteria commonly used to describe primary follicular atresia in most mammals (Asami, 1920; Brambell, 1956; Erickson, 1966; Rajakoski, 1960). Oocyte degeneration always resulted in atresia except in extremely small follicles (Brambell, 1956; Rajakoski, 1960); whereas, follicular atresia was reported to have preceded oocyte degeneration in the cow (Marion et al., 1968). In rabbits, small follicles degenerated as a result of phagocytosis of the oocyte by granulosa cells (Asami, 1920).

Growing Follicular Atresia

Atresia in growing follicles has been characterized mainly by hyaline clumping and degenerative alterations similar to those depicted for primary follicle atresia (Rajakoski, 1960). The earliest signs of atresia in the bovine were loosening and sloughing of the granulosal layer, disintegration of the membrana propria, formation of Call-Exner bodies and configurational changes in the thecal cells (Marion et al., 1968).

Vesicular Follicle Atresia

Atresia of the follicle wall was concomitant with oocyte degeneration (Brambell, 1956). Moreover, oocyte degeneration always preceded follicular atresia (Nalbandov, 1964). This class of follicles was also considered atretic when fibroblastic invasion of the antrum appeared

(Erickson, 1966). In guinea pigs (Schmidt, 1942) and rabbits (Asami, 1920), the first evidence of degeneration occurred in the granulosa cells.

The criteria for vesicular follicle atresia in the cow are: (1) a decreased mitotic index in the granulosa layer (Rajakoski, 1960), (2) clumping of nuclear chromatin (Erickson, 1966), (3) dissemination of the granulosa cells, (4) sloughing of cells into the follicular cavity, (5) follicle shrinkage, (6) eventual thecal cell degeneration, and (7) phagocytic invasion of the oocyte (Asami, 1920; Schmidt, 1942).

Large follicle degeneration in the human has been described as follows (Allen et al., 1930): (1) a cloudy or gelatinous liquor folliculi, (2) dispersed granulosa cells, (3) granulosal villi, and (4) cumulus disruption and granulosal cell invasion into the oocyte. In large bovine follicles a rapid type of atresia occurred (Marion, et al., 1968; Rajakoski, 1960) in which the granulosa rapidly degenerated and thecal cells extended inward and eventually filled the follicular cavity (Brambell, 1956). As atresia began in large bovine vesicular follicles, lysosomal enzymic activity increased and granulosa cells underwent luteinization (Lobel and Levy, 1968).

Cystic Follicular Atresia

Cystic follicular atresia in the bovine ovary has been described as follows (Marion et al., 1968; Rajakoski, 1960; Wiltbank et al., 1953): (1) the membrana propria remained intact, (2) the granulosa was maintained as a single layer of cells, and (3) there was no noticeable reduction in follicle size. However, Marion et al. (1966) reported

that follicular expansion occurred with a concomitant stretching of the theca interna.

Vesicular atresia occurred in various sized follicles with a subsequent increased diameter twice that calculated during the onset of atresia (Marion et al., 1966; Rajakoski, 1960). In 135 cows, 11.3 percent possessed atretic cystic follicles greater than 25 mm in diameter (Wiltbank et al., 1953). Line of breeding, reproductive period, stage of postpartum interval, month, and individual variations all affected the incidence of large atretic follicles. Breeding efficiency was also affected significantly in cows with degenerated large follicles (Wiltbank et al., 1953).

Causes of Atresia

In mammals follicular atresia has been postulated to be the result of an inadequate differentiation of the theca interna produced by inefficient LH stimulation (Hisaw, 1947). Schmidt (1942) surmised that if estrogen was produced in the theca interna and was responsible for granulosa growth, then inadequate theca differentiation would result in granulosa deformation (Hisaw, 1947). During the primate cycle, E formation has been linked with the onset of atresia (Sturgis, 1950).

Follicular atresia, according to the literature reviewed, appears completely random and involves complex structural degeneration. Normal growth and atresia occur at a seemingly equal ratio; however, this aspect has not been adequately researched. Hormonal control of atresia is suggested (Hisaw, 1947; Rajakoski, 1960; Schmidt, 1942; Sturgis, 1950), but complete dilineation of this problem has not been

undertaken. Purity of hormonal preparations (Hisaw, 1947; Rajakoski, 1960; Schmidt, 1942; Sturgis, 1950) can be subjected to criticism as well as their attempts to attribute follicle growth responses to "pure" LH, "pure" FSH, and/or E.

Criteria for the characterization of atresia has been provided by many authors (Brambell, 1956; Choudary et al., 1968; Erickson, 1966; Hisaw, 1947; Rajakoski, 1960). These individuals used varied classifications which served to confuse the literature and made any attempt toward standardization of characteristics for atresia improbable.

III. MICROSTRUCTURAL EVENTS CORRELATED WITH GRANULOSA CELLS

Structure

Granulosa cells of the monkey have been observed with electron micrographs (Crisp and Channing, 1972). Those from large pre-ovulatory follicles on Days 11 and 12 were described as small polyhedral shaped cells 10 microns in diameter. The cells contained a low cytoplasmic to nuclear ratio, had a high mitotic rate, and possessed a dense nuclear heterochromatin pattern. The cytoplasm contained endoplasmic reticulum, ribosomes, mitochondria, and lipid droplets (Crisp and Channing, 1972). Bovine granulosa cells contained lipid bodies which were more concentrated and larger in atretic follicles than in normal follicles (Priedkalns and Weber, 1968). These observations suggested inactivity in steroid synthesis during atresia (Short, 1964). Prior to ovulation, granulosa cell cytoplasm was reported as being rich in "ergastoplasm" (granular endoplasmic reticulum) and free ribosomes. These suggested activity of a protein secretory and mitotic nature (Birdbeck and Mercer, 1961).

Zonulae occludentes were also observed as a connecting network between granulosa cells (Crisp and Channing, 1972; Espey and Stutts, 1972; Merk et al., 1973).

Rabbit granulosa cells were collected from estrous follicles for histochemical examinations (Guraya, 1968a, b). They were high in lipid bodies, mitochondria, and cytoplasmic RNA. After stimulation with HCG, granulosa cells were in contact with blood capillaries and frequent mitotic divisions were observed.

Microstructural Events

The electron microscope was used to show that microvilli of the egg interlaced within the zona pellucida to microvilli of the granulosa (Anderson et al., 1974; Adams and Hertig, 1964). This relationship was described as important in terms of oocyte nutrition and informational transfer (Baker and Neal, 1972; Priedkalns and Weber, 1968).

Medium-sized cow and pig follicles were cultured and examined (Foote and Thibault, 1969). In this experiment it was observed that oocytes remained in the dictyate stage for the duration of granulosa cell viability. Their work suggested that the granulosa cells were responsible for maintenance of mature oocytes in the dictyate stage of development (Foote and Thibault, 1969).

Whole follicles containing ova, transplanted into the eye of rats did not luteinize, while follicular tissue devoid of an oocyte did luteinize (Falck, 1959). Ovectomy of a single follicle on the pig or rabbit ovary allowed transformation into tissue morphologically similar to luteal tissue (Nalbandov, 1972). Granulosa cells of rats

were cultured with and without oocytes (Nekola and Nalbandov, 1971). Luteinization and fibroblast formation occurred in those granulosa cells which were devoid of oocytes. This suggested that luteinization occurred independent of hormonal control and that the ovum produced a luteostatic substance that prevented luteinization of the granulosa cells (Nalbandov, 1972; Nekola and Nalbandov, 1971).

Cytoplasmic structures were transferred via zonulae occludentes which connected granulosa cells in the rabbit follicle (Espey and Stutts, 1972). These structures contained endoplasmic reticulum, lipid droplets, and mitochondria (Espey and Stutts, 1972); nevertheless, no physiological function has been proven for these features. Espey and Stutts (1972) suggested: (1) transportation of nutrients across the granulosa layer, (2) conveyance of follicular fluid into the antrum, or (3) the perpetuation of membrane potentials across the granulosa cells as theoretical functions of the cytoplasmic junctions.

Intercellular junctions similar to zonulae occludentes but identified as nexuses have been observed in granulosa tissue of rat ovaries (Merk et al., 1973). Dreifuss et al., (1966) as reported by Merk et al. (1973) demonstrated that nexuses electrically coupled adjacent cells and were possibly important in intercellular communication. It was further suggested that nexuses played a role in granulosa cell development by allowing transversion of nucleotides, especially cyclic 3', 5' adenosine monophosphate (cAMP) across the cells (Merk et al., 1972). An interesting feature was the significant appearance of eosinophils, plasma cells, and mast cells in follicles during the preovulatory period of follicular development, suggesting an immune-type

response during ovulation (Priedkalns et al., 1968).

IV. ENDOCRINE FUNCTION OF GRANULOSA CELLS

The endocrine function of granulosa cells has been subjected to close scrutiny (Bjersing, 1967; Channing and Kammerman, 1973; Crisp and Channing, 1973; Erickson et al., 1974; Hisaw, 1947; Ryan and Short, 1965). Culturing techniques and sensitive steroid assays have led to a realization of the complexity of granulosa cells in steroid production. Recent researchers have investigated hormonal binding sites (Channing and Kammerman, 1973), hormonal control of follicle growth and structural alterations (Guraya, 1968a, b; Laster, 1972; Merk et al., 1972; Ryle, 1972), luteinization (Crisp and Channing, 1972; Jones and Nalbandov, 1972), and steroidogenic activity of granulosa cells (Channing, 1969; Erickson et al., 1974; Lacroix et al., 1974; Moor, 1973; Younglai, 1972b).

Binding Sites

Porcine granulosa cells from small (1-2 mm diameter), medium 3-5 mm diameter), and large (6-12 mm diameter) follicles were incubated with ^{125}I-HCG (Channing and Kammerman, 1973). Granulosa cells from large follicles bound 10 to 1,000 times more ^{125}I-HCG (623,000 $\pm$ 44,890 cpm/5x10^6 cells, n=12) when compared to those of small follicles (7,554 $\pm$ 1,983 cpm/5x10^6 cells, n=16) of the same ovary. Granulosa cells from medium-sized follicles bound slightly more ^{125}I-HCG than those from small follicles. Kammerman et al. (1972) reported that in vitro HCG and LH were bound to similar receptor sites which appeared to be

different from FSH receptor sites. LH stimulation of granulosa cells from small porcine follicles (1-2 mm diameter) did not increase cellular cAMP; however, LH did increase cAMP levels in granulosa cells from large (6-12 mm diameter) follicles (Kolena and Channing, 1972). This suggested the inability of LH to stimulate cAMP or P secretion by porcine granulosa cells from small follicles was due to a deficiency of LH receptors (Channing and Kammerman, 1973). Low LH receptor numbers reduced the adenyl cyclase response and steroidogenesis (Erickson et al., 1974; Marsh et al., 1972).

Steroidogenesis

Equine granulosa and theca cells from pre-ovulatory follicles (3-5 mm diameter) were incubated with labeled testosterone (testosterone 4-^{14}C) (Ryan and Short, 1965). Granulosa cells converted testosterone to E_1 or E_2 at a higher yield than thecal cells. Testosterone was also converted into E by a granulosa cell tumor from a human female (Marsh et al., 1962) and in rats (Oakey and Stitch, 1967).

Falck (1959) implanted granulosa and thecal tissue, conjointly and individually, into the eyes of several rats. A section of vaginal mucosa was used to indicate E stimulation. Vaginal cornification occurred only in the tissue that contained both cell types (Falck, 1959). That suggested E production required granulosa and theca cells; whereas, P synthesis required only luteinized granulosa cells (Falck, 1959; Short, 1964). However, Marsh et al. (1962) investigated a granulosa cell tumor from a human female and contended that E production occurred mainly in luteinized granulosa cells.

Granulosa cells from four estrous mares at slaughter were incubated with labeled ^{3}H-pregnenolone and ^{14}C-androstenedione (Younglai, 1972). Conversion of androstenedione to E_2 and E_1 was minimal; whereas, the conversion of pregnenolone to P_4 was higher. Porcine granulosa cells could not produce E from pregnenolone (Bjersing, 1967). This report agrees with other data (Channing and Grieves, 1969; Short, 1964; Younglai and Short, 1970) that equine granulosa cells have an active 3-B-hydroxysteroid dehydrogenase system and a reduced aromatizing system (Bjersing, 1967). In contrast, other authors (Channing, 1969; Lacroix et al., 1974; Ryan and Short, 1965) reported that equine and bovine granulosa cells had a good aromatizing activity. Several investigators concluded that granulosa cells converted pregnenolone to P_4 (Bjersing, 1967; Short, 1964; Younglai, 1972; Younglai and Short, 1972) and that E was formed from androstenedione or testosterone (Lacroix et al., 1974; Oakey and Stitch, 1967). However, E was reported not to be formed from P (Bjersing, 1967; Channing, 1969; Ryan and Short, 1965). This suggested a two-cell cooperation from the thecal and granulosa layers in steroid hormone biosynthesis (Bjersing, 1967).

Rabbit granulosa cells after stimulation by DBC, gonadotrophins, or PG secrete neither E_1 or E_2 from one to 30 days old, nor P_4 from newborn to seven days old (Erickson et al., 1974). However, granulosa cells from seven to 30-day-old rabbits did respond to gonadotrophins and DBC stimulation by increasing P_4 secretion 5-10 times. Granulosa cells from preantral follicles of 200-day-old rabbits did not secrete P_4. This suggested that granulosa cells differentiate the capacity to secrete

P and to respond to hormone stimulation only as antral formation occurred (Erickson, 1974).

Hormonal Control of Follicular Growth

Twenty-one Holstein heifers were treated with porcine or bovine FSH (Laster, 1972). All treated animals had more follicles with a diameter larger than 5 to 10 mm than did the control group. Ovaries of infantile mice were cultured with no hormone, LH, or FSH for two or five days (Ryle, 1972). Tritiated thymidine was injected into the culture medium during the last 24 hours of culture. Autoradiographs demonstrated that cell division was stimulated by FSH in follicles of all sizes; however, LH did not stimulate division in primary, one, or two-cell layered follicles (Ryle, 1972).

Thirty-two sexually immature female rats of which 24 were hypophysectomized were subjected to E treatment (Merk et al., 1973). There was an increased number of large follicles and a reduction in the number of small follicles after estrogen treatment. A comparison between E treated versus non-treated granulosa cells of hypopysectomized and intact rats was made. The rough endoplasmic reticulum cisternae were more pronounced in the estrogen treated cells of intact rats and suggested an increased metabolic activity (Merk et al., 1972). Increased numbers of nexuses were also reported in hypophysectomized and intact E_2 treated rats and suggested a more confluent intercellular organization. Merk et al. (1972) speculated that the function of estrogen in the rat was to prepare the granulosa cells in vivo for further differentiation via pituitary hormones. Granulosa cells stimulated by E ceased to

function as isolated units and became an intercellularly connected network (Merk et al., 1972).

Luteinization

Luteinization was induced in vivo in transplanted granulosa cells from rats by injections of DBC and LH (Ellsworth and Armstrong, 1973). The follicles were incubated with DBC, LH, or in Krebs-Ringer bicarbonate buffer system, then transplanted into the kidney capsules of hypophysectomized female rats. Follicles subjected to LH or DBC decreased their nexuses between cells, luteinized, and produced P; whereas, those cells in buffer solution regressed (Ellsworth and Armstrong, 1973; Nalbandov, 1972). Prolactin administration increased P_4 content of the LH and DBC-incubated transplants. Ovulation did not occur in the ectopic follicles of rats (Ellsworth and Armstrong, 1973); however, rabbit follicles ovulated when injected in vivo with LH or FSH or in combination (Jones and Nalbandov, 1972). It was not reported whether luteinization occurred as a consequence of oocyte degeneration (Nekola and Nalbandov, 1971).

Actinomycin D and LH administered simultaneously prevented luteinization in rabbit follicles (Keyes et al., 1972). Prostaglandins E_1 and E_2 activated adenyl cyclase in ovarian tissue (Kuehl et al., 1970). An increased cAMP production upon PG administration (Kolena and Channing, 1972) and luteinization of follicles by prostaglandin E_2 occurred. Prostaglandins, cAMP, FSH and LH appear interrelated in the luteinization process of follicular cells.

Summary

Hormonal control of follicular growth has yet to be delineated. The postulation that the ovary has two sets of controls (Hisaw, 1947), one internal and one external, has been somewhat elucidated. External control via the pituitary hormones, LH and FSH, facilitate an increased steroidogenesis and growth. However, recent evidence (Gospodarowicz et al., 1974) suggests the possibility of mitogenic agents other than LH in commercially prepared hormones. The ovum's luteostatic secretion (Nalbandov, 1972) or a product-substrate reaction involving estrogen control of intercellular communication (Merk et al., 1972) are indicative of internal controls.

The two-cell theory of estrogen synthesis (Falck, 1959), even though assessed indirectly, still has credence with the possible exception of the horse (Younglai, 1972b). The explicit location of estrogen or progesterone steroidogenesis would seem an easy problem to decipher; however, impure cell populations, in vitro culturing problems, and passive ovum control of follicle cells have hindered progress in this area.

V. STEROID LEVELS IN THE FOLLICLE

Steroids in follicular fluid have been reviewed in comparative species (Edwards, 1974), specifically, in cattle (Short, 1962b) and in sheep (Seamark et al., 1974).

Follicular fluid is composed of excretions from the granulosa and thecal cells plus an exudate from the plasma (Edwards, 1974). Its importance is not only as a medium for late ova nourishment but as

a reflector of cellular secretory activity. Estrogen, androgens, P, and their metabolites have been identified in FF (Short, 1964). This section is concerned with E and P levels as they relate to ovarian and follicular morphology as well as the physiology of the estrus or menstrual cycle.

Relation of Estrogen and Progesterone to Ovarian Morphology

Estrogen was quantitated by GLC and was reported higher (9.2 ug/g of follicle) (England et al., 1973) in the largest follicle in the ovary without a CL than in the ovary with a CL in Angus heifers. This situation reversed on Day 14 (3 vs 6.1 ug/g) and suggested a possible local effect of CL-associated P on follicular E secretion. Supporting this contention, Baird and Fraser (1974) reported that 95 percent of the circulating E originated from secretions either by the preovulatory follicle or the postovulatory CL from a single ovary in women.

Estradiol 17B was the major E in FF of cattle (Short, 1962) while in cattle and humans, ovarian follicles were suggested as the major source of E during the follicular phase of the cycle (England et al., 1973; Giorgi, 1967). Removal of the largest follicle on the bovine ovary resulted in a decline of peripheral E levels (Bjersing et al., 1972). This report supported the hypothesis that large follicles are the major site of production of E and P during the proliferation phase, as opposed to the idea of the CL being the major site of P production during the luteal stage of the cycle (Sanyal et al., 1974; Short, 1962). Brand (1970), according to Edwards (1974), reported E_2

fluctuations characteristic of follicle growth waves in sheep. Statistical analyses of the apparent peaks were not reported; hence, it is not clear how populations respond or vary. Low quantities of steroids were also found in atretic bovine follicles when compared to normal follicles (Short, 1962).

Size and Steroid Content

Steroid levels of E and P are 10 to 100 times more concentrated in FF than plasma (Abraham, 1970; Baird and Frasher, 1974; Eiler and Nalbandov, 1973; England, et al., 1973; Korenman et al., 1973). Follicular fluid from 44 bovine follicles of various volumes (.1-6 ml) was aspirated and assayed (Korenman et al., 1973). A coefficient of correlation of -.632 for P vs E, and their ranges (E=0-136 ng/ml of FF; P=0-879 ng/ml) were reported. From 13 ovarian samples during the early proliferative phase (Days 3-9) of normally menstruating women aged 24 to 44 years, a radioimmunoassay technique was used to quantitate E and P levels in various-sized follicles (5-21 mm diameter) pooled into three periods representing the 27-day menstrual cycle (Sanyal et al., 1974). The mean steroid values for 22 follicles (5-10 mm diameter) increased progressively (P=66-130 pg/ul; E_1=36-172 pg/ul; E_2=38-262 pg/ul). Thirty-six follicles from twelve late, proliferative phase ovarian samples (Days 10-14) showed that P, E_1, and E_2 concentrations increased (50-200 pg/ul) in follicles 4 to 21 mm in diameter. Estradiol 17B reached a maximum concentration (1,354 pg/ul) in 14 to 21 mm follicles. Progesterone, E_1, and E_2 showed little fluctuations and remained below 100 pg/ul in all follicles (5-13 mm) during the luteal

stage (Days 15-27) of the cycle. Follicles 1 to 4 mm in diameter had P and E concentrations (P=45±5 ug/ml; E=17±2 ug/ml) less than those in follicles 4.1 to 11 mm in diameter (P=386±59 ug/ml; E+116±4 ug/ml) in 180 pooled porcine FF samples (Eiler and Nalbandov, 1973).

Estrogen and Progesterone in Preovulatory Follicles

Many investigators (Ross et al., 1970; Sanyal et al., 1974; VandeWiele et al., 1970; Yussman et al., 1970) have reported a preovulatory rise of P and E in humans and cattle in plasma and FF (Lunaas, 1964). Gonadotrophins also accumulated in preovulatory follicles (Bjersing et al., 1972) of sheep and stimulated an increased level of E and P in FF (Edwards, 1974). However, Short (1962; 1964) reported that while concentrations of steroids in the bovine declined in preovulatory follicles, the levels varied immensely among follicles and between animals. Progestins quantitated by CPB and E quantitated by RIA were assayed in FF of sexually mature New Zealand white rabbits following mating (Younglai, 1972α). A significant rise at three hours (E=60.2±4.6-271.3±3.7 pg/ml of FF; P=.75±.13-1.53±.26 ng/mg) and a decline at six hours (E=30.3±7.8 pg/mg; P=.40±.16 ng/mg) after coitus was noted. This correlated well with the LH-stimulated E_2 depletion reported in pig follicles (Eiler and Nalbandov, 1973) and observations that E_2 increased twofold in the ovarian vein of rabbits within three hours ofter coitus (Hilliard and Eaton, 1971). Luteinizing hormone given in the peripheral circulation (40 ug/kg) or intrafollicularly (100 ug/follicle) stimulated a decline in E_2 in seven hours in porcine follicles (Eiler and Nalbandov, 1973). The conclusions were that LH

stimulated a release of E_2 and P into the ovarian vein rather than stimulating steroid synthesis by granulosa cells as suggested by Channing (1969). No mention, however, was made as to the stage of the porcine cycle at the time of LH injection, the effect LH had on P concentration (other than it being docimetric), or if steroid synthesis was stimulated prior to seven hours.

Estrogen and Progesterone During the Cycle

Follicular fluid was aspirated from six gilts laparotomized every three days of the 21-day estrous cycle (Eiler and Nalbandov, 1973). Estrogen began to peak on Day 17 (147±40 ng/ml) and remained high until the second day of estrus (27±3 ng/ml). Progesterone rose from Day 8 to Day 11 (32±3-408±95 ng/ml) and remained high through the second day of estrus. Estrone, E_2, and P_4 levels were quantitated in FF of ovaries from women during different stages of the menstrual cycle, via RIA techniques. Mean progesterone values from pooled follicular fluid samples rose from Day 4 to Day 11 (154-1042 ng/ml), then dropped sharply and remained low between Days 14 to 27. Mean concentrations for E_2 were constant (1000 ng/ml) from Days 4 to 9, rose (1300 ng/ml) on Day 12, declined to baseline values (500 ng/ml) by Day 14, and remained at base until Day 27. Estrone remained constant (250 ng/ml) from Days 4 to 11, peaked on Day 12 (500 ng/ml), then fell to baseline (250 ng/ml) by Day 14, remaining low through Day 27 (Sanyal et al., 1974).

Steroids in FF of humans (Sanyal et al., 1974), hogs (Eiler and Nalbandov, 1973), and sheep (Seamark et al., 1974) are extremely

concentrated, fluctuate with the estrus cycle, maintain a proportionality with the size of the follicle, and support the peripheral levels of hormones. It appears that E and P synthesis, accumulation, and release by the follicle are controlled by gonadotrophins, mainly LH.

VI. STEROIDS IN BOVINE PLASMA

Plasma E and P levels have been simultaneously quantitated in cycling cattle (Batson, 1972; Corah et al., 1974; Dobson et al., 1973; Dobson et al., 1975; Echternkamp and Hansel, 1973; Glencross et al., 1973; Hendricks et al., 1971; Lemon et al., 1975; Wetteman et al., 1972). This section of the review will focus on E and P levels in the bovine throughout the estrous cycle. Special emphasis will be paid to CL growth as it relates to P and E production since follicle growth and its relationship to steroid production has been discussed in the previous section.

Corpus Luteum Size and Plasma Progesterone

Corpus luteum growth, CL progesterone content, and ovarian venous P content correlated positively with peripheral levels of P (Erb and Stormshak, 1961; Foley et al., 1964; Gomes et al., 1963; Hafs and Armstrong, 1968; Mares et al., 1962; Swanson et al., 1972). The mean CL weights from 40 normally cycling beef and dairy cattle were highly correlated with the stage of the estrous cycle (Foley, 1964). In most cattle studied, the CL gains in weight and diameter (0-6.8g) from Day 2 to Day 14 remained constant until Day 16 to 18, then declined precipitously in weight (6.8-0g) (Erb and Stormshak, 1961; Erb et al.,

1971; Foley, 1964; Gomes et al., 1963; Hafs and Armstrong, 1968; Mares et al., 1962; Swanson et al., 1972). Progestin levels appear to correlate with CL growth and development throughout the cycle. However, insensitive assays and variability between results of authors prohibit any summary of these results (Erb and Stormshak, 1961; Erb et al., 1971; Gomes et al., 1963; Hafs and Armstrong, 1968; Mares et al., 1962).

Most recently it has been reported that P content increased from Day 3 to 12, remained constant through Day 16, then regressed (Erb et al., 1971).

The RNA/DNA ratios in CL dropped significantly from Day 13 to Day 17 (1.36-1.01 mg/g of CL) and was closely associated with the P peak (Mares et al., 1962). However, the concentration of RNA and DNA throughout the cycle did not differ significantly. In contrast, Hafs and Armstrong (1968) reported that DNA increased from Day 2 until ovulation (1.78±.22-4.40±.75 mg/g), somewhat approximating a growth curve. The ratios of RNA/DNA were highest from Day 2 to Day 7 (1.23±13-1.11±.12 mg/g), then dropped steadily from Day 11 to estrus (.82±.05-.63±.02 mg/g).

The CL has been shown to increase in size after ovulation, peak approximately by Day 11 or 12, and begin regression by Day 16 to 19. Progesterone content of the ovary, the periphery, and the ovarian venous pattern (Dobrowolski et al., 1968) closely mimick this sequence.

Plasma Progesterone

Progesterone has been reviewed prior to 1963 by Gomes and Erb (1963) and recently by Batson (1972) and Hansel and Echternkamp (1972).

In a summary of data on 30 Holstein heifers (2-3 yr.) and cows (3-5 yr.), peripheral P_4 was quantitated via CPB and GLC analysis throughout their 21-day estrous cycles. Progesterone rose from Day 4 to 12 (4-5 ng/ml), remained constant through Day 16, then dropped precipitously and remained low (.25 ng/ml) until one to two days after estrus. Progestin was quantitated via CPB during five estrous cycles of seven nonlactating dairy cows (Hendricks et al., 1970a). Progesterone remained low from Day 0 to Day 2 (1 ng/ml), peaked by Day 14 (6 ng/ml), then began its decline. Similar results have been reported in beef cattle (Hendricks et al., 1971; Sprague et al., 1971), in seven heifers (Snook et al., 1971), in dairy cattle (Glencross et al., 1973; Stabenfeldt et al., 1969; Wettman et al., 1972), and in mature cows (Dobson and Dean, 1974). Basic differences in the results of these investigators were not in the overall pattern of secretion throughout the cycle but in the exact levels and days that P peaked, declined, then rose again. These slight differences are probably due to breed age, size differences, methods of assay, and estrous cycle fluctuations.

Lemon et al. (1975), sampling every two hours for five days in four dairy cattle, and Kazama and Hansel (1970), sampling every six hours prior to, during, and after estrus, reported no preovulatory surge in P. Ayalon and Shemesh (1974), nevertheless, reported that in nine dairy cows transient peak of P (2.28 ng/ml) occurred 16 hours before the onset of estrus.

Plasma Estrogen

Estrogen during the bovine cycle has been reviewed (Batson,

1972; Hansel and Echternkamp, 1972) and reported in the literature to be inversely proportional to P during proestrus and estrus in the bovine. Estradiol 17B began to rise two to three days prior to estrus, peaked at estrus (16.15±5.17 pg/ml) (or shortly before, 9.27±2.78 pg/ml), then declined rapidly after estrus (Echternkamp and Hansel, 1973). Many investigators have reported similar results (Henricks et al., 1971; Shemesh et al., 1972; Wetteman et al., 1972). However, Hoffman (1972) found levels of 100 pg/ml during the luteal phase and 350 pg/ml at estrus using RIA and quantitating total E via non-chromatographic procedures. A secondary peak (4-8 pg/ml) has also been observed four to six days after estrus (Batson, 1972; Dobson et al., 1973; Echternkamp and Hansel, 1973; Glencross et al., 1973). Estrone and E_2 were quantitated via a flourescence assay during the estrous cycle of 22 dairy cows (Mason et al., 1972). Peaks occurred on Days 19 to 20 for E_1 (226.9 ng/100 ml) and E_2 (117.5 ng/100 ml) and on Days 6 to 8 for E_1 (150 ng/100 ml). Estrone and estradiol 17α were assayed in five cows via RIA (Dobson and Dean, 1974). Estradiol 17α showed inconsistent fluctuations throughout the cycle (20-30 pg/ml), and E_1 remained very low (4 pg) with little variation (Dobson and Dean, 1974; Glencross et al., 1973). Hansel and Echternkamp (1972) reported that E_1 levels in dairy cows were elevated (>30 pg/ml) on Days 1 to 5, 13 to 16, and one day before or during estrus. However, a small number of cattle was used in these quantitations.

Estradiol-17B levels remained low until P declined, and the onset of estrus varied from 28 to 40 hours after P reached nadir (<.5 ng/ml) (Lemon et al., 1975). Sampling every two hours for five

days at estrus demonstrated a series of fluctuating peaks that maximized on the day of estrus at 25 pg/ml. Sexual receptivity was reported more highly correlated with the LH peak when estrus occurred simultaneously (Lemon et al., 1975).

Estrogen quantitations suggest: (1) a rise in E_2 two to three days prior to estrus, (2) a peak just before (24-4 hours) or during estrus, (3) a precipitous decline on the day of estrus, and (4) a secondary peak five to seven days after estrus. Estradiol 17B remains low throughout the rest of the cycle. Estrone and $E_2\alpha$ are highly variable and await further data before patterns can be elucidated.

CHAPTER III

EXPERIMENTAL PROCEDURE

I. OBJECTIVES OF EXPERIMENT

The objectives of this experiment were to compare E and P levels in the peripheral circulation to steroid levels in FF, (2) to compare E and P levels within small, medium, and large follicles, and (3) to obtain behavior heat dates along with morphological ovarian descriptions as a basis for these comparisons. These comparisons are of special interest because selective control of the future ovulatory follicle in single ovulating animals (such as bovine, human, sheep) is not well understood. The potential contribution of different size follicles in the bovine and their relative levels of steroid concentration has not been hitherto reported.

II. ANIMALS AND SAMPLING

The animals used (180 Hereford heifers, 12-18 months of age) had been selected for comparisons of their growth curves following two levels of feeding (silage and grain). These animals were assigned to various pens based on selection and grading criteria. They were all on concrete flooring and housed in 30 pens (13x30 feet), containing six animals each. The growth experiment was terminated when a pen of heifers averaged 12 mm of back-fatness after "somascoping."

The heifers were observed at least twice daily for signs of

estrus (standing, riding, discharge, standing alone) with all characteristics being recorded until the animals were slaughtered. KaMar Heatmount Detectors (KaMar, Inc., Steamboat Springs, Colo.) were used to assist in estrus detection and were replaced each time the heifers were weighed (1-2 weeks) if there had been a previous color change.

All animals were slaughtered at East Tennessee Packing Plant. After decapitation, blood samples were collected in 50 ml plastic heparinized tubes from each heifer and placed on ice. Within one to two hours after slaughter, the blood was centrifuged (800 g for 30 minutes) and the plasma stored in glass tubes (-20 degrees C) until assayed. Within 30 minutes after slaughter, the ovaries were collected, washed, tagged according to cow, placed in plastic containers, and kept on ice. After the ovaries were returned to the lab, they were weighed and classified according to morphological parameters. The CL were then removed and weighed, followed by aspiration of follicles and separation of the FF according to volume: (1) small (<.1 ml), (2) medium (.1<M>.4ml), and (3) large (>.4 ml). All aspirated surface follicles from a pair of ovaries were counted, pooled, and then kept frozen until they were assayed.

III. MATERIALS

Phosphate buffered saline plus gelatin (PBSG) (.1 M, pH 7.5) was used in the P and E assays and prepared in the following manner:

.696 grams K_2HPO_4

.138 grams $NaH_2PO_4 \cdot H_2O$

4.380 grams NaCl

.190 grams EDTA

.500 grams Gelatin (Knox unflavored)

500.000 ml Double distilled water

This was heated to 50 degrees C for 10 minutes then stored at 0-5 degrees C.

Dextran-coated charcoal solution was used in both assays and was prepared in the following manner:

250.000 mg Norit A charcoal

25.000 mg Dextran T-70

100.000 ml PBSG

The charcoal was stirred for 10 minutes after preparation, then stored at 0-5 degrees C.

Concentration changes in the preparation of charcoal produced no significant changes in its capacity to bind free steroid.

All solvents were reagent grade except for benzene which was spectrophotometric grade and redistilled (obtainable from Burdick and Jackson Labratories, Muskegon, Michigan). Ether (MCB) was stored in pint bottles, reopened only once, then discarded. Benzene was stored in one gallon glass bottles which were fitted with a dispenser when initially opened. Blank values for the solvents are shown in Tables II and III.

Progesterone and Estradiol 17B were obtained from Sigma and used without further purification.

Progesterone 1, 2, 6, 7-H^3 and Estradiol-17B-2, 4, 6, 7-H^3 were obtained from New England Nuclear.

Their purities were checked via thin layer chromatography.

Glassware in the assays consisted of RTU culture tubes (12x75 mm.), used without prior cleaning. Binding of E_2 and P_4 to glass was checked and found to be approximately 10 percent for P_4 and 18 percent for E_2.

The steroid dilutions were made with the Pipetman P-200 (Gilson). The variability of the instrument at various settings is shown in Table I.

IV. PROGESTIN ASSAY PROCEDURE

The P RIA procedure was adapted from the methods of Morgan and Cook (1972) and Tsou (1974). The P anti-serum was developed by Dr. F. A. Murray, presently at Wooster Agriculture Institute, and Dr. R. L. Murphree, Animal Science Department, via inoculation of an ewe (No. 917) with 20B-hydroxyprogesterone conjugated to bovine serum albumin at the 3 position. Abraham's (1969) procedure for preparation of the anti-serum was followed by treating the collected serum in the following manner: (1) absorption of the serum with BSA (500 ug per ml), (2) purification with four volumes of .4 percent rivanol (2-ethoxy-6, 9-diaminoacridine lactate) at a pH of 6.8, and (3) absorption with 1-5 volumes of .5 percent dextran-coated charcoal.

The treated antiserum was stored in PBSG (1:10) in 4 ml vials at -20 degrees C (Tsou, 1974).

A toluene-base counting solution containing 23 percent Triton-X (octyl phenoxy polyethoxyethanol) from Sigma was used as the scintillation fluid. The constituents of the toluene-base cocktail were 4 grams of

Table I

Pipetting Efficiency

Amount Pipetted	Mean	N	Standard Error	Range	Coefficient of Variation (%)
10	543.86	10	13.31	491.0- 630.2	7.74
20	1000.60	10	15.00	936.0- 1094.2	4.74
60	2803.50	10	34.15	2644.2- 2918.0	3.85
100	4267.10	10	31.55	4075.6- 4364.4	2.33
200	8363.40	10	63.37	7968.4- 8618.6	2.40
600	25156.00	10	197.45	24099.4-25827.8	2.48

Amount pipetted = microliters of estrogen (labeled) added.

Mean = cpm.

PPO (2, 5-Diphenyloxazole) and .5 grams of POPOP (1, 4-bis-2-5-Phenyloxazolyl benzene) per liter of toluene.

The following RIA method for P was used:

1. A P_4 stock solution was prepared in 100 percent ethanol (100 ng per ml) and diluted with ethanol to obtain a working solution (10 ng per ml). A standard curve with quantities of 0, 100, 500, 1000, and 2000 pg's was constructed and assayed in duplicate from the working solution.
2. Pooled cow plasma, organic solvent blanks, and labeled and unlabeled P_4 recoveries were determined for each assay (page 47). Pooled cow plasma samples and organic solvent blanks were used to check for variability and interfering compounds in the assay. Labeled P_4 (5,000-10,000 cpm) was added to each of four 13x100 mm RTU culture tubes and scintillation vials to check for recovery rates. The labeled P_4 was dried and 200 ul of cow plasma or 10 ul of FF were added to the tubes.

   Cold P_4 (500 pg) was evaporated in two 13x100 mm RTU culture tubes followed by the addition of steer plasma (200 ul). Contents of all extraction tubes were mixed and allowed to incubate for 10 minutes to enhance diffusion of labeled and unlabeled steroid.
3. After the addition of 3 ml of petroleum ether to each tube, the samples were vortexed for 30 seconds, and centrifuged at 800 g for 10 minutes to enhance separation of the ether phase from the plasma and FF phases. Immediately following centrifugation, the tubes were immersed in an acetone-dry

ice bath and the ether phase decanted into 12x75 mm disposable culture tubes. After thawing the plasma and FF were extracted a second time.

4. Ether was evaporated in a vacuum oven (27 psi) at 40-50 degrees C. After evaporation the P were concentrated in the bottom of the tubes by rinsing with 1 ml of ether. At this time the standard curve was prepared and dried in the vacuum oven along with the unknowns.

5. A final dilution (1:100) of the antiserum solution was prepared after the addition of .8 ml of labeled P_4 diluted (1:1000) in PBSG. This mixture was incubated for 30 minutes at room temperature before use in the assay, resulting in approximately 10,000 cpm per 300 ul of the PBSG-Ab solution. After incubation (0-5 degrees C for 4-5 hours) 300 ul of the PBSG-Ab solution was pipetted into each culture tube containing the unknowns and standards. The culture tubes containing labeled P_4 were solubilized in .5 ml of 100 percent ethanol and decanted into scintillation vials. After PBSG-Ab addition the samples were vortexed for 3-5 seconds, centrifuged at 800 g for two minutes, and covered with parafilm. The samples were then incubated 14-16 hours at 0-5 degrees C.

6. After incubation (14-16 hours) .5 ml of cold Dextran-coated charcoal solution was pipetted into each sample except the ones containing only PBSG-Ab to which water had been added. All samples were vortexed, then centrifuged at 800 g in the cold (0-5 degrees C) for 10 minutes. The supernatant was

decanted into scintillation vials containing 10 ml of the aforementioned cocktail, shaken, and placed in a liquid scintillation counter for 30 minutes prior to counting.

7. Each sample was counted for five minutes and the external standard was used to correct for quenching of various samples. The mean counting efficiency for P was 32 percent.

The cpm from the P_4 standards were used to calculate a regression line (100-2,000 pg) which was used in calculating the quantity of P in the unknown samples. The amount of P in an unknown is inversely related to the cpm.

Figure 1 illustrates the simple linear regression line for 43 consecutive standard curves. The standard errors indicate the variation among all assays. Each point is represented by 43 duplicate determinations with the coefficient of variation ranging from 10.28 percent (500 pg) to 15.48 percent (2,000 pg). All determinations for unknown values were made using this composite line.

The final antiserum dilution was determined by constructing standard curves using 1:100, 1:300 and 1:500 concentrations of antiserum (Figure 2). The 1:100 dilution was selected as it exhibited the greatest sensitivity over a range of 100 to 2000 pg.

V. ESTROGEN ASSAY PROCEDURE

The E assay procedure was adapted from the methods of Batson (1972) and Tsou (1974). The antiserum [1] was diluted 1:100 with PBSG

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[1] The estrogen antiserum was obtained from Dr. Burton V. Caldwell (Yale University).

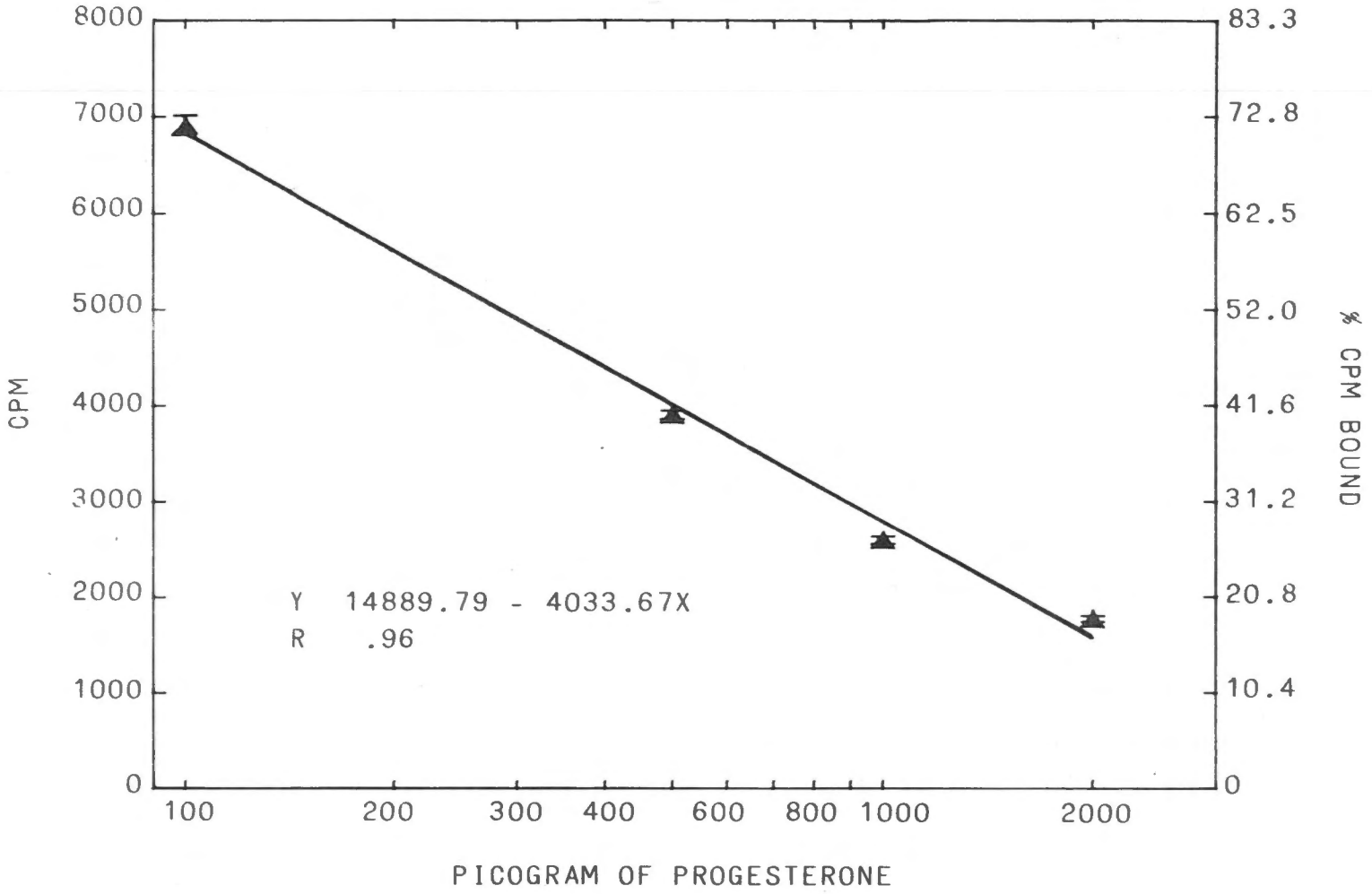


Figure 1. A linear regression line constructed for 43 consecutive assays for progestins. Each point represents 86 separate determinations with associated standard errors.

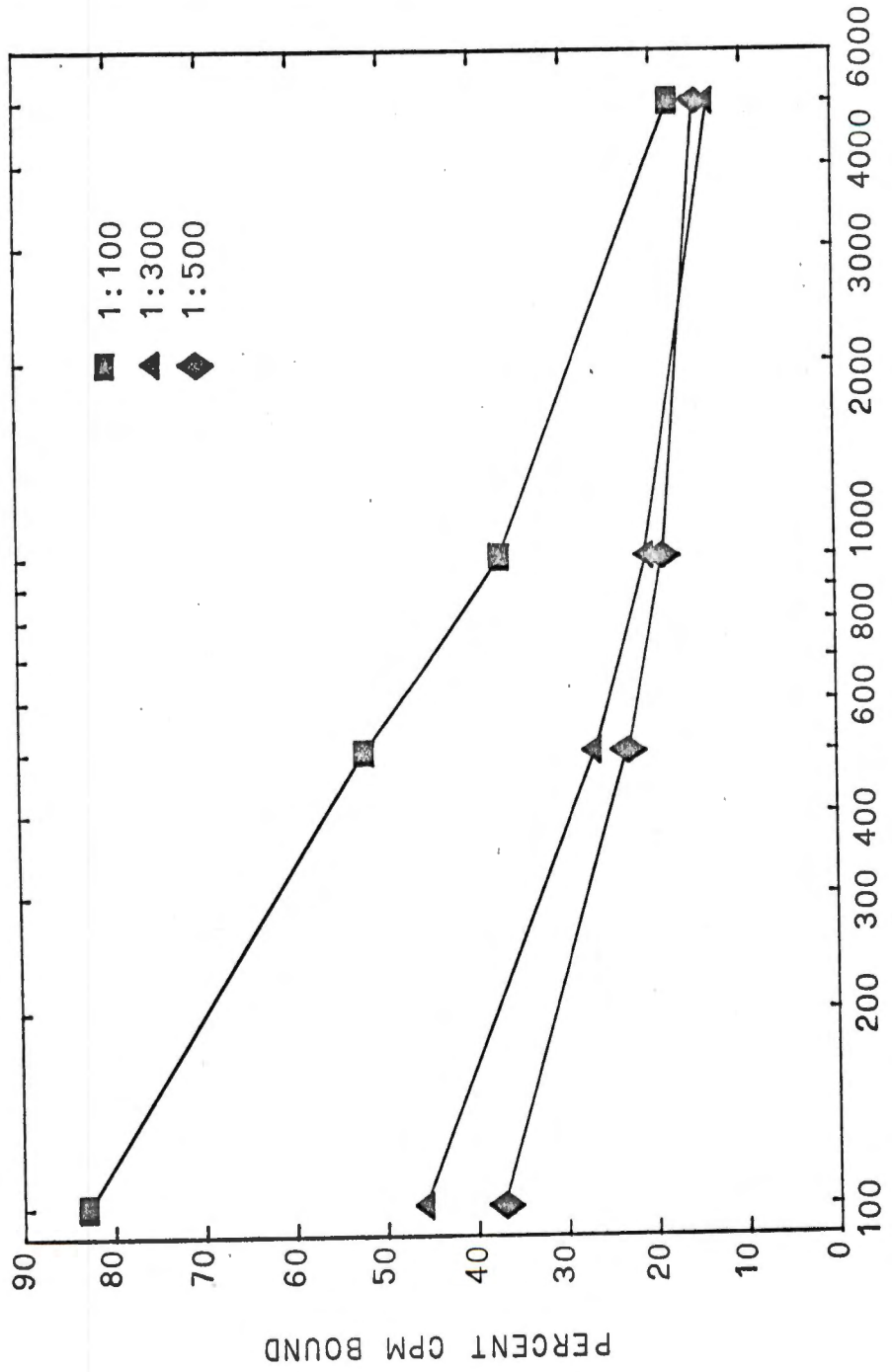


Figure 2. Progestin standard curves constructed using various dilutions of antiserum.

and stored at -20 degrees until used.

Benzene was used instead of ether to extract E from the plasma and FF because of a reduction in blank values. The scintillation cocktail was the same as that used for the P assays.

The following RIA method for E was used:

1. An E_2 stock solution was prepared in 100 percent ethanol (100 ng per ml) and diluted with ethanol to obtain a working solution (1 ng per ml). A standard curve with quantities of 0, 2, 4, 8, 20, 60, 100, 200 and 300 pg's was constructed and assayed in duplicate from the working solution.
2. Labeled and unlabeled E_2 recoveries and organic solvent blanks were determined for each assay. Labeled E_2 (10,000-15,000 cpm) was added to each of 4, 13x100 mm culture tubes and scintillation vials to check for recovery rates via benzene extraction. After evaporation 1 ml of cow plasma and 10 ul of FF were added to these tubes.

   Cold E_2 at various levels (20, 60, 100 pg) was added to 13x100 mm tubes followed by the addition of 1 ml of charcoaled cow plasma. All tubes were mixed and allowed to incubate for 10 minutes. The results of the extractions are shown on page 49.
3. After the addition of 6 ml of benzene to each tube, the samples (plasma-2 ml, FF-10 ul) were vortexed for one minute and centrifuged (1000 g for 30-60 minutes) to separate the aqueous and organic phases. The benzene phase was

pipetted into 13x100 mm tubes. The extraction was carried out only once with results reported on pages 48 and 49.

4. Benzene was evaporated in a vacuum oven (27 psi) at 40-50 degrees C. After evaporation the E were concentrated in the bottom of the tubes by rinsing with .5 ml of benzene. The samples were then aliquoted in duplicate (plasma-200 ul, FF-50 ul) into 12x75 mm tubes. These along with the standard curve were dried in the vacuum oven.
5. Estradiol 17B (10,000-15,000 cpm) dissolved in 30 ul of PBSG was pipetted into each tube, followed by the addition of 270 ul of PBSG-Ab resulting in a final dilution of 1:60,000 for the assay antiserum. The samples were vortexed, covered with parafilm, and incubated at 0-5 degrees C for 12 hours. At this point the culture tubes containing labeled E_2 were solubilized in .5 ml of benzene and poured into scintillation vials to check for extraction efficiency.
6. After incubation (12 hours) free steroid was separated from bound steroid in the same manner as noted in the P assay (Dextran-coated charcoal). The samples were then centrifuged at 800 g (0-5 degrees C) for five minutes. The supernatant was then decanted into 5 or 10 ml of the aforementioned cocktail, shaken and placed in a Beckman LS-230 liquid scintillation counter for 30 minutes prior to counting. Each sample was counted for five minutes, and the external standard was used to correct for quenching of various samples. The mean counting efficiency for ^{3}H-E was 32 percent.

The cpm from the E_2 standards were used to calculate a regression line (2-300 pg). This line was used in calculating the quantities of the unknown samples. The amount of estrogen in an unknown is inversely related to the cpm.

Figure 3 illustrates a simple linear regression line for 11 consecutive standard curves. The standard errors indicate the variation between all assays. Each point is represented by 11 duplicate determinations with the coefficient of variations ranging from 10.72 percent (200 pg) to 17.81 percent (60 pg). All determinations for unknown values were made using this composite regression line.

The final antiserum dilution was determined on both batches of antiserum by constructing standard curves using various concentrations of antiserum (Figure 4). In the first batch of antiserum a 1:30,000 dilution was used, and in the second batch a 1:60,000 dilution was selected. Sensitivity over a range of 2-300 pg for the first batch of antiserum is represented by Figure 4.

VI. STATISTICAL ANALYSIS

The P and E data had their analysis based on SASS computations using an IBM 360 computer. The following comparisons were devised A Priori: (1) P and E in the peripheral plasma by MGU, (2) P and E in small, medium, and large follicles by MGU, (3) ovarian and CL weights, follicle numbers, and follicle volume versus MGU, and (4) a comparison of behavior heat dates versus MGU.

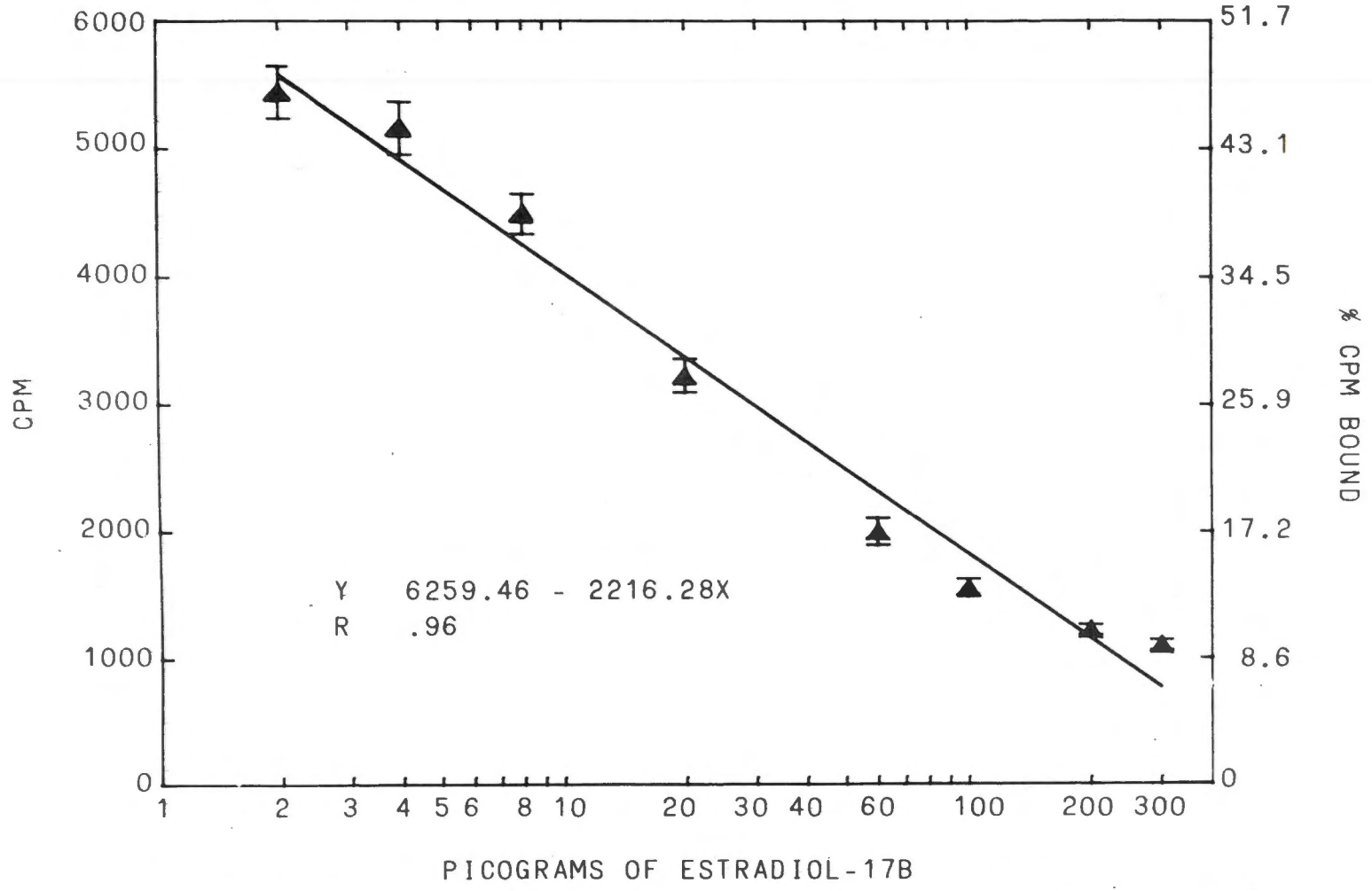


Figure 3. A linear regression line constructed for 11 consecutive assays for estrogens. Each point represents 22 separate determinations with associated standard errors.

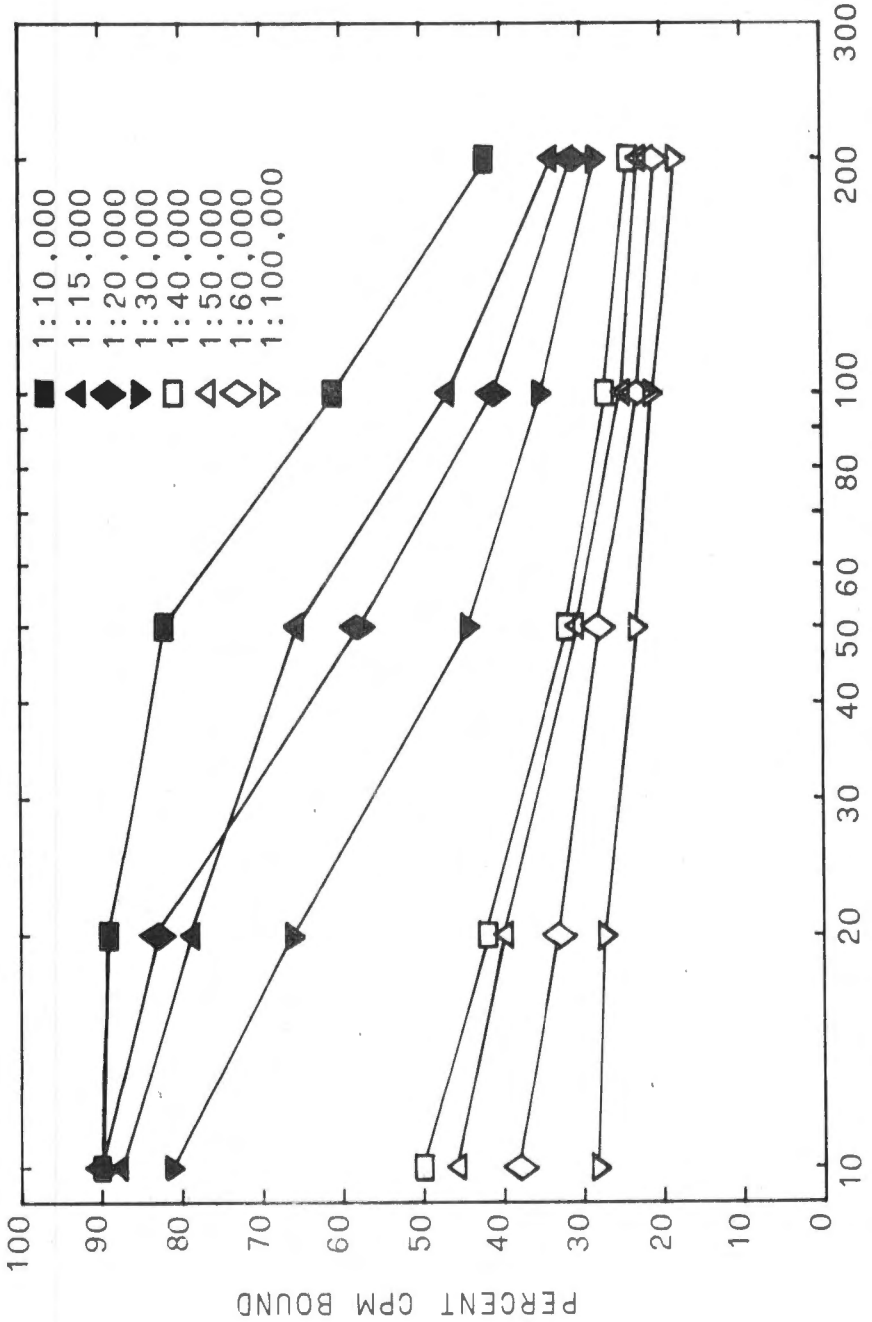


Figure 4. Estrogen standard curves constructed using various dilutions of antiserum.

CHAPTER IV

RESULTS AND DISCUSSION

I. EVALUATION OF ASSAY METHODS

Extraction Efficiency and Precision

The extraction efficiencies for labeled and unlabeled P_4 were greater than 90 percent (Table II); therefore, no corrections were made for P_4 concentrations. Estradiol 17B was extracted only once with the mean for unlabeled extraction being 80 percent and labeled 82 percent (Tables III and IV). In this instance, corrections were made for extraction losses by using the labeled recovery percentage.

Determinations were made on the solvents used in the assays to obtain a blank value. Blank values which were indicative of interfering compounds in the assay were subtracted from the unknowns if they were above the sensitivity level of the standard curve. In the P assay the mean blank value for ether was .076 ng, and in the E assay it was 5.21 pg for plasma and 2.82 pg for the FF when benzene was used for extractions. The blank values in the E assay were subtracted from the unknown values; whereas, no corrections were made for blank values in the P assay.

Antiserum

Hill plots (Mahler and Cordes, 1966) were performed on the E and P antiserum in order to determine association constants (K_A) and cooperativity (Figure 5). The K_A for P antiserum is 3.07×10^9, and E is 5.25×10^6 per mole. Both antisera have a Hill coefficient of 1

TABLE II

PROGESTIN ASSAY CONTROLS

	N	Mean	Standard Deviation	Standard Error	Range	Coefficient of Variation (%)
Percent Extraction of Labeled Progesterone from Steer Plasma	33	89.09	9.32	1.62	70.89 - 107.94	10.36
Percent Extractrion of Labeled Progesterone from Follicular Fluid	26	96.33	14.51	2.84	77.73 - 146.34	15.06
Solvent Blank (Ether) (picograms/6 ml ether)	30	76.98	43.12	7.87	12.97 - 165.94	56.00
Pooled Cow Plasma (Picograms/ml)	25	827.15	163.96	32.79	615.97 -1154.06	21.27
Steer Plasma (picograms.ml)	27	92.16	57.95	11.06	13.73 - 205.27	62.00
Steer Plasma plus 500 pg of Progesterone (Amount Extracted) (picograms.ml)	33	561.67	167.80	29.21	268.35 -1053.34	37.09

N = number of duplicate determinations.

All samples extracted twice with benzene.

TABLE III

ESTROGEN ASSAY CONTROLS

	N	Mean	Standard Deviation	Standard Error	Range	Coefficient of Variation (%)
Percent Extraction of Labeled E_2 from Pooled Cow Plasma	15	82.85	6.32	1.32	73.10 - 92.80	8.00
Solvent Blank (Benzene)						
a. Plasma	26	5.21 pg	3.35	0.66	1.33 - 13.14	64.00
b. Follicular Fluid	26	2.82 pg	2.49	0.49	0.30 - 11.31	88.00

N = number of duplicate determinations.

All samples extracted once with 6 ml of benzene.

Table IV

Cold Extraction of Estrogen with Benzene

Steroid Added to Charcoaled Plasma (Picograms)	N	Steroid Extracted (Picograms)	Number of Extractions
20	5	32	1
60	5	62	1
100	5	80	1

N = number of duplicate determinations.

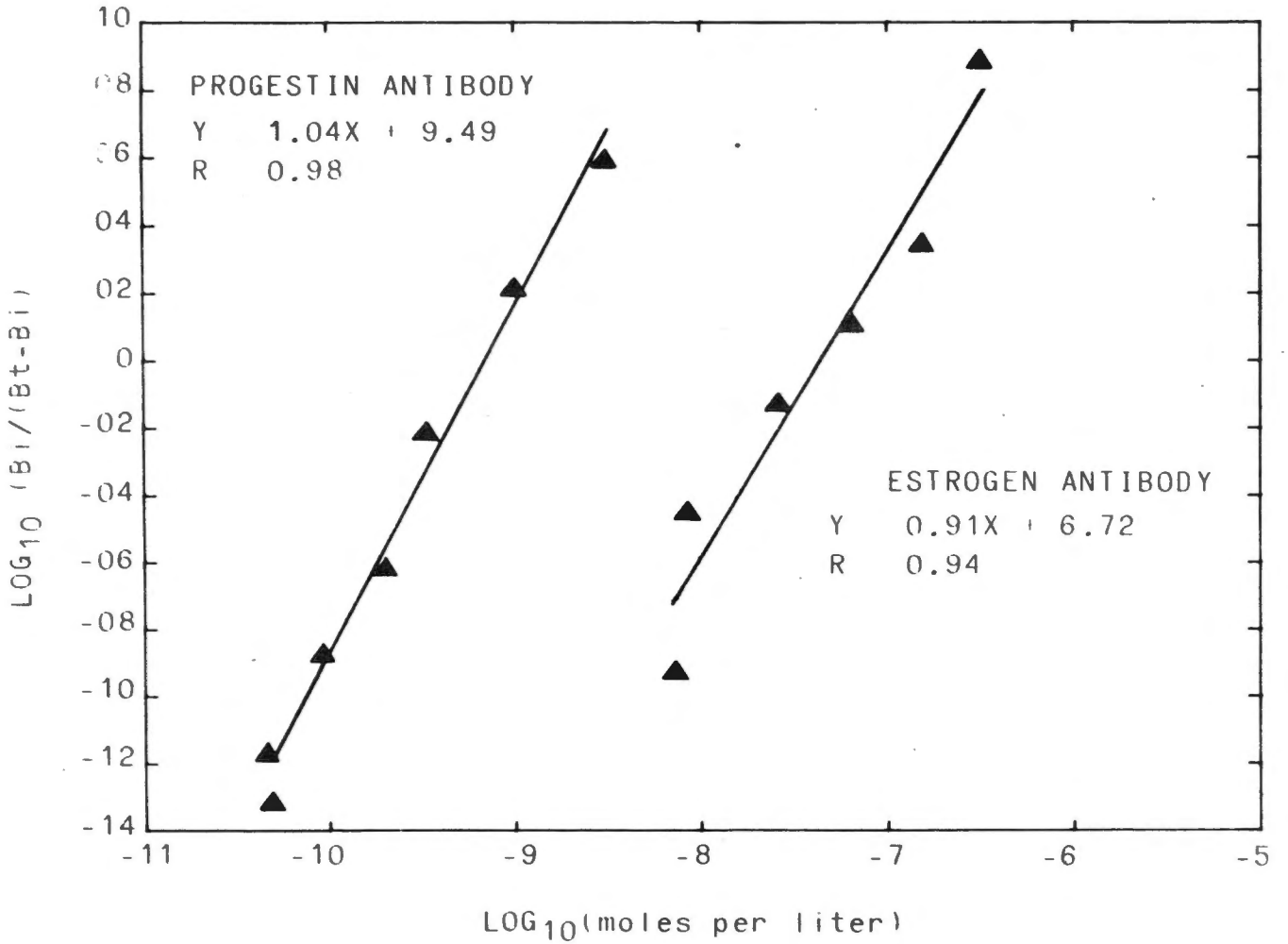


Figure 5. Hill plots.

suggesting that one binding site on each antibody is available per molecule of ligand bound.

Specificity of the Estrogen and Progestin Antisera

Estrogen and progestin were quantitated without further purification. The antiserum was checked for cross reactivities with various steroids (Tables V and VI). The percentage cross reactivity is defined as 50 percent displacement of X (E_2 or P_4) by a cross reacting steroid (Y) multiplied by 100 (Abraham, 1970).

Sensitivity of the Standard Curve

Sensitivity is defined as the smallest amount of steroid statistically distinguishable from zero (Reaves and Calhoun, 1970). The sensitivities of the E_2 and P_4 standard curves were determined by assaying quadruplicate samples at varying concentrations (Figures 6 and 7). The t-test was used to distinguish these values from zero. The sensitivity of the E_2 standard curve was determined to be between 2 and 4 pg ($P<.01$) and P_4 between 100 and 200 pg ($P<.01$). The lower limit of sensitivity for the E_2 standard curve was determined to be approximately 3 pg whereas P_4 was 200 pg.

II. EVALUATION OF BEHAVIOR HEAT DATES

Behavioral heat is defined as an activity characteristic of estrus, in this instance, one animal standing firmly to be ridden by another animal. A frequency histogram of cycle lengths is shown in Figure 8 and indicates a high incidence of short (4-14 days) (27

Table V

Cross Reactivity Against Progesterone Antibody

Steroid	Maximum Amount Assayed	Percent Cross Reaction at 50% Displacement
20B Hydroxyprogesterone	40 ng	100.00
17α Hydroxyprogesterone (17α-Hydroxypregn-4-ene-3,20 dione)	40 ng	0.00
20α HydroxyΔ^4-pregnen-3-one (20α-Hydroxypregn-4-ene-3,20 dione)	40 ng	20.00
5α-Pregnan-3b,20b-diol	40 ng	0.00
3α,20α-dihydroxy-5b-pregnane	1 ug	0.00
11α-hydroxypregn-4-ene-3,20 dione	1 ug	0.79
5B-pregnan-3α,20α-diol glucuronide	40 ng	0.00
5B-pregnan-3α,20α-diol diacetate (pregnanediol diacetate)	40 ng	0.00
5B-pregnan-3α,20α-diol (pregnandiol)	40 ng	0.00
3B,30B-dihydroxypregn-5-ene	1 ug	2.10
21-hydroxypregn-4-ene-3,20 dione	1 ug	0.00
6B-hydroxypregn-4-ene-3,20 dione	1 ug	0.88
11-B-hydroxypregn-4-ene-3,20 dione	1 ug	0.61
3B-20α-dihydroxypregn-5-ene	1 ug	0.00
6α-hydroxypregn-4-ene-3,20 dione	260 ng	7.90
5B-pregnan-3α,17α,20α-triol	10 ug	0.00
Prednislone	10 ug	0.00

Table V (continued)

Steroid	Maximum Amount Assayed	Percent Cross Reaction at 50% Displacement
Pregnenolone (3B-hydroxy-5-pregnen-20-one)	100 ng	0.00
Testosterone (17B-hydroxy-4-androsten-3-one)	100 ng	0.00
Δ^4 Androstendione (4-Androstene-3,17-dione)	100 ng	0.00
1-Dehydrotestosterone (Δ^1, 4-Androstandien-17b-ol-3-one)	10 ug 10 ug	0.00 0.00
5 -Androstan-17b-ol-3-one (Androstanolone, Stanolone, 5 - Dihydrotestosterone)	10 ug	0.00
Etiocholan-3 -ol-17-one (3α-Hydroxy-5b-androstan-17one, 5B-Androstan-3α-ol-17one)	10 ug	0.00
17α Estradiol	10 ug	0.00
Estradiol 17B	100 ng	0.00
Estriol	100 ng	0.00
Estrone	50 ug	0.00
Cholesterol	1 mg	0.00

Table VI

Cross Reactivity Against Estrogen Antibody

Steroid	Maximum Amount Assayed	Percent Cross Reaction at 50% Displacement
Estrone	200 pg	86.95
Estriol	200 pg	6.66
Progesterone	2 ng	0.00
Testosterone	1 ng	2.50
Cholesterol	100 ng	0.00

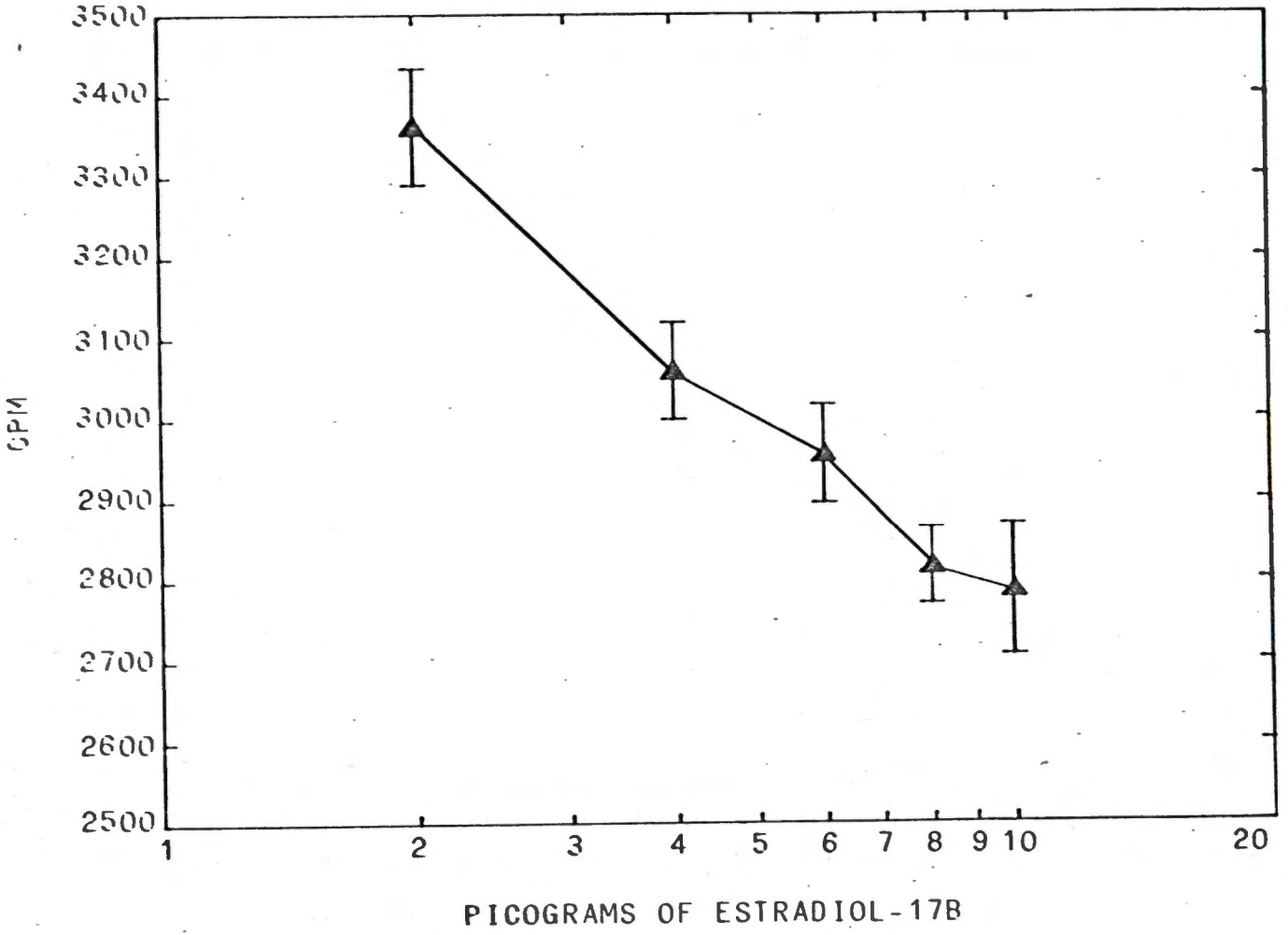


Figure 6. Estradiol 17B sensitivity curve.

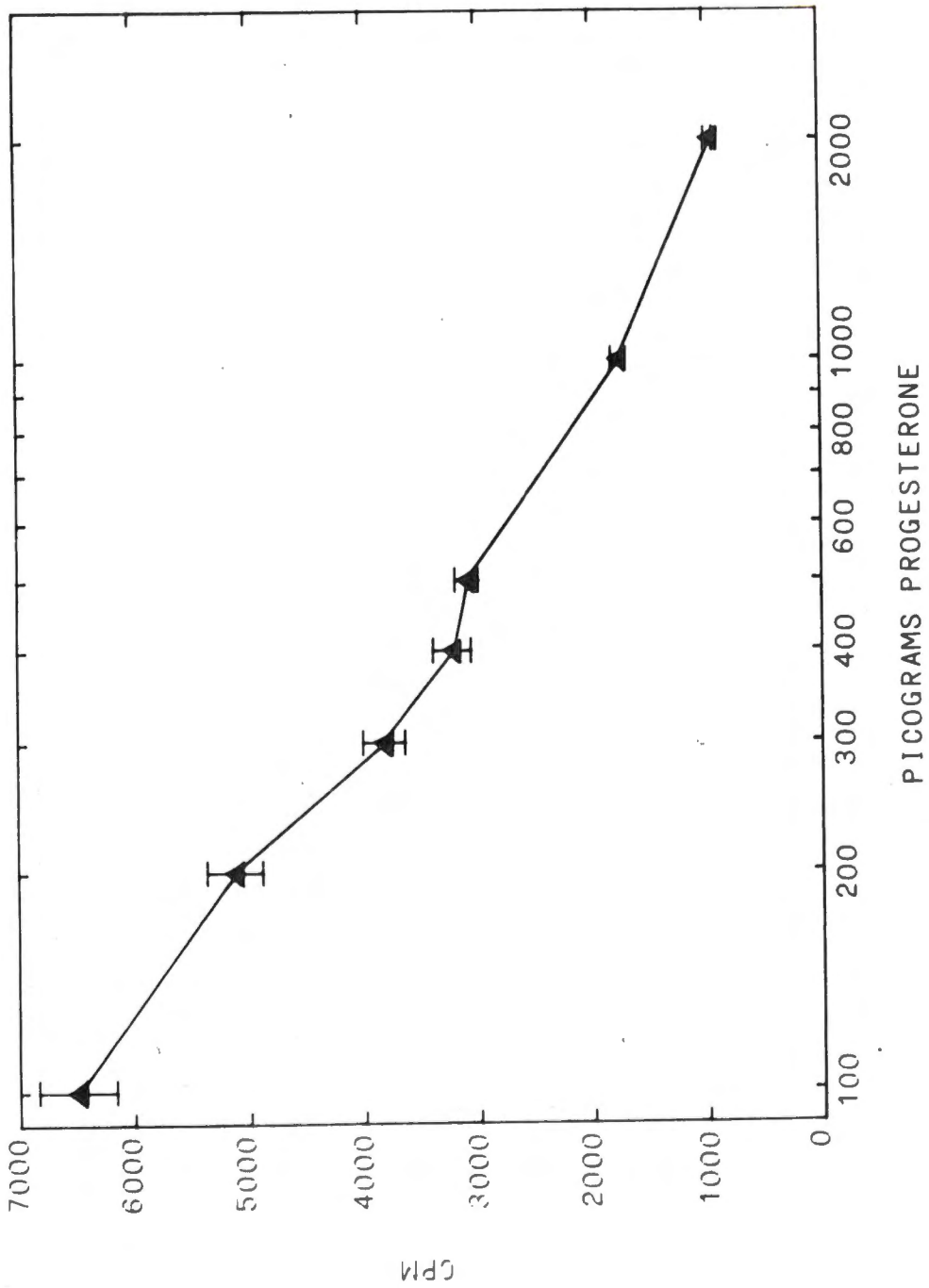


Figure 7. Progesterone sensitivity curve.

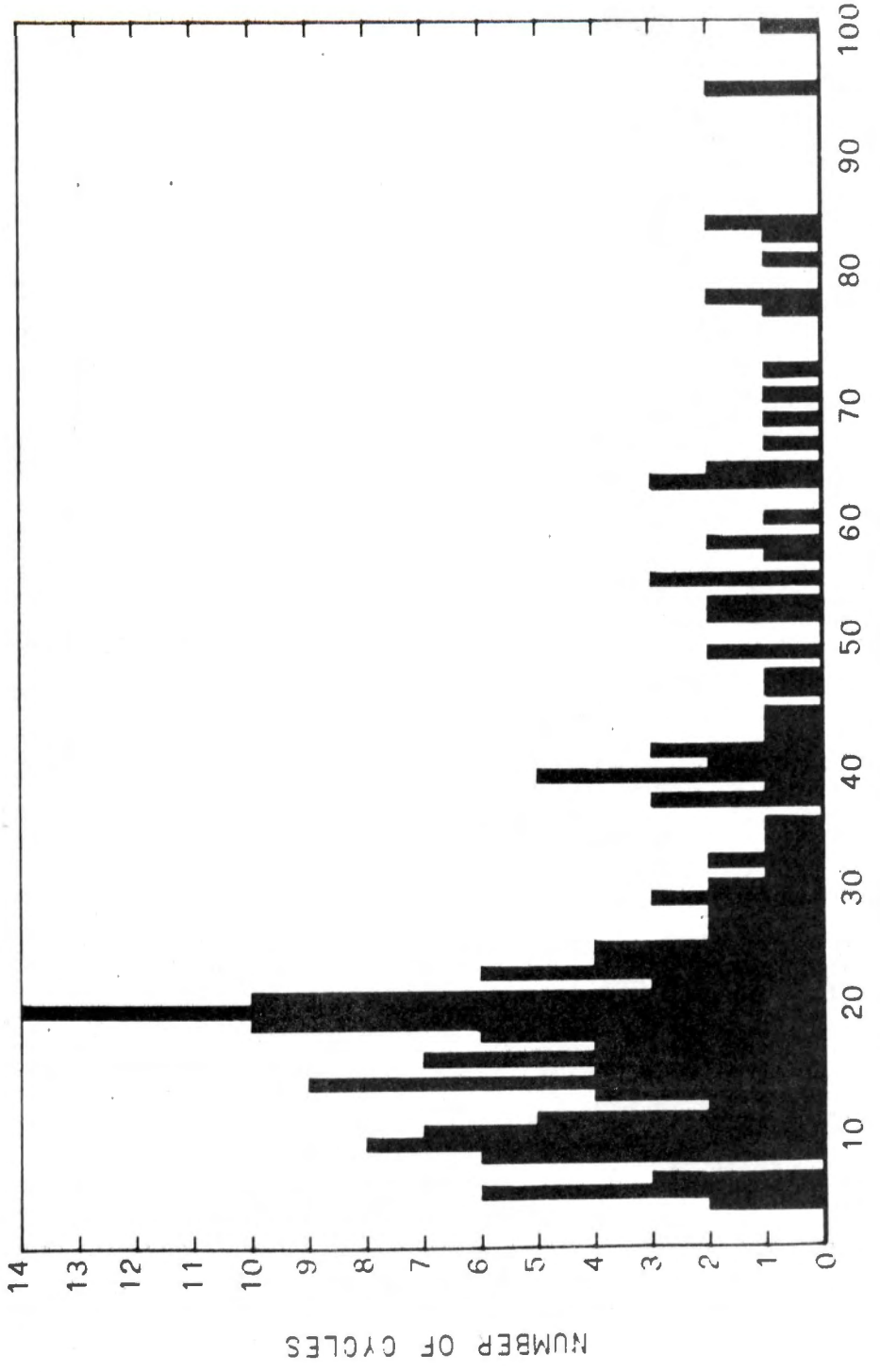


Figure 8. A frequency histogram of all cycle lengths of the heifers.

percent) and long cycles (>26 days) (37 percent). Observations of estrous cycle length varied from 4 to 100 days in length with a mean cycle length of 21 ± 21 days for 192 cycles. There was a tendency for heifers exhibiting concurrent short cycles to sum to normal cycle lengths (15-25 days) and for heifers exhibiting long cycles to either be multiples of normal cycles or normal plus short cycle lengths. Table VII depicts the methods of detection of activities and their distribution. There were nine pens in which little behavioral activity (estrus or riding) (<5) was recorded, comparing favorably with those pens that had less animals riding. This suggests an initial non-random distribution of heifers that could result in pens of animals being void of heifers that have a riding tendency. The end result was that pens with no "riders" or few "riders," even though possessing animals in estrus, would have no observable signs demonstrated; or, if non-randomness can be assumed, then a disproportionality of immature animals is also likely. These problems, compounded with the possibility of stress due to overcrowding and being housed on concrete flooring, made comparisons using estrus behavior unlikely (Table VIII).

The only other report of observations on feedlot heifers was by Wagnon et al. (1972). They concluded that short cycles may have been indicative of stress in the animals. The heifers he used were of approximately the same age (15 months) but were not grouped in pens, and a vasectomized bull was used to assist in estrus detection rather than using KaMar markers and other heifers. Cycle lengths have been reported to vary immensely especially in large samples of animals. Chapman and Casida (1937) reported that 40 percent of 690 normal cows

TABLE VII

Behavioral Activities of Heifers

Activity Observed	N	Percentage of Total Activities
1. Standing	106	24.20
2. KaMar Heatmount Detector color change with no standing observed	140	31.96
3. KaMar Heatmount Detector color change and standing observed	16	3.65
4. KaMar Heatmount Detector lost	11	2.51
5. Standing and riding observed by the same heifer	34	7.76
6. KaMar Heatmount Detector color change and riding observed by the same heifer	13	2.97
7. KaMar Heatmount Detector color change, riding, and standing observed by the same heifer	3	0.69
8. Riding	115	26.26

N = numbers of cows observed during the experiment.

There were 28 cows that never exhibited any of the above mentioned activities and 6 cows which were pregnant. Standing was observed 159 times (49.23 percent) and no standing but a Kamar Heatmount Detector color change was observed 164 times (50.77 percent).

Table VIII

Behavior Activities Within Pens

Pen	Feed Type	Days Observed	Total Estrus	Total Riding	Animals With Zero Activity	Immature MGU
A1A	silage	116	3	2	2	1
A2A	grain	88	2	-	3	3
A3A	grain	102	3	2	1	1
A4A	silage	151	16	9	-	1
A5A	grain	109	6	1	1	2
A6A	silage	123	9	8	-	-
A7A	grain	109	6	2	1	1
A8A	silage	144	8	6	1	-
A9A*	silage	123	8	7	1	-
A10A*	silage	172	16	8	-	-
A11A	silage	172	15	8	1	1
A12A	grain	116	10	2	1	-
A1B	silage	172	18	8	-	1
A2B	grain	109	10	6	-	1
A3B*	silage	186	33	11	-	-
A4B*	silage	144	14	6	-	-
A5B	grain	88	4	1	2	-
A6B	silage	115	8	10	-	-
B1A*	silage	144	15	16	-	-
B2A*	silage	179	23	9	-	-
B3A	grain	95	3	-	3	3
B4A	silage	179	20	11	-	-
B5A	silage	172	20	13	-	1
B6A	grain	115	11	7	1	2
B7A	grain	81	3	-	3	-
B8A	silage	166	17	3	-	-
B9A	grain	115	5	-	1	1
B10A	silage	144	10	7	-	2
B11A	grain	81	3	-	3	1
B12A	silage	102	1	3	3	1

*These animals were not originally included in the growth experiment.

MGU (immature) depicts animals that had no corpus luteum upon morphological examination.

All pens had 6 animals.

had cycles that were not in the normal range (17-24 days). Ellenberger and Lohmann (1946) according to Salisbury and VanDemark (1961) found 30 percent of 274 cycles observed in cows not in the normal range (17-25 days). Finally, a study utilizing 4,885 cows found 44 percent not in the normal range (18-25 days) (Mueller and VanDemark, 1951). Salisbury and VanDemark (1961) concluded that the modal cycle is 21 $\pm$ 4 days; however, a substantial amount of cows return to estrus at intervals outside the implied "normal" range (17-25 days).

In each pen, one particular heifer usually did most of the riding.

III. EVALUATION OF MORPHOLOGICAL GROWTH UNITS

Morphological growth units (MGU) were based on the progressive growth and regression of the CL. These changes have been well reviewed by Hammond (1927); moreover, a previous pilot study with heifers was used to describe patterns of growth and regression of the CL of heifers in this experiment.

The seven MGU classifications for the CL are as follows:

I. 0.5 cm in diameter, hollow with blood clots and an open top red in color.

II. One cm in diameter, still not solid but fleshy and soft to growing solid in the center, with external clots and red apical cells.

III. One cm in diameter, growing solid and filling in toward the center, no clots but red apical cells present in the center, and the tip not completely grown over with no

visible external vasculature.

IV. One and one half cm in diameter, growing solid to completely solid center, no clots or red cells, but brown apical cells in the center, and grown over with no external vasculature.

V. Two cm in diameter, very solid with orange to tan apical cells in the center, along with a grown over top usually absent of vasculature.

VI. Two cm in diameter, very solid with orange or tan apical cells and a grown over top with a prominent vascular bed appearing.

VII. Two cm regressing to 1 cm in diameter, solid center with a yellow to white top and the vascular bed disappearing.

Each pair of ovaries were classified as being at a particular growth stage after slaughter. Besides grouping, all ovaries and CL were checked for abnormalities (Table IX).

A frequency distribution based on a 21 day estrous cycle was constructed (Table X) with numbers of cows at one particular development period indicative of the length of time the CL remains at that stage. This procedure has been well reviewed by Mitchison (1971). Table X indicates that the heifers remained in MGU V and VI longer than the other MGU stages. Disproportionality in CL development has been alluded to by many authors when describing why cycles may vary in length, but never before supported by a frequency distribution of this type.

When comparing the MGU and behavior estrous information collected from 47 heifers that were slaughtered within 21 days of

Table IX

Morphological Growth Codes

Classification of the Ovary, CL, or Reproductive Tract	Percent of Total	N
Normal (absence of blood and cysts in the CL)	76	137
Small cysts found in the CL	7	13
No CL (immature)	12	21
No reproductive tracts*	1	2
Large cystic follicles	1	1
Pregnant	3	6

N = number of animals.

*At slaughter there were no reproductive tracts found in the animals, suggesting that they were possibly freemartins or that the tracts were extremely small and overlooked.

Table X

Morphological Growth Units

MGU Code	N	Percentage of Total (146)	*
I	14	9.59	2.01
II	11	7.53	1.58
III	6	4.11	0.86
IV	15	10.27	2.16
V	51	34.93	7.34
VI	31	21.23	4.46
VII	18	12.33	2.59

N = number of heifers.

* = number of days the CL remains at a specific MGU in a 21 day or unit development pattern is assumed.

MGU = morphological growth units.

their last observed estrus, the following comparisons were noted: (1) 17 heifers (36 percent) were coded exactly alike, (2) 23 (49 percent) were within 1 MGU of each other, and (3) 7 (15 percent) were within 2 MGUs. All cows that were pregnant were coded as such, and 19 of 23 heifers (83 percent) that were coded as immature (no CL) also were never observed to exhibit any type of estrous behavior. There were 28 heifers that had no estrous behavior observed, 12 of 28 were coded as immature, 14 were coded MGU V or VI, and 2 were coded I or III.

Because of the erratic cycle lengths determined from behavioral observations (Figure 8), steroid levels compared with days of a 21-day cycle could not be evaluated. Therefore, comparisons were based on morphological criteria on ovarian development which were suggested from observations (Erb and Stormshak, 1961; Foley et al., 1964; Gomes et al., 1963; Hafs and Armstrong, 1968; Mares et al., 1962; Swanson et al., 1972) in the literature and reported to correlate positively with peripheral levels of P. Consequently, MGU are used for comparisons of E and P levels at various developmental stages of the CL rather than using the erratic estrus data. Although this author maintains that the two should be comparable, it must be pointed out that two of the most widely accepted determinants for estrus behavior (1) KaMar Heatmount Detectors and (2) observed standing behavior when used in the absence of a vasectomized bull or in close crowding or pen conditions may lead to erroneous evaluations (P. B. Coulson, personal communication).

IV. EVALUATION OF PROGESTIN AND ESTROGEN DATA IN THE PERIPHERAL PLASMA AND FOLLICULAR FLUID

Peripheral Plasma Progestins and Estrogens

Estrogens and progestins varied inversely throughout the CL growth cycle (Figure 9 and Table XI). Progestins were significantly different ($P<.0001$) throughout the MGU; however, estrogens were not ($P<.23$). High variation for E secretion among cattle and cross reactivity of E_1 and E_3 (Table VI, page 54) with the E antiserum probably account for the major portion of the variation; consequently, this masks any statistically discernable differences in E between MGU. However, E concentration at MGU VII is significantly different ($P<.05$) from MGU III when a t-test comparison is made. Figure 9 shows patterns and levels which are comparable to those already reported in the literature (see Review of Literature). The values with statistics are shown in Table XI, again demonstrating close similarity of peak and nadir levels with those reported by other authors.

Progestins remained low (1.6 ng per ml) through MGU II, peaked and remained high (≅8 ng per ml) by MGU IV and V, then declined precipitously by MGU VII (Figure 9). Estrogens remained high in MGU I (11.8 pg per ml) and MGU II (9.45 pg per ml), declined and remained low (<6 pg per ml) through MGU III, IV, and V, then peaked again by MGU VI (14.92 pg per ml) and remained high (11.82 pg per ml).

Corpus Luteum Size and Plasma Progestin Concentration

The CL growth pattern was significantly different ($P<.001$) among MGU, but total ovarian weight ($P<.26$) and total ovarian weight

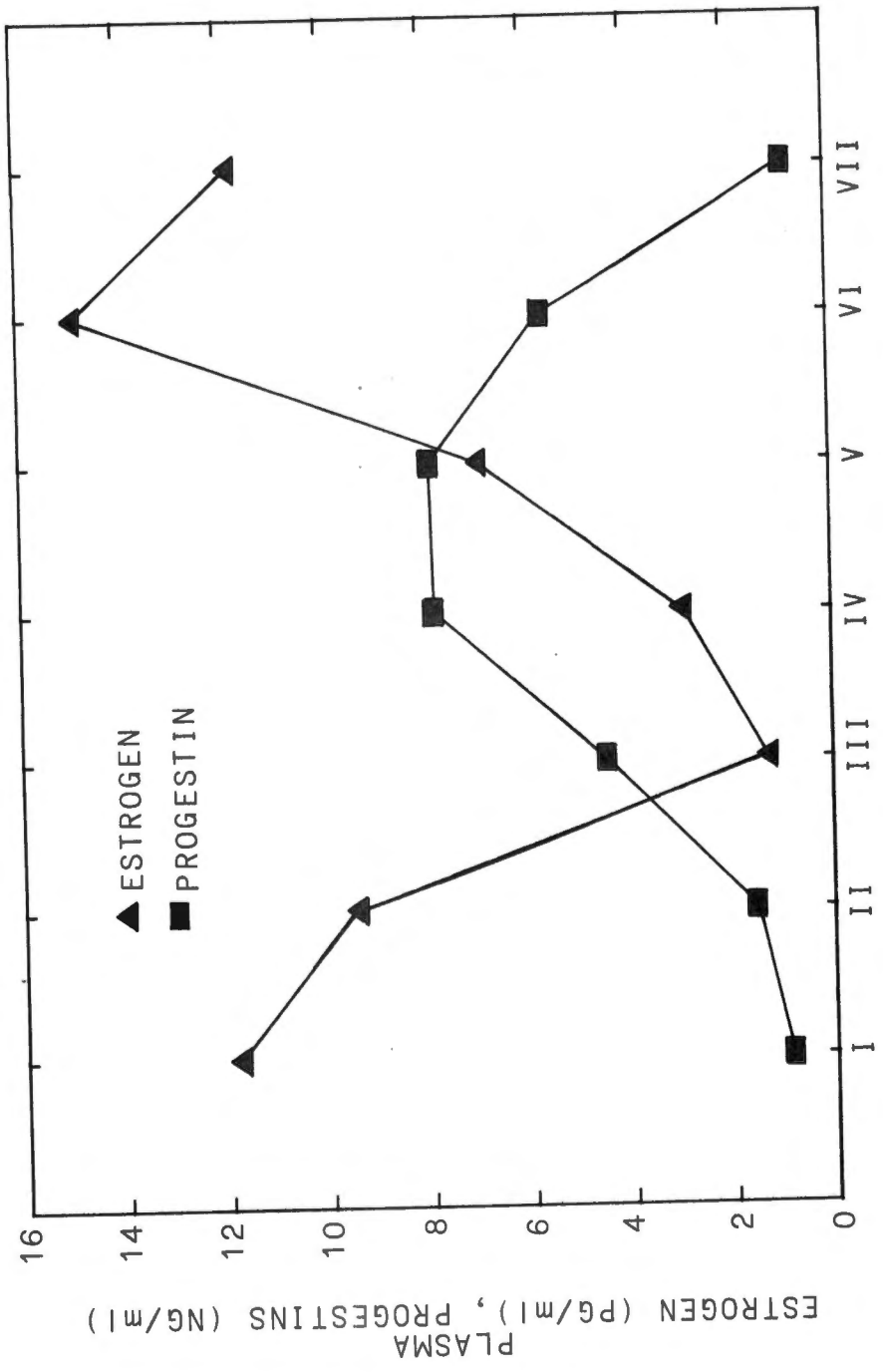


Figure 9. Estrogen and progestin levels (means) in the peripheral plasma.

Table XI

Steroid Levels in the Peripheral Plasma

MGU	Mean	N	Standard Error	Range	Coefficients of Variation (%)
			Progestin		
I	.86	15	0.12	0.14- 1.67	54.86
II	1.56	11	0.23	0.31- 2.73	48.73
III	4.49	6	1.24	1.92-10.45	67.40
IV	7.86	15	0.75	4.29-14.08	36.87
V	7.92	49	0.49	0.52-15.81	43.46
VI	5.69	32	0.50	1.05-10.99	49.31
VII	0.85	17	0.15	0.17- 2.52	71.65
			Estrogen		
I	11.80	4	5.33	5.17-27.70	90.27
II	9.45	3	4.61	2.62-18.25	84.64
III	1.29	2	0.06	1.22- 1.36	7.69
IV	2.95	4	0.92	1.40- 4.99	61.95
V	6.96	10	1.62	2.16-17.57	73.36
VI	14.92	6	6.09	0.04-34.16	95.18
VII	11.82	2	3.49	8.34-15.31	41.70

N = Number of cows.

Mean = nanograms per ml for progestins and picograms per ml for estrogens.

MGU = morphological growth units.

minus CL weight ($P<.51$) did not vary significantly among MGU (Table XII and Figure 10). The CL growth pattern closely mimicks the P output in the plasma substantiating previous results by other authors (Dobrowolski et al., 1968; Erb and Stormshak, 1961; Foley et al., 1964; Gomes et al., 1963; Hafs and Armstrong, 1968; Mares et al., 1962; Swanson et al., 1972). Corpus luteum growth and P output have also been reported to be highly correlated with the stage of the estrous cycle (Foley et al., 1964).

The CL began to increase at MGU II, peaked at MGU IV, then began its regression at MGU VI. This closely follows the pattern reported for cycling animals in which the CL increases in size after ovulation, peaks approximately by Day 11 or 12, then regresses between Day 16 to 19 (see Review of Literature).

Estrogen and Progestin in the Follicular Fluid

Estrogens and P were quantitated in FF of small, medium, and large vesicular follicles. The results for E and P levels in FF of small, medium, and large follicles are shown in Figure 11 and Tables XIII and XIV. There were significant differences ($P<.0001$) in steroid concentrations between small, medium, and large follicles; however, only P per ml of FF varied significantly ($P<.05$) among MGU. Estrogens were extremely variable within MGU classifications; consequently, no significant differences ($P<.89$) were noted among MGU. Within the small follicles P peaked by MGU III (286.60 ng per ml), then began a steady decline in concentration through MGU VII back to baseline values (145.00 ng per ml). Progestins from medium follicles remained low

Table XII

Ovarian and Corpus Luteum Weights

MGU	Mean	N	Standard Error	Range	Coefficient of Variation (%)
			Total Ovarian Weights		
I	8.49	9	0.77	5.60-13.63	27.16
II	11.19	11	0.63	6.78-14.54	18.68
III	10.93	6	0.64	9.29-13.80	14.29
IV	12.17	15	0.87	8.78-21.48	27.71
V	12.61	48	0.43	8.59-21.24	23.89
VI	12.51	31	0.47	8.32-19.27	20.72
VII	11.14	14	0.58	7.97-15.09	19.42
			Corpus Luteum Weights		
I	0.77	9	0.07	0.41- 1.05	27.16
II	1.01	11	0.16	0.40- 2.21	53.72
III	2.09	6	0.08	1.79- 2.28	9.52
IV	3.57	15	0.28	1.17- 5.25	30.62
V	3.60	48	0.11	2.23- 5.25	20.51
VI	3.15	31	0.16	1.31- 4.72	27.55
VII	1.39	14	0.16	0.73- 2.01	27.92
			Ovarian Weights Minus Corpus Luteum Weights		
I	7.71	9	0.79	4.70-12.83	30.59
II	10.18	11	0.61	5.56-12.35	20.03
III	8.84	6	0.60	7.31-11.57	16.63
IV	8.61	15	0.72	6.13-17.00	32.24
V	9.01	48	0.39	5.06-16.11	30.36
VI	9.36	31	0.45	5.91-15.30	26.84
VII	9.76	14	0.59	6.63-14.02	22.70

N = number of cows.

Mean = grams per pair of ovaries.

MGU = morphological growth units.

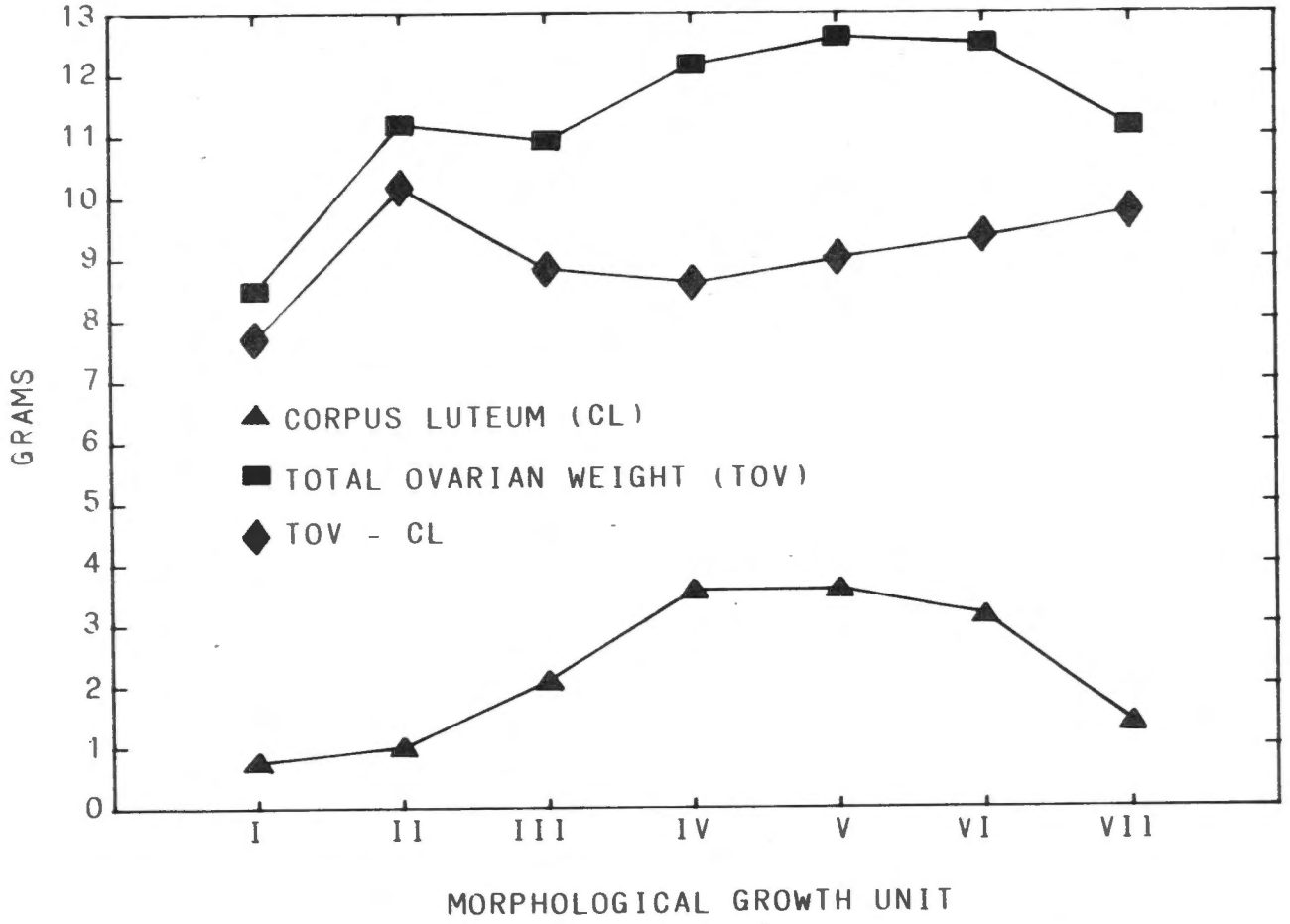


Figure 10. Ovarian and corpus luteum weights (means in grams per pair of ovaries). Corpus luteum weight was subtracted from ovarian weights (TOV-CL) to check for changes in ovarian weight without corpus luteum influence.

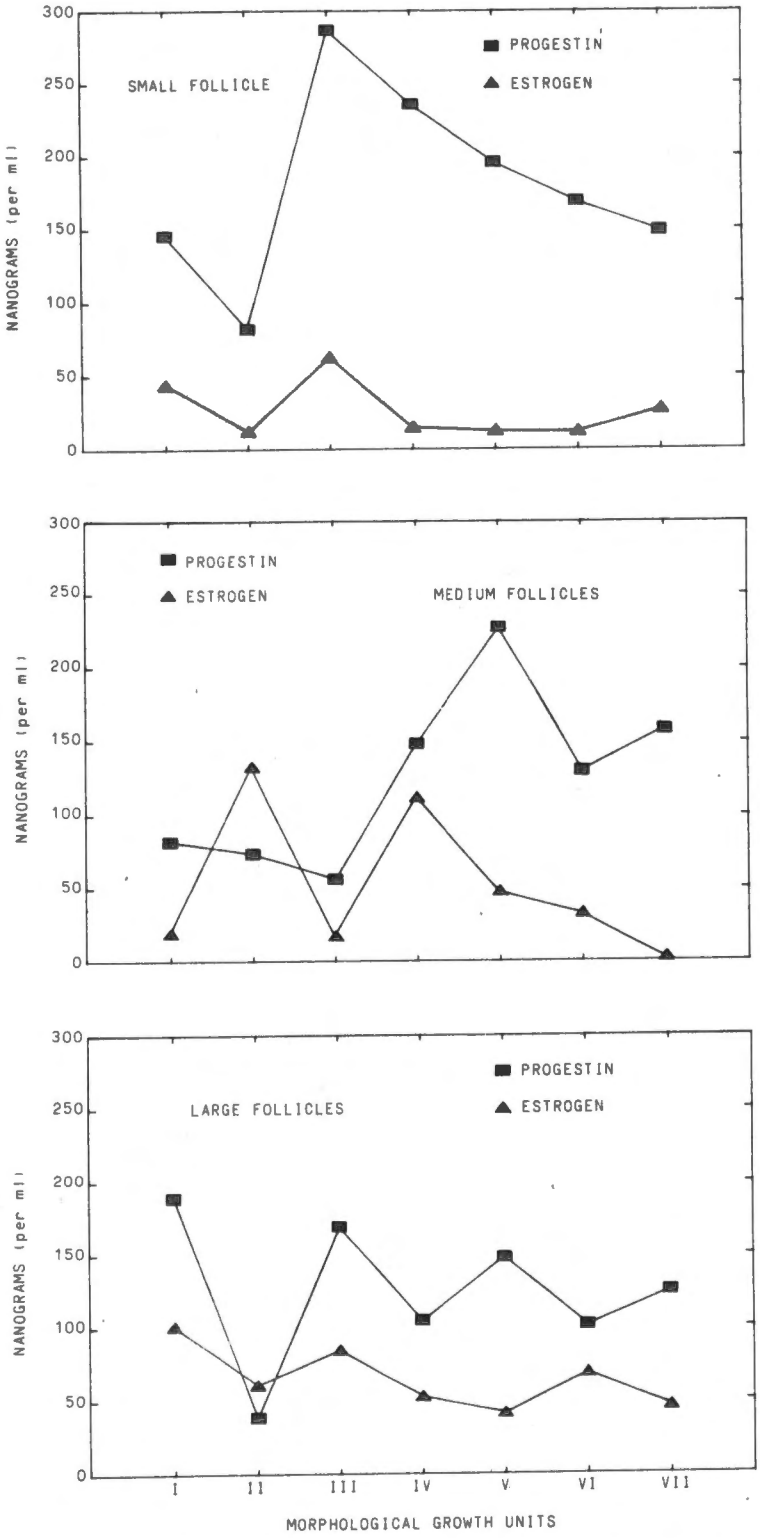


Figure 11. Estrogens and progestins in small, medium, and large follicles normalized on a per ml basis.

Table XIII

Progestin Content Per ml of Follicular Fluid

MGU	Mean	N	Standard Error	Range	Coefficient of Variation (%)
			Small Follicles		
I	145.86	15	26.27	37.35-335.63	69.76
II	81.93	11	18.36	23.00-189.34	74.32
III	286.60	6	102.67	47.94-693.37	87.75
IV	235.78	14	52.95	43.76-644.98	84.02
V	195.73	44	21.77	57.26-883.70	73.79
VI	168.90	29	20.30	4.05-457.92	64.74
VII	148.63	17	34.51	26.42-631.27	95.75
			Medium Follicles		
I	82.05	5	6.35	64.23- 98.21	17.32
II	73.88	4	29.81	33.01-161.40	80.68
III	56.22	2	12.68	53.55- 68.90	31.89
IV	148.07	7	49.41	29.79-335.83	88.29
V	227.19	23	42.87	16.88-697.02	90.51
VI	129.88	10	29.66	52.64-306.51	72.22
VII	157.91	4	44.02	26.31-210.66	55.75
			Large Follicles		
I	189.03	2	126.05	62.98-315.08	94.30
II	38.54	5	5.07	23.49- 51.67	29.40
III	169.12	5	72.21	18-57-368.48	95.47
IV	104.41	11	24.17	8.58-277.37	76.78
V	148.00	33	20.07	17.93-453.99	77.90
VI	101.18	24	12.80	18.41-313.54	62.00
VII	124.84	10	18.97	58.23-255.05	48.06

N = number of cows.

Mean = nanograms of progestins per ml of follicular fluid.

MGU = morphological growth units.

Table XIV

Estrogen Content Per ml of Follicular Fluid

MGU	Mean	N	Standard Error	Range	Coefficient of Variation (%)
			Small Follicles		
I	44.13	4	41.69	1.71-169.18	188.92
II	12.58	5	5.00	0.76- 25.53	89.05
III	6.26	3	2.15	2.48- 9.92	59.41
IV	15.04	8	4.85	0.76- 34.23	94.15
V	12.60	25	2.67	0.00- 52.46	106.04
VI	12.19	11	4.53	1.83- 40.99	123.28
VII	27.18	10	19.00	0.61-194.08	24.04
			Medium Follicles		
I	19.37	3	4.60	12.94- 28.29	41.78
II	133.02	2	58.98	74.05-192.00	62.70
III	17.49	1	-	-	-
IV	111.90	3	63.84	12.56-231.02	98.81
V	47.87	13	21.11	0.33-257.04	158.99
VI	32.74	4	14.45	0.69- 67.73	88.28
VII	2.38	2	0.42	1.96- 2.80	24.75
			Large Follicles		
I	100.93	2	66.44	34.53-167.33	93.04
II	60.63	5	29.69	16.34-175.46	107.51
III	84.40	4	25.43	39.42-155.13	60.26
IV	52.74	6	27.58	1.13-152.30	128.09
V	41.26	25	9.56	0.40-188.65	115.90
VI	68.59	15	19.42	0.62-256.87	109.64
VII	45.90	11	14.11	0.65-139.53	101.93

N = Number of cows.

Mean = nanograms of estrogen per ml of follicular fluid.

MGU = morphological growth unit.

(<85 ng per ml) through MGU III, peaked by MGU V (227.19 ng per ml), then dropped and remained low (<150 ng per ml) by MGU VI and VII. This pattern somewhat mimicked the peripheral P secretion shown in Figure 9 for the plasma. Progestins in large follicles were extremely variable (8.58 to 453.99 ng per ml) with no discernable pattern evident. Tables XV and XVI plus Figure 12 display E and P concentrations normalized on a per follicle basis. This normalization predicts the actual amount of steroid in the small, medium, and large follicles. There were significant differences ($P<.0001$) in steroid secretions among small, medium, and large follicles; however, they did not vary significantly among MGU ($P<.26$). Estrogens were highly variable within MGU and between all sizes, thus no discernable (statistically) patterns were evident. Progestins remained low in the small follicles (<7 ng per ml), and showed changes in the medium follicles that mimicked the plasma pattern. The P in the large follicles were variable (5.39 to 501.42 ng per ml) but did increase 50 percent over previous high levels by MGU VII. The ranges and variation of E and P content compare favorably with those reported by Korenman et al., (1973) and Short (1962). However, these are the only reports of P and E concentrations in bovine follicles that could be used for comparisons. Sanyal et al., (1974) and Eiler and Nalbandov (1973) have reported the patterns of E and P in FF of humans and hogs to vary similar to the peripheral pattern. This was not apparent in this experiment except for medium-sized follicles. There was a statistically significant difference noted ($P<.0001$) in E and P concentration between small, medium, and large follicles, but no significant differences were detected among the growth classifications

Table XV

Progestin Content Per Follicle

MGU	Mean	N	Standard Error	Range	Coefficient of Variation (%)
			Small Follicles		
I	6.95	4	1.16	3.97- 9.06	33.43
II	1.35	5	0.38	0.45- 2.62	63.11
III	7.38	3	2.66	3.79- 12.58	62.57
IV	6.16	8	2.75	0.52- 24.84	126.11
V	3.71	24	0.55	0.91- 13.26	73.07
VI	6.44	11	0.92	2.20- 11.00	47.34
VII	2.98	10	0.69	0.43- 8.31	73.15
			Medium Follicles		
I	17.07	3	4.35	9.49- 24.55	44.10
II	10.88	2	0.98	9.90- 11.86	12.75
III	10.34	1	-	-	-
IV	50.38	3	25.06	17.48- 99.57	86.14
V	55.91	13	18.89	6.69-207.03	121.85
VI	21.92	4	3.45	16.42- 32.01	31.45
VII	7.89	1	-	-	-
			Large Follicles		
I	91.68	2	63.86	25.82-157.54	101.59
II	22.25	5	4.22	11.75- 31.00	42.37
III	94.48	4	43.83	11.14-184.24	92.79
IV	101.29	6	32.67	12.40-249.63	79.00
V	88.30	24	21.31	5.39-501.42	118.21
VI	58.32	13	12.24	12.89-188.12	75.70
VII	158.89	10	36.10	39.53-444.97	71.85

N = number of means.

Mean = nanograms per follicles.

MGU = morphological growth units.

Table XVI

Estrogen Content Per Follicle

MGU	Mean	N	Standard Error	Range	Coefficient of Variation (%)
			Small Follicles		
I	1.46	4	1.39	.050- 5.64	190.03
II	0.22	5	0.08	.016- 0.38	84.49
III	0.09	3	0.04	.032- 0.18	77.12
IV	0.29	8	0.11	.011- 0.76	105.34
V	0.22	24	0.05	.000- 0.94	109.10
VI	0.33	11	0.13	.051- 1.23	128.26
VII	0.39	10	0.25	.009- 2.55	200.98
			Medium Follicles		
I	4.05	3	1.67	1.290- 7.070	71.48
II	39.91	2	17.69	22.210- 57.600	62.70
III	2.62	1	-	-	-
IV	32.55	3	18.79	3.770- 69.310	102.89
V	14.81	13	6.89	0.039- 77.110	167.88
VI	9.80	4	4.35	0.137- 20.320	88.70
VII	0.84	1	-	-	-
			Large Follicles		
I	48,91	2	34.75	14.160- 83.670	100.49
II	30.90	5	14.45	9.810- 87.730	104.49
III	49.27	4	10.52	28.670- 77.570	42.68
IV	38.21	6	22.01	0.653-137.070	141.08
V	20.33	24	4.59	0.120- 94.320	110.72
VI	42.74	13	12.12	0.372-128.440	110.69
VII	47.17	10	11.44	0.058- 86.730	73.60

N = number of cows.

Mean = nanograms per follicle.

MGU = morphological growth units.

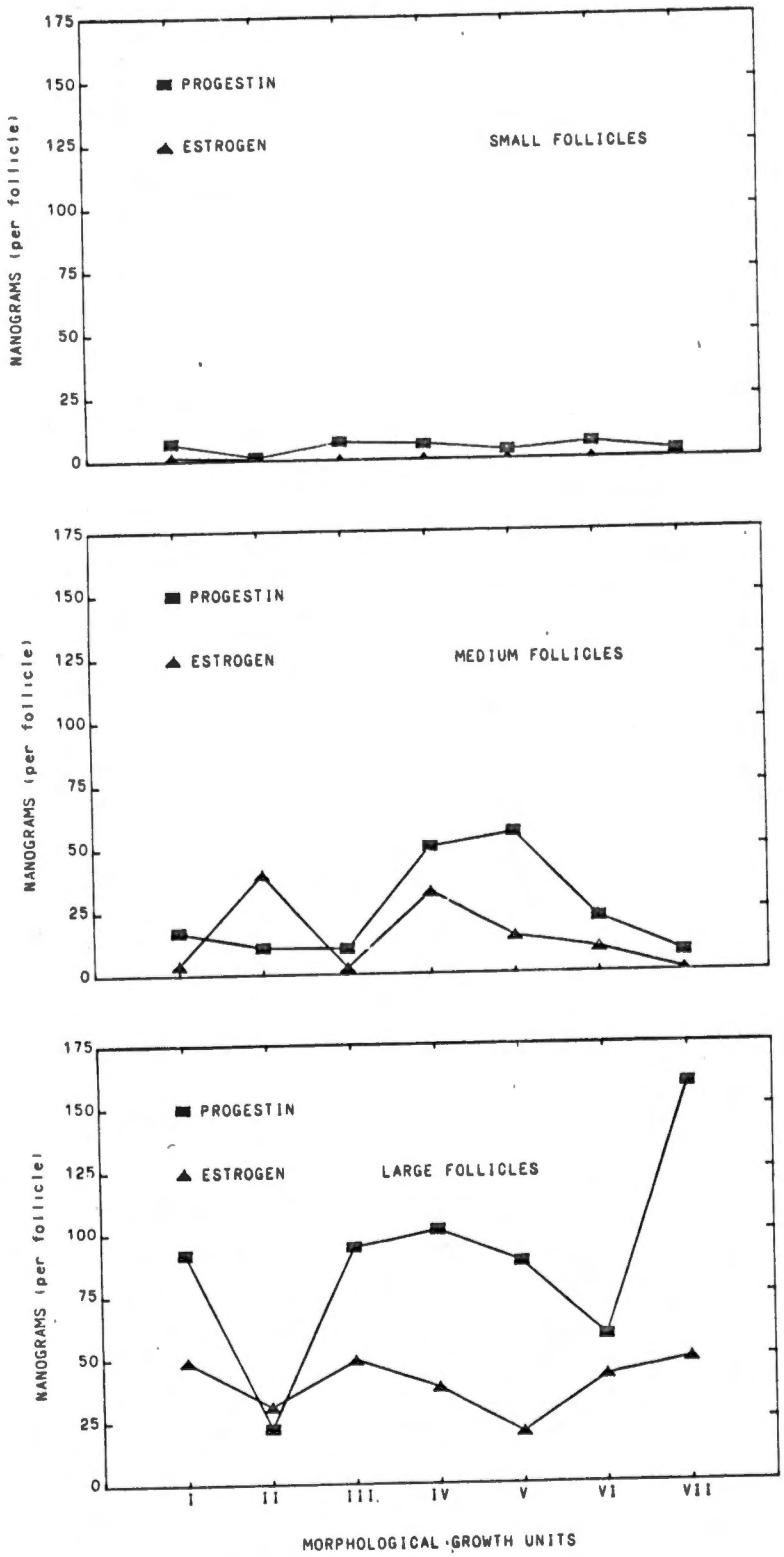


Figure 12. Estrogens and progestins in small, medium, and large follicles normalized on a per follicle basis.

(MGU) except in P content per ml of FF ($P<.05$).

There was no significant interaction of MGU and follicle size with E, P, or follicle numbers (Table XVII), but there was with follicle volume ($P<.0001$) (Figure 13 and Table XVIII). The volume of small follicles remained constant throughout the MGU; whereas, medium and large follicles increased in volume. Table XVII displays the mean number of follicles per pair of ovaries throughout the MGU. Follicle numbers per pair of ovaries remained constant between follicles (1 per pair of ovaries) for large follicles. There were 1 to 6 medium follicles per pair of ovaries with the most variable stages being MGU I (2-6 follicles) and V (1-5 follicles). The mean numbers of small follicles varied from 12 to 100 per pair of ovaries. There were greater numbers of follicles at MGU II (51.60) and MGU VII (46.00) than other stages.

The high amount of variation detected in steroid content can probably be attributed to atresia and animal differences. Atresia was not accounted for in follicle classifications, therefore, when combined with intense animal differences could produce the high variation noted. It may have been possible to separate steroid concentrations in the follicles into quantitative categories and reduce variation; however, relationships between atresia and steroid output have not been demonstrated.

Table XVII

Total Number of Follicles Per Pair of Ovaries

MGU	Mean	N	Standard Error	Range	Coefficient of Variation (%)
			Small Follicles		
I	25.50	4	2.22	20.00- 30.00	17.89
II	51.60	5	6.46	39.00- 76.00	28.00
III	26.67	3	0.88	25.00- 28.00	5.73
IV	33.25	8	8.80	12.00- 78.00	74.85
V	40.33	24	5.43	13.00-100.00	66.01
VI	31.91	11	4.03	15.00- 60.00	41.92
VII	46.00	10	7.48	16.00- 86.00	51.44
			Medium Follicles		
I	3.33	3	1.33	2.00- 6.00	69.28
II	1.00	2	-	1.00- 1.00	0.00
III	1.00	1	-	-	-
IV	2.00	3	0.58	1.00- 3.00	50.00
V	1.54	13	0.31	1.00- 5.00	73.23
VI	1.50	4	0.50	1.00- 3.00	66.67
VII	1.00	1	-	-	-
			Large Follicles		
I	1.00	2	-	1.00- 1.00	0.00
II	1.00	5	-	1.00- 1.00	0.00
III	1.00	4	-	1.00- 1.00	0.00
IV	1.00	6	-	1.00- 1.00	0.00
V	1.08	24	0.06	1.00- 2.00	26.06
VI	1.08	13	0.16	1.00- 2.00	25.75
VII	1.00	10	-	1.00- 1.00	0.00

N = number of cows.

Mean = number of follicles per pair of ovaries.

MGU = morphological growth units.

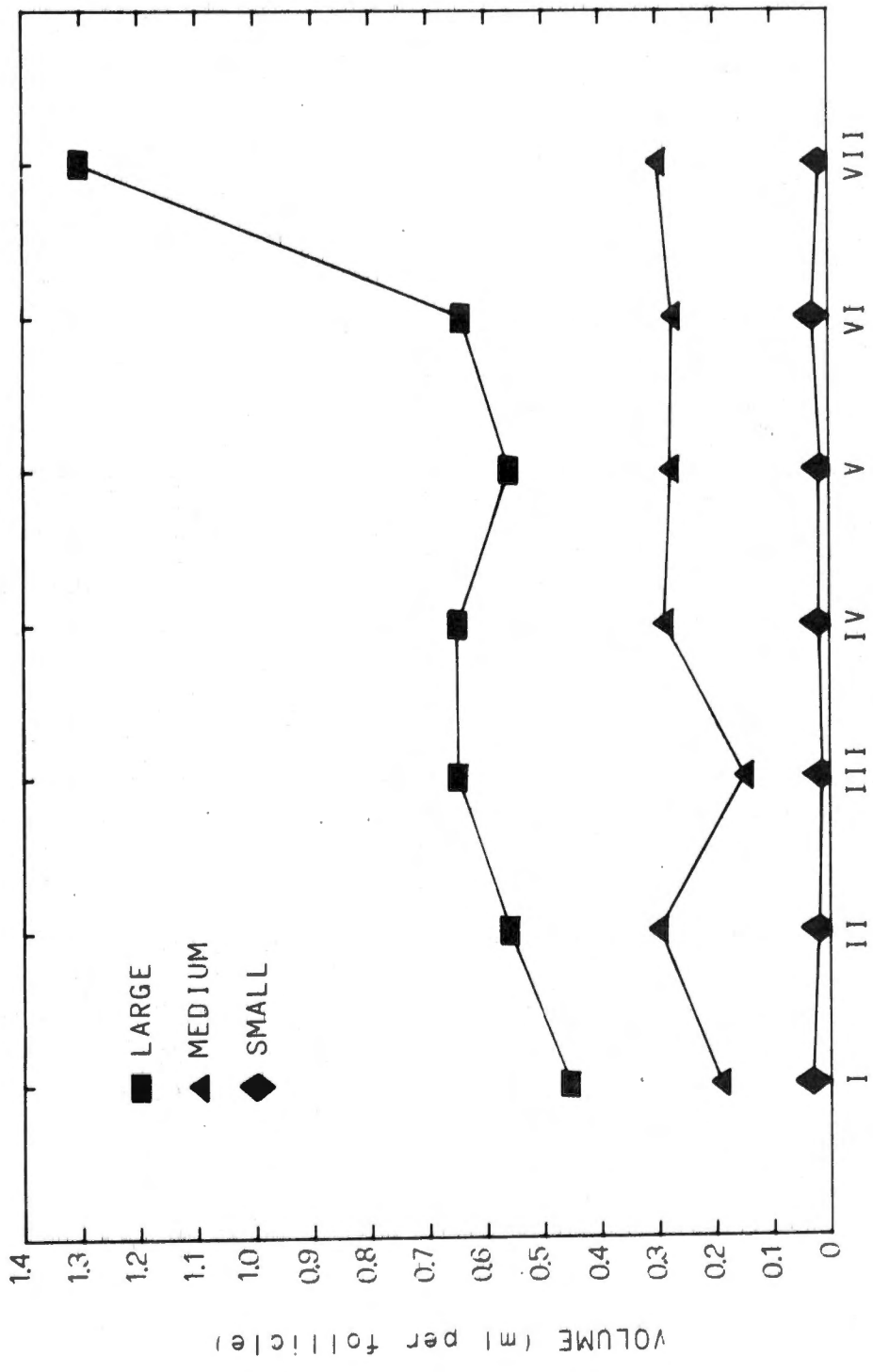


Figure 13. Volume of follicular fluid in small, medium, and large follicles.

Table XVIII

Volume of Follicular Fluid Per Follicle

MGU	Mean	N	Standard Error	Range	Coefficient of Variation (%)
			Small Follicles		
I	.032	4	.003	.027-0.390	18.63
II	.020	5	.002	.014-0.023	17.38
III	.015	3	.002	.013-0.018	21.07
IV	.020	8	.004	.005-0.043	58.00
V	.017	24	.001	.006-0.034	34.20
VI	.029	11	.003	.020-0.045	28.57
VII	.017	10	.001	.012-0.023	20.30
			Medium Follicles		
I	.192	3	.046	.100-0.250	41.43
II	.300	2	-	.300-0.300	0.00
III	.150	1	-	-	-
IV	.289	3	.011	.266-0.300	6.66
V	.278	13	.021	.120-0.400	26.71
VI	.275	4	.025	.200-0.300	18.18
VII	.300	1	-	-	-
			Large Follicles		
I	.455	2	.045	.410-0.500	13.99
II	.560	5	.051	.400-0.700	20.36
III	.650	4	.119	.500-1.000	36.62
IV	.650	6	.072	.500-0.900	27.09
V	.560	24	.048	.300-1.300	42.18
VI	.642	13	.045	.400-1.000	24.99
VII	1.303	10	.250	.600-3.400	60.69

N = number of cows.

Mean = ml of follicular fluid.

MGU = morphological growth units.

CHAPTER V

SUMMARY AND CONCLUSIONS

There were 180 Hereford heifers used in this experiment. The animals were slaughtered when a pen (6 animals) had a mean of 12 mm of backfatness after "somascoping." Blood and FF were sampled and assayed simultaneously for E and P content. Ovarian weights, CL weights, and follicle numbers were also recorded. Each pair of ovaries was then classified as to stage of growth (MGU) and inspected for abnormalities.

Estrogen and P assays were performed on the plasma and corresponding FF samples. The extraction efficiency for P was greater than 90 percent for two extractions, and E was 80 percent for one extraction. The P antiserum exhibited high specificity; whereas, the E antiserum cross reacted considerable with other steroids. Composite curves were used in the E and P assays for the determination of unknown values. The coefficients of variation for the values of the E standard curve ranged from 10.72 percent (200 ng) to 17.81 percent (60 pg) and for the P curve from 10.28 percent (500 pg) to 15.48 percent (2,000 pg). The lower limit of sensitivity of the E standard curve is 3 pg, whereas, P was 200 pg, in the radioimmunoassay.

The first objective of the experiment was to compare E and P levels in the peripheral circulation to steroid levels in the FF. Progestins and estrogens in the plasma varied inversely throughout

the CL growth phases and their patterns, levels, and ranges compared favorably with those previously described in the literature. Progestins and E from small, medium, and large follicles had patterns, levels, ranges, and variabilities different and independent from plasma steroid levels except for the patterns noted in medium follicles which appeared to mimick plasma patterns of steroid secretions.

The second objective was to compare E and P levels within small, medium, and large follicles. There were significant differences ($P<.0001$) in E and P levels and patterns from different size follicles. Progestins varied significantly ($P<.05$) among MGU; however, E was quite variable and showed no statistical significance among MGU. The high amount of variation detected in steroid levels can probably be attributed to atresia and animal differences. Within the size categories, steroid concentrations in the FF varied independently of volume changes. Follicle numbers per pair of ovaries remained constant for large follicles (1 follicle per pair of ovaries) but varied within the small (12-100) and medium (1-6) classifications.

The third objective was to obtain behavioral estrous dates along with morphological ovarian descriptions. Morphological ovarian descriptions (MGU) were used as a basis for comparisons of E and P levels in the peripheral circulation to steroid levels in FF and for comparisons between follicle sizes. Behavioral estrous cycles were extremely variable with 27 percent having short cycles (<15 days) and 37 percent having long cycles (>26 days); consequently, these were not used as a basis for comparisons of steroid secretions. Approximately 50 percent

of the animals were observed in standing estrus while the other half were not observed standing, but did display a color change in the KaMar Heatmount Detector. The variable estrous cycles were probably attributable to an initial non-random allotment of heifers into the pens, disproportionality of immature animals, stress from overcrowding and being on concrete flooring, and a possible effect of ration on the heifers.

Within the MGU classifications, there was a disproportionality in numbers of heifers in MGU V and VI. This suggests that prior to and at the beginning of regression are the most variable stages in the CL morphology; thus, this emphasizes an explanation for variability in the cycle lengths that was observed in the heifer experiment. Further substantiation of the validity of using CL changes as a basis for comparisons is suggested by CL growth and P output into the plasma comparing positively.

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He received the Bachelor of Science degree with a major in agriculture and biology in 1969. While at Austin Peay, he was a member of Delta Tau Alpha, an honorary agriculture fraternity. From 1969 until 1971 he served in the United States Army.

In the fall of 1971 he received an early-out from the Army to accept an assistantship from the Dairy Department at the University of Tennessee. One year later he received an NDEA Fellowship for one year, then in 1973 participated in an NSF Grant with Dr. P. B. Coulson. While at the University of Tennessee he was awarded the Bill Brown Memorial Award and John R. Fain Scholarship for academic accomplishments. He is presently a member of Phi Kappa Phi and Gamma Sigma Delta and is presently engaged in research towards the Doctor of Philosophy degree in Animal Science. After completion of his graduate work, he will accept a postdoctoral traineeship (NIH) with Dr. Rees Midgely at the University of Michigan.