ABSTRACT

ENDOCRINE, REPRODUCTIVE AND MAMMARY DEVELOPMENT OF HEIFERS AND RATS DURING THE ESTROUS CYCLE

by Yagya Nand Sinha

The relationship between the endocrine function and reproductive and mammary development of bovine and murine female during the estrous cycle was investigated. Endocrine function was studied by measuring luteinizing hormone (LH) and prolactin in the pituitary gland, metabolic status of the uterus and ovarian weight changes. Reproductive and mammary development was assessed by deoxyribonucleic acid (DNA), ribonucleic acid (RNA), protein, lipid and collagen content of the organs as well as histological observations.

The length of the estrous cycle of heifers averaged 20.6 $\pm$ 0.2 days. The age of vaginal opening of rats averaged 36.7 $\pm$ 0.13 days. Vaginal opening coincided with proestrus or estrous smears in 58% of the rats. The first (5.5 days) and second (5.2 days) estrous cycles were significantly longer than the third (4.8 days) and fourth (4.9 days) estrous cycles.

Uterine DNA of rats did not change between proestrus (0.88 mg/100 g BW) and estrus (0.85 mg/100 g BW)

or metestrus (0.70 mg/100 g BW) and diestrus (0.70 mg/100 g BW) but declined 18% between estrus and metestrus, suggesting a significant loss of uterine cells during the early luteal phase of the cycle. Cumulatively, uterine DNA increased from the first through the third estrous cycles but not thereafter. Uterine weight, RNA, RNA:DNA ratio and lipid content were maximum at proestrus, declined at estrus and metestrus but increased during diestrus. Pituitary LH concentration, which averaged 0.72, 0.31, 0.27 and 0.33 μg/mg at proestrus, estrus, metestrus and diestrus, respectively, closely paralleled the changes in the RNA:DNA ratio of the uterus.

Mammary DNA of heifers increased from 97.8 mg/100 lb BW at proestrus (day 20) to 213.7 mg/100 lb BW at estrus, then declined during metestrus (day 2, 169.1 mg/100 lb BW) and diestrus (days 4-18, 142.8 mg/100 lb BW), suggesting proliferation of mammary cells during the estrogenic phase of the cycle and loss of cells during the progestational phase. Mammary RNA and RNA:DNA ratio followed a pattern similar to mammary DNA. Mammary protein, collagen and lipid content also increased during the estrogenic phase of the cycle but, unlike the nucleic acid content, they did not decline until after metestrus. Pituitary prolactin concentration increased linearly from a minimum of 0.012 IU/mg on day 2 of the estrous cycle to 0.045 IU/mg on the day of estrus. Prolactin concentration paralleled

mammary development during the estrogenic phase of the estrous cycle but not during the progestational phase.

Mammary DNA of rats increased 8% between proestrus and estrus but did not change thereafter. Mammary RNA and RNA:DNA ratio, however, increased between proestrus and estrus, remained constant at metestrus but declined 9% at diestrus, suggesting stimulation of mammary growth during the estrogenic phase of the cycle and involution during the luteal phase. Cumulatively, mammary DNA increased from the first through the fourth estrous cycles but not thereafter. Pituitary prolactin concentration during proestrus (0.032 IU/mg) and metestrus (0.041 IU/mg) were significantly greater than during estrus (0.015 IU/mg) and diestrus (0.015 IU/mg). Thus, like the heifer, pituitary prolactin concentration of rats paralleled increases in mammary growth during the estrogenic phase but not during the progestational phase of the cycle.

It is concluded that the pubertal development of the reproductive and mammary apparatus of the heifer and the rat is completed within a few estrous cycles. And, although there are intrinsic differences in the mechanism of reproductive and mammary growth, both are closely related to the estrous cycle.

ENDOCRINE, REPRODUCTIVE AND MAMMARY DEVELOPMENT OF HEIFERS AND RATS DURING THE ESTROUS CYCLE

By

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A THESIS

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DEDICATED TO

my father

Sri Baidyanath P. Sinha

BIOGRAPHICAL SKETCH

I was born at Rohua, Muzaffarpur, Bihar, India, on October 21, 1936. I received my high school education at Muzaffarpur Zilla School and passed the Matriculation Examination in February, 1952. In July, 1952, I entered Langat Singh College, Muzaffarpur and passed the Intermediate in Science examination in February, 1954. The same year I entered Bihar Veterinary College, Patna and graduated in March, 1957.

I worked for the government of Bihar, India, as a Veterinary Assistant Surgeon at Nathnagar from April, 1957, to March, 1958; as a Veterinary Assistant Surgeon in charge of the Artificial Insemination Center at Surajgarha from April, 1958, to September, 1959; and as a research assistant in Biological Products at the Livestock Research Station, Bihar, Patna, from October, 1959, to September, 1961.

I joined the graduate school at Michigan State University in September, 1961, and received the Master of Science degree in the Department of Dairy in June, 1964, and the Doctor of Philosophy degree in September, 1967. My primary area of interest is reproductive and mammary physiology.

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CHAPTER I

INTRODUCTION

Based upon DHIA records, over 850,000 cows in the United States are lost each year due to infertility--an annual loss of $170 million to the U. S. dairymen. Little is known about the direct causes of these reproductive failures, especially those related to physiological and endocrine factors. But part of this infertility is probably due to aberrations of the estrous cycle which markedly influences development of the reproductive organs and mammary glands of the female. A knowledge of the normal endocrine and reproductive changes during the estrous cycle will be necessary in order to diagnose these aberrations. Therefore, one objective of this study was to define the normal patterns of endocrine and reproductive development during the estrous cycle of the pubertal female.

The animals that are lost each year due to reproductive failures could still be used for milk production artificially by means of hormonal treatment. But all attempts in this direction have been beset by the problem of inconsistent response by the individual animals. Since milk production depends greatly on the extent of initial

development of the mammary gland, part of the reason for this variability may be inadequate mammary development prior to hormonal therapy due to aberrant functioning of the endogenous hormones of the animal. Therefore, a clear understanding of the factors necessary for normal mammary development before the onset of pregnancy is essential for successful control of milk production. In addition, although it is recognized that much of the pubertal growth of the mammary gland occurs under the influence of the estrous cycle, the magnitude of this relationship has never been quantified. Thus, the second objective of this study was to determine the normal pattern of mammary development and its controlling mechanisms during the estrous cycle of the pubertal female.

According to a recent survey by the Dairy Department, Michigan State University, the economic pressure and the cost of production in dairy farm operations will continue to rise in the coming years. This will demand increased efficiency in the operation of the dairy farms in the future. At present, it costs about $350 to raise a dairy heifer to 24 months, the usual age at first calving. A reduction in the age of first calving of heifers may, therefore, provide one avenue to improve efficiency. Thus, a third objective of this study was to determine the age of sexual maturity of the female in terms of endocrine, reproductive and mammary development.

Finally, it was the purpose of these experiments to compare the development of the reproductive organs and the mammary glands during the estrous cycle. Although the reproductive organs and the mammary apparatus are believed to be influenced commonly by the hormones of the estrous cycle, their growth in the same animal has never been compared. Such a comparison should help elucidate the differences in the controlling mechanisms of their growth.

Heifers and rats were used as the experimental animals. Endocrine function was studied by measuring the hormones luteinizing hormone (LH) and prolactin in the pituitary gland, metabolic status of the uterus and ovarian weight changes. Reproductive and mammary development was assessed by the use of the biochemical parameters deoxyribonucleic acid (DNA), ribonucleic acid (RNA), protein, lipid and collagen as well as histological observations. The DNA served as an estimate of cell numbers, RNA and protein as indices of metabolic activity and collagen and lipid as measures of connective tissue components of the organs.

CHAPTER II

REVIEW OF LITERATURE

A. Definition of Puberty and Estrous Cycle

Although growth is a gradual process, between birth and adulthood the animal undergoes a phase of rapid bodily and sexual development commonly known as puberty. Puberty has been variously defined by many authors; for example, "puberty is the time at which reproduction first becomes possible" according to Asdell (1946) whereas "appearance of the first estrus is tantamount to attainment of puberty" according to Desjardins (1966). But most definitions suffer from the fact that they depict puberty as a one-stroke phenomenon. Recently, however, Donovan and van der Werff ten Bosch (1965) have defined puberty as "the phase of bodily development during which the gonads secrete hormones in amounts sufficient to cause accelerated growth of the genital organs and the appearance of secondary sexual characters." This definition envisages the advent of puberty in advance of the occurrence of vaginal operning or menarche which is merely a visible landmark in the process of attainment of puberty and extends it to the stage when the reproductive functions become fully perfected. It is during this period of

attaining puberty that the animal exhibits for the first time the sexual phenomenon known as estrus.

The word "estrus" or "estrum" is a Latin adaptation of the Greek term: oistros. This is defined by the Oxford English dictionary as "something that stings or goads one on, a stimulus, vehement impulse, frenzy" and in its specific biological meaning as the "sexual orgasm" and the "rut of animals."

The British scientist Heape (1900), for the first time, adopted the masculine form of the term to describe the "special period of sexual desire of the female," distinguishing it from the rutting season of the male. He also employed it in its neuter form, in combination with such qualifying prefixes as pro-, di-, met-, an- and as the adjective "estrous." Heape's terminology met with universal approval and in the years since its first introduction it has been used widely by reproductive physiologists throughout the world.

But estrus is only a brief phase in the reproductive cycle of the adult female which consists of stages such as (1) follicle growth, (2) ovulation, (3) progravidity, (4) gravidity, (5) parturition, and (6) postpartum nurture (Everett, 1961). Although the above phases constitute a complete cycle, in the absence of mating or insemination the animal does not experience pregnancy and the cycle is interrupted. In such a case, the animal exhibits a

sequence of rhythmical changes known as the "estrous cycle." The highlight of this event is the period of estrus at which time the female is receptive to the male.

B. Periods of the Estrous Cycle

Animals in which estrus occurs only once during the sexual seasons are called "monoestrus" (for example, the bitch). Those which show a regularly recurring estrous cycle throughout their reproductive life are said to be "polyestrus" (for example, the cow and the rat). A third type of animal shows a recurrence of the estrous cycle only during certain seasons of the year and are known as "seasonally polyestrus" (for example, the mare and the ewe). Yet in each case, the estrous cycle, based on certain changes, is generally characterized by four phases: proestrus, estrus, metestrus and diestrus.

Proestrus, the period of preparation, is characterized by follicular growth of the ovary and growth of the tubular genitalia. It is the stage of mounting estrogenic activity and lasts for 2-3 days in the cow (Salisbury and VanDemark, 1961) and about 12 hours in the rat (Asdell, 1965).

The next period, estrus, the period of desire, marks the climax of the process. It is characterized by psychic manifestations of heat and it is only at this time that the female is willing to receive the male. It lasts for 12-18 hours in the cow (Salisbury and VanDemark, 1961) and about 13 hours in the rat (Asdell, 1965).

If conception or pseudopregnancy does not occur, estrus is followed by metestrus which is characterized by a sudden decline of sexual activity. During this period estrous changes in the generative system and estrogenic activity subside. It lasts for 3-4 days in the cow (Salisbury and VanDemark, 1961) and about 6 hours in the rat (Asdell, 1965).

Finally, diestrus is the period at which the activity of the corpus luteum is paramount. The uterus undergoes marked changes in preparation for the nourishment of the embryo. It lasts for 14-15 days in the cow (Salisbury and VanDemark, 1961) and about 57 hours in the rat (Asdell, 1965).

Although the above is a convenient division of the estrous cycle, more recently it has also been defined on the basis of the hormone effective at the time. Thus, the term "follicular" or "estrogenic" phase is frequently used to describe the part of the cycle when the influence of the estrogenic hormones is predominant and "luteal" or "progestational" when the corpus luteum develops and progesterone becomes the predominating hormone (Salisbury and VanDemark, 1961). These terminologies are especially useful in the study of comparative reproductive physiology.

C. Vaginal Smear During the Estrous Cycle

The four phases of the estrous cycle--proestrus, estrus, metestrus and diestrus--are associated with cyclic

changes in almost all parts of the female reproductive tract including the ovaries and the mammary glands. But it is the discovery of their occurrence in the vaginal smear of the guinea pig (Stockard and Papanicolaou, 1917) that opened the way to the detailed investigation of the estrous cycle in several species. The two classical studies that quickly followed were those of Long and Evans (1922) on the rat and of Allen (1922) on the mouse. Ever since, examination of the vaginal smear has provided an easy and fairly accurate method for following the estrous cycle in living animals and has contributed much to the advancement of our knowledge of reproductive physiology.

The basic constituents of the vaginal smear are nucleated epithelial cells, cornified cells and leukocytes (Allen, 1922; Long and Evans, 1922), although by the use of special staining, various subgroups of these three cell types can be recognized (Hartman, 1944). The relative proportion of each of these three cell types varies characteristically during different phases of the estrous cycle and forms the basis for identification of the stage of the cycle.

During proestrus, the vaginal smear consists largely of small, round, lightly staining, nucleated epithelial cells which occur singly or in small sheets. Leukocytes are totally absent (Long and Evans, 1922).

At estrus the vaginal smear is "cheesy" and consists exclusively of non-nucleated cornified cells which stain a brilliant orange-red with Shorr's stain. This phase is occasionally divided into two stages (Long and Evans, 1922); during the second stage cornified cells are more abundant, although the female no longer accepts the male. In late estrus cornified cells begin to diminish and the leukocytes appear in the vaginal smear. At the same time, large basophilic epithelial cells with vesicular nuclei (so-called "Shorr" cells; Hartman, 1944) appear.

During metestrus and early diestrus the smear is thick and consists mostly of leukocytes and a few cornified cells, Shorr cells and basophilic epithelial cells.

During diestrus the vagina usually contains a little mucus in which are entangled a large number of leukocytes and a few nucleated basophilic and sometimes vacuolated epithelial cells (Mandl, 1951a).

Although the vaginal smear is a reliable technique for diagnosing stage of the estrous cycle in the rat and also some other species, it cannot be used accurately in the case of the cow. Changes in the vaginal mucus occur, but they are not as clear cut in the cow as they are in many other species, probably because of the low hormone level in the cow at all times and also the complex nature of the vaginal epithelium (Hansel, Asdell and Roberts, 1949). Hammond (1927), for the first time, studied the

smears taken from the vulvar region of the vagina of heifers. According to him, smears a few hours before heat are characterized by few vaginal epithelial cells present owing to their dilution with mucin. For about 6 days after the onset of heat smears from the vagina generally show abundant leukocytes and often red blood cells 2 to 4 days after the beginning of heat. During the remainder of the cycle only vaginal epithelial cells occur with now and then an occasional leukocyte.

Since the part of the vagina closest to the vulva consists largely of mucus-secreting cells where true cornification does not occur, Hansel et al. (1949) studied vaginal smears aspirated from the cervical end of the vagina but still did not observe any sharply distinguishable pattern. And whatever changes they observed varied so much between individuals as to render it useless as a diagnostic tool.

In contrast, naked eye observations of the vaginal discharge at different stages of the cycle are fairly characteristic in the cow (Hammond, 1927). The vagina is comparatively dry during the greater part of the cycle; a day or two before heat it becomes rather moist, at the beginning of heat it shows a flow of clear fluid mucus; and toward the middle or end of the heat the flow contains small cheesy yellowish-white lumps. About the end of heat the mucus becomes thicker and just after heat frequently

whitish in color and very much less in amount. In most heifers this is followed about the second or third day after the beginning of estrus by a flow of mucus stained with blood which usually lasts from 6-20 hours but may continue intermittently for 3 days.

It must be noted, however, that although prevalence of a certain type of cell in the vagina is highly correlated with the ovarian cycle of the animal, it is by no means a completely foolproof index of the physiological state of the animal. For example, estrus in the rat may occur in the absence of a cornified smear, and vice-versa (Young, 1941). According to Blandau, Boling and Young (1941), the "copulatory response" is a better test of sexual receptivity. Cyclic vaginal changes, similar in general character and duration, though not in intensity to those present in normal animals, also occur in ovariectomized rats (Mandl, 1951b).

D. Uterine Changes During the Estrous Cycle

Despite occasional aberrations, the estrous cycle forms the nucleus of mammalian reproductive function and the uterus and other reproductive organs undergo marked morphological and biochemical changes during its various phases.

Increases in water content of the uterus during the estrogenic phase of the cycle have been observed by many workers (Astwood, 1939). Uterine weight--dry as well as

wet--increases dramatically during the estrogenic phase in virtually all species studied (Astwood, 1939; Dirschel, Schriefers and Bruener, 1955; Olds and VanDemark, 1957), as does volume and length of uterine horns in the rat (Cullen and Harkness, 1964). These physical changes are accompanied by increases in such biochemical entities as DNA (Drasher, 1952), nitrogen (Morgan, 1963) and non-collagen protein (Mortan, 1963), all signifying growth of the organ. But these changes represent growth of all components of the uterus: epithelial, myometrial as well as connective tissue. Since each of the above components may respond differently under different physiological conditions, some experiments have directed attention to the study of individual components.

In order to study alterations in the connective tissue framework, hydroxyproline, an index of collagen (Neuman and Logan, 1950) and hexosamine, an index of ground substance (Allison and Smith, 1965) have been measured. Contrary to the growth parameters (weight, DNA and nitrogen) the collagen content of the uterus decreased to low values during estrus from maximal values during diestrus (Morgan, 1963; Smith and Kaltreider, 1963). This suggested a catabolism and/or reduction in the formation of connective tissue in transition from diestrus to estrus, but simultaneous increases in nitrogen and non-collagen protein suggested a replacement by non-connective tissue elements.

On the other hand, hexosamine continued to increase during this same transition period (Morgan, 1963), indicating that laying down of ground substance proceeds during the growth of each type of tissue component.

Other parameters of uterine metabolism also show cyclic fluctuations. RNA and RNA:DNA ratio (indices of protein synthetic activity) in mouse uterus attained maximal levels during estrus and minimal values during diestrus (Drasher, 1952). Oxygen consumption in the rat uterus was greatest during late diestrus and proestrus (Saldarini and Yochim, 1967). ß-glucuronidase activity in the mouse uterus (Leathem, 1959), but not in the rat (Saldarini and Yochim, 1967), reached high levels during proestrus and declined during diestrus, although the relationship between this enzyme and the growth process is not clear.

Reduced diphosphopyridine nucleotide (DPNH) oxidase is an essential mediator of several DPN-linked dehydrogenation reactions and, together with lactic dehydrogenase, controls the reversible oxidation and reduction of lactate and pyruvate at the end of the glycolytic sequence of reactions. Both of these enzymes in the rat uterus displayed maximal activity during proestrus and early estrus and minimal activity during diestrus (Bever, Velardo, Telfer, Hisaw and Goolsby, 1954). Another study (Rosa and Velardo, 1959) revealed similar changes in two of the

other oxidative enzyme systems of the uterus: DPN-diaphorase and succinic dehydrogenase.

Lipid components of the uterus also vary during the estrous cycle. Lecithin and free cholesterol, but not cholesterol esters, increased in the pig uterus during the progestational phase of the cycle (Okey, Bloor, and Corner, 1930). In the mouse uterus, progesterone injection slightly enhanced lipid concentration, whereas estrogen reduced it significantly (Leathem, 1959).

The function of uterine glycogen has always been more or less of a mystery. Because it appeared in large amounts in the primate endometrium in the progestational phase, a time when the uterus prepares to receive the egg, it was considered a nutritive agent (Van Dyke and Chen, 1936). But in the rat, glycogen probably plays a different role, since it was present in high concentration during the estrogenic phase and low at the time of implantation (Boettiger, 1946). Glycogen is also deposited in large amounts in the myometrium and is thus considered a source of energy for contraction (Telfer, 1953).

The morphological changes that occur in the uterus during the estrous cycle have been reviewed extensively by Eckstein and Zuckerman (1956) and the account that follows has been adapted mainly from them.

In the cow, during proestrus, the epithelium lining the uterine cavity is tall columnar and pseudostratified

with basal nuclei. Rarely it may be ciliated. But the glandular epithelium is not as tall as that lining the uterine cavity. The basal glands are spiral and the stroma very vascular. Although no extravasation of blood occurs, the epithelial cells lining the uterine cavity may contain blood pigment.

At estrus, the glandular epithelium of the uterus is taller; the stroma more edematous. And scattered throughout the stroma are many cells including erythrocytes. Two days after estrus the basal glands are more coiled and filled with secretion, while the stroma is less edematous. Glandular hypertrophy and vascularity of the stroma are most marked 8 days after estrus and signs of regression appear about the 15th day.

In the rat, during proestrus, uterine horns fill with watery fluid and the epithelium is lined with cuboidal cells. Estrus is marked with the signs of degeneration of the epithelium such as loss of the basement membrane, vacuolar degeneration and leukocytic invasion. But denudation of the uterine epithelium does not occur; few or no mitoses are ever seen. Uterine glands are small at this time but there are no visible degenerative changes either in the glands or in the stroma.

During metestrus, degeneration and regeneration proceed together and the uterus rapidly returns to the condition typical of diestrus. During diestrus, the uterus

is small, avascular and has a slit-like lumen. It is lined by a simple columnar epithelium. Frequent mitoses occur in the endometrial glands of the mouse uterus during late diestrus (Allen, 1922). But in the rat, mitoses could not be observed in the glands and only rarely in the surface epithelium (Allen, 1931).

E. Changes in the Mammary Glands During the Estrous Cycle

To the best of my knowledge there have been practically no biochemical studies of the mammary glands during the estrous cycle. The only study available is by me (Sinha, 1964) in which I measured the DNA content of mammary glands of rats killed at specific ages without regard to stage of the estrous cycle. When the data were later pooled according to stage of the estrous cycle the results were not conclusive. Yet the literature abounds with observations on the gross morphological changes in the mammary glands of numerous species during the estrous cycle (Turner, 1939). And in general, all agree that like the uterus, the mammary gland undergoes profound changes during different phases of the estrous cycle.

In species with short estrous cycles, such as rats (Sutter, 1921) and mice (Bradbury, 1932; Turner and Gomez, 1933a; Cole, 1933) the duct system proliferated with the onset of the first estrous cycle while each subsequent estrus caused a further slight burst of growth resulting in a gradual cumulative growth of the duct system with the

succession of estrous cycles. During proestrus buds formed at the ends of the ducts while in early estrus the ducts distended with fluid and the buds elongated and sprouted. Regression and collapse of the duct system occurred during metestrus and diestrus. But alveoli and intralobular ducts did not form in the mammary glands during the estrous cycle.

In animals with long estrous cycles, such as guinea pigs and cows, where some growth of the alveoli would be expected due to the long progestational phase of the cycle, very few, if any, alveoli form. Hammond (1927) has described changes in the mammary glands of virgin heifers during the estrous cycle. Like rats and mice, the lumen of the ducts were large and filled with secretion just before estrus and the epithelium was cuboidal. Eight days post-estrus, the lumina were small and the epithelial cells were almost columnar. But he did not find any alveoli at any stage of the cycle in virgin heifers. Nor was any observed in goats (Turner and Gomez, 1936) or guinea pigs (Turner and Gomez, 1933b).

In the rabbit, which is in constant estrus, indicating an indefinite follicular phase, marked development of the duct system with terminal enlargements occurred (Ancel and Bouin, 1911), but in the ferret, which is also in constant estrus (Hammond and Marshal, 1930), no significant development of the duct system occurred.

F. Changes in the Hormones of the Ovary, Anterior Pituitary and Hypothalamus During the Estrous Cycle

Most of the changes described in the uterus and the mammary glands are brought about by ovarian hormones, estrogen and progesterone, secreted in varying amounts during the estrous cycle. Although relatively little is known about the exact levels of secretion of these hormones during the normal estrous cycle, the general pattern of their secretion is apparent in several species.

Estrogens. Follicular fluid of the cow ovary contained ten times more estrogenic activity during the follicular phase than during the luteal phase (Paredis, 1950), and estrogens in the peripheral blood plasma followed a similar pattern (Ayalon and Lewis, 1961). But measurement of estrogen activity in the urine of cycling cows has not yielded consistent results (Mellin and Erb, 1965). In the sow, however, urinary estrogen excretion invariably was greatest just before or at the onset of estrus (Velle, 1958; Raeside, 1963; Liptrap and Raeside, 1966). Presumptive evidence that the level of estrogen secretion increases with the enlargement of the follicles was also provided by the demonstration that more and more estrogen was required to maintain perineal turgescence in baboons (Gillman and Gilbert, 1946).

In animals with short estrous cycles, such as rats and mice, while no actual data are available, it has

generally been assumed that estrogen is secreted maximally at the time of estrus, based on the observation that cornified vaginal smears and estrus behavior occur simultaneously. But since it takes 48 to 72 hours for vaginal epithelium to proliferate and then degenerate into cornified cells, estrogen must be elaborated several hours before estrus (Young, 1961). The dilation of the uterus with fluid late in proestrus (Astwood, 1939) is also evidence that effective estrogen secretion had been elaborated 24-30 hours earlier.

Progesterone. The concentration of progesterone in bovine corpora lutea showed an initial rise between day 1 and 5, remained constant until day 9 or 10 and then increased to a peak on day 14 and 15 followed by a decline to the next estrus (Mares, Zimbleman and Casida, 1962; Gomes, Estergreen, Frost and Erb, 1963). Concentration of progesterone in ovarian venous plasma (Gomes et al., 1963) or systemic plasma (Henricks, Oxenreider, Anderson and Guthrie, 1967; Plotka, Erb, Callahan and Gomes, 1967) followed a similar pattern. And finally, in vitro synthesis of progesterone in the corpus luteum was maximum 4 to 13 days post-estrous, declined between day 14 and 18 and no synthesis could be detected in 19-day-old corpus luteum (Armstrong, Black and Cone, 1964).

Similarly, in the sow, corpus luteum progesterone concentration and ovarian venous plasma progesterone concentration increased until day 12 to 15 of the cycle and declined thereafter (Masuda, Anderson, Henricks and Melampy, 1967). The corpus luteum synthesized progesterone *in vitro* 4 to 16 days post-estrus but not on day 18 (Duncan, Bowerman, Anderson, Hearn and Melampy, 1961).

Concentration of progesterone in the ovarian venous plasma of the ewe (Edgar and Ronaldson, 1958) followed a pattern similar to that of the cow (Gomes *et al.*, 1963). In the goat, concentration of progesterone in arterial plasma was low during estrus but rose to high levels in the luteal phase (Heap and Linzell, 1966). Interestingly, the mammary glands absorbed 20% of this secretion of estrogen. In the rat, according to a preliminary study (Porter, Siiteri and Yates, 1967), progesterone concentration in the ovarian venous plasma showed maximal levels during proestrus and declined during estrus, metestrus and diestrus in that order. But according to another report (Hashimoto and Melampy, 1967) two peaks of progesterone --one during proestrus and the other during metestrus-- were observed.

Luteinizing Hormone (LH). The ovarian hormones, estrogen and progesterone, are not the only hormones which fluctuate with the phase of the estrous cycle. Among the

pituitary hormones, LH has been most widely studied because of the availability of a simple and sensitive assay. In general, LH secretion increases during the follicular phase of the cycle and is low at other times in almost all species studied.

In the cow, the LH content of the pituitary dropped significantly after the onset of estrus (Rakha and Robertson, 1965a), whereas LH concentration in the blood plasma rose rapidly 6-17 hours prior to ovulation (Anderson and McShan, 1966) suggesting marked enhancement of LH secretion at the time of ovulation. In the pig, LH content of the pituitary (Parlow, Anderson and Melampy, 1964) as well as LH concentration of the blood plasma (Anderson and McShan, 1966; Liptrap and Raeside, 1966) showed essentially the same pattern. Similarly, LH content of the pituitary of the sheep (Robertson and Hutchinson, 1962; Rakha and Robertson, 1965b; Robertson and Rakha, 1965) and the monkey (Simpson, van Wagenen and Carter, 1956) and LH concentration in the plasma of the human (Ross, Odell and Rayford, 1967) were elevated at the time of ovulation.

Much work on LH secretion has been done in the rat. On the basis of earlier studies (Everett, 1961) involving hypophysectomy and the use of central nervous system blocking drugs, an ovulatory surge of LH release between a relatively short interval of time on the afternoon of proestrus was postulated. Recently direct measurements

of LH in the pituitary (Mills and Schwartz, 1961; Schwartz and Caldarelli, 1965; Anderson and McShan, 1966) have confirmed the hypothesis. In addition, they have shown that LH is probably secreted at a fairly high level for a more sustained interval of time than indicated by earlier studies.

In the fowl, however, there may not be just one peak of LH secretion. Two (one at ovulation and the other 8 hours prior to ovulation) or three (21, 14 and 8 hours prior to ovulation) peaks of LH in the pituitary (Heald, Furnival and Rookledge, 1967) and the plasma (Nelson, Norton and Nalbandov, 1965), respectively, have been reported. One peak (8 hours before ovulation) is probably involved in causing ovulation, but the function of the others is not clearly understood.

Follicle Stimulating Hormone (FSH). In contrast to LH, few direct estimates of FSH secretion during the estrous cycle have been made. But in general, FSH secretion parallels LH secretion except that peak FSH secretion precedes peak LH secretion by a few hours to a few days, depending upon the species.

In the cow (Rakha and Robertson, 1965a) and sheep (Santolucite, Clegg and Cole, 1960; Robertson and Hutchinson, 1962; Rakha and Robertson, 1965b; Robertson and Rakha, 1965) pituitary content of FSH dropped during estrus; in the pig (Parlow, Anderson and Melampy, 1964)

FSH increased between day 4 and 18 but decreased markedly between day 18 and 1 of the cycle, all suggesting enhanced FSH secretion during estrus. In the rat, however, conflicting reports have appeared: pituitary FSH content decreased on the day of proestrus according to one report (Gans, deJones, van Rees, van der Werff ten Bosch and Wolthuis, 1964) whereas it decreased on the day of estrus according to others (Soliman and Nasr, 1962; McClintock and Schwartz, 1967).

Prolactin. The only species in which prolactin has been shown unequivocally to have a luteotrophic function are rats and mice (Meites and Nicoll, 1966). But in these species, because of short duration, corpora lutea are not believed to be functional during the estrous cycle (Simpson, 1959). Moreover, in species in which corpora lutea are definitely known to be functional during the estrous cycle, such as the cow, the role of a luteotrophin has been assigned more and more to LH during recent years (Hansel, 1966). Thus, the function of prolactin secreted during the estrous cycle is intriguing and its other prominent target organ--mammary gland--acquires special significance in this context. Nonetheless, several reports indicate that prolactin is probably secreted in various species during the estrous cycle.

In the sheep (Day, Anderson, Hazel and Melampy, 1959) prolactin potency of the pituitary gland showed a

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linear increase with advancing stages of the estrous cycle. Although Clark and Baker (1964) did not observe any difference in the prolactin content of the rat pituitaries during the estrous cycle, Reece and Turner (1937) noted greater concentration of prolactin in the rat pituitary during proestrus and estrus than during metestrus and diestrus. Indirect evidence that prolactin secretion may increase during proestrus was suggested by the transient depletion of cholesterol observed in the corpora lutea of rats during proestrus (Everett, 1945).

A satisfactory method for measuring prolactin in blood plasma has been hard to come by. But recently, by the use of a radio-immunoassay, a surge of prolactin in the blood plasma of mice during proestrus and estrus has been reported (Kwa and Verhofstad, 1967). On the other hand, hyperemia of corpora lutea formed in the intraocular ovarian isografts of mice suggested increased prolactin secretion during metestrus (White and Browning, 1962).

Like the rat, guinea pig pituitaries contained greater amounts of prolactin during estrus than during diestrus (Reece, 1939). And in women (Simkin and Arce, 1963), prolactin activity appeared in the blood during the first five and last ten days of the menstrual cycle.

Other Pituitary Hormones. Information on the secretion of adrenocorticotrophic hormone (ACTH), thyroid stimulating hormone (TSH) and growth hormone

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(GH) during the estrous cycle is scarce. Simpson, van Wagenen and Carter (1956) did not observe any change in the pituitary concentration of ACTH, GH and TSH during the menstrual cycle of the monkey but the assays used were not quite rigorous. That TSH secretion may indeed be influenced by the estrous cycle is manifested by an increase in thyroid function during estrus in the rat (Feldman, 1956; Brown-Grant, 1962a) and guinea pig (Brown-Grant, 1962b) and during proestrus in the mouse (Boccabela and Alger, 1961). Adrenal weights of the rat were significantly greater during estrus than during any other stage of the cycle (Bearn, Gould and Jones, 1960), suggesting cyclic fluctuations in ACTH secretion as well.

Hypothalamic Factors. Since hypothalamic factors control the release and/or synthesis of almost all pituitary hormones, fluctuations in their secretion during the estrous cycle would be expected. But research in this area is only in its infancy at the present time. Of all the hypothalamic factors, the LH-releasing factor (LRF) has been studied during the estrous cycle. The content of LRF in the hypothalamus increased during late diestrus followed by a sharp decline during proestrus (Ramirez and Sawyer, 1965; Chowers and McCann, 1965) synchronizing with the pattern of LH secretion during the estrous cycle.

G. The Hypothalamic-pituitary-ovarian Relationship During the Estrous Cycle

The secretion of the ovarian sex steroids is controlled by the pituitary hormones, particularly FSH, LH and prolactin; FSH and LH synergize to stimulate estrogen secretion (Everett, 1961) whereas LH and/or prolactin influence progesterone synthesis (Meites and Nicoll, 1966). The blood levels of sex steroids in turn regulate the secretion of pituitary gonadotrophins (van Rees, 1964; Everett, 1964), the phenomenon commonly known as "feedback" inhibition. The only exception is prolactin, which is not subject to this classical "feedback" regulation (Meites, 1966).

Only a few years ago, the anterior pituitary gland was considered to be the supreme regulator of gonadal function. But recently this view has undergone considerable change. Today, it is generally acknowledged that the activity of the anterior pituitary is controlled by the hypothalamus. Harris (1948) described the adenohypophysis as a gland being under neural control but deprived of neural connections. Thus, neurohormones elaborated by the hypothalamus and transported to the anterior pituitary via the hypophyseal portal system are essential for the discharge of hormones from the anterior pituitary. Consequently, the hypothalamus serves as an integrator of the external and internal environment of the organism.

Of rather recent origin is the concept of "auto-feedback" inhibition whereby circulating levels of some pituitary hormones have been shown to regulate their own secretion. Evidence for ACTH (Motta, Mangili and Martini, 1965), LH (David, Fraschini and Martini, 1966) and GH (Krulich and McCann, 1966) has been presented in the past two years whereas in the case of other pituitary hormones, it remains to be explored.

The intricate network of endocrine control systems does not end at this point, however. Reproductive processes in the cow as well as other domestic species indicate a physiological and possibly endocrine dependence involving not only the hypothalamus, pituitary and ovaries but also the uterus. The effect of the uterus on the life span of the corpus luteum and length of the estrous cycle is well demonstrated in the sow, cow and the ewe (Anderson, 1966). Thus, the regulatory mechanisms controlling the estrous cycle are not simple, isolated phenomena but are composed of a series of interrelated endocrine and physiological adjustments.

CHAPTER III

MATERIALS AND METHODS

A. Experimental Animals, Determination of the Stage of the Estrous Cycle and Slaughter Procedures

Animals used in this study consisted of two species: bovine and murine. Inasmuch as the protocol differed in each case, it will be described separately.

Bovine[1]

Thirty-five female dairy calves of the Holstein breed born between June 29 and July 19, 1964, were purchased from farms in Wisconsin within a week after birth. All calves were sired by registered Holstein bulls and born to production-tested dams. They were housed in individual pens and fed an average of 8 lb of milk, 5 lb of hay and 2.5 lb of grain per day until four months of age. Subsequently, they were moved into box stalls and received 5 lb of grain and 12 lb of hay until 6 months of age. Finally, they occupied a loose housing barn and were fed silage and hay free choice until the time of sacrifice.

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[1]The reproductive and some endocrine parameters of these heifers were measured by A. J. Hackett and will be published under his name.

Beginning in August 1965 each heifer was observed for estrus twice daily--between 7:30-8:30 a.m. and 4:30-5:30 p.m. The occurrence of estrus was judged by the following criteria: (1) standing when mounted by other animals, (2) repeated attempts to mount other animals, (3) swollen external genitalia, (4) copious vaginal mucous secretion and (5) general restlessness.

The stages of the estrous cycle studied comprised days 0 (day of estrus), 2, 4, 7, 11, 18 and 20 of the cycle. Five heifers were included in each group. On the day of sacrifice, the animals were transported from the farm to the University Meats Laboratory at around 5:30 a.m. Soon after arrival, each heifer was weighed and the killing usually commenced by 7 a.m. and was completed by 11 a.m. Heifers were slaughtered by stunning with a captive-bolt gun followed by exsanguination.

The mammary gland was removed immediately and the left and right udder-halves were separated along the median suspensory ligament. The left udder-half was stored in 0.25 M sucrose at -20 C for nucleic acid, collagen, protein and lipid analyses. A piece of mammary tissue from the right front quarter was stored in Heidenhain's fixative (Appendix I) for histology.

Within 5 minutes of exsanguination, the pituitary gland was also removed and dissected free of the adherent tissue. The anterior pituitary was separated from the posterior lobe, weighed, frozen on Dry Ice and stored at -20 C until assayed for prolactin.

Murine

Female Sprague-Dawley rats, obtained at 25 days of age from Spartan Research Animals, Haslett, Michigan, were used throughout this study. The animals were housed in a temperature (24 ± 1 C) and light (6 a.m. to 6 p.m.) controlled room and were given food (Appendix II) and water ad libitum. Six rats occupied one cage having a floor space of 16.5 x 9.5 inches. Beginning at 30 days of age, the rats were checked twice daily for vaginal opening. The stage of the estrous cycle was determined by daily examination of the vaginal smear following vaginal opening.

The first five estrous cycles after the opening of vagina were included in this study. The stages of the estrous cycle identified were proestrus, estrus, metestrus and diestrus. In general, presence of small nucleated epithelial cells in the vaginal smear was designated as proestrus, while non-nucleated cornified cells constituted estrus. Metestrus was marked with a mixture of cornified cells, large nucleated epithelial cells and some leukocytes, whereas diestrus consisted predominantly of leukocytes. While no distinction was made between rats coursing 4- or 5-day cycles, each group was assigned 12 rats chosen at random.

Rats were killed by decapitation between 1 and 3 p.m. on the day of sacrifice. Body weights of the rats were recorded just prior to sacrifice. Uteri were dissected

free of fat and mesentery and weighed immediately after blotting free of any luminal fluid. A piece of the uterine horn was placed in Bouin's fluid (Appendix I) for histology and the rest of the tissue was stored in 0.25 M sucrose at -20 C for nucleic acid and lipid analyses. The six abdominal-inguinal mammary glands were removed and placed in 95% alcohol for subsequent nucleic acid analysis.

In addition, the anterior pituitaries were taken out within 2 minutes after decapitation, weighed and stored at -20 C for the bioassay of LH and prolactin. The weights of the trimmed ovaries were recorded in each case.

B. Assay of Biochemical Parameters

The biochemical parameters measured in this study included DNA, RNA, collagen, protein and lipid. DNA and RNA were measured in the mammary gland of heifers and rats and the uterus of rats. Collagen and protein were measured only in the heifer mammary glands, whereas lipid was measured in the heifer mammary glands as well as the rat uteri.

(a) Nucleic Acids

Nucleic acids analysis was performed by a modified (Tucker, 1964) Schmidt and Thanhauser (1945) procedure with slight changes incorporated to suit each type of tissue involved.

Bovine

The mammary glands of the heifers were thawed and the fatty connective tissue surrounding the gland was trimmed off as close to the parenchyma as possible. The trimmed mammary tissue was weighed, cut into small pieces and minced in a meat grinder.[1] A 10-15 g sample of the minced mammary tissue was homogenized for 2 mintues in 1:20 volume of cold distilled water at top speed in a Cenco PB-5 Waring Blendor.

Duplicate 2 ml samples of the homogenate were removed into polypropylene tubes and 8 ml of 95% alcohol was added. The samples were incubated at least 12 hours at room temperature with continuous shaking and were then centrifuged in a Servall RC-2 centrifuge at 1-3 C. All centrifugations were performed at 32,000 x g for 20 minutes. The supernatant fluid was poured off[2] and 9 ml of methanol-chloroform (2:1) was added to the residue. After incubation at room temperature for at least 24 hours, the samples were centrifuged, supernatant fluid poured off[3] and 9 ml of ether was added to the residue. The samples were then incubated for at least 12 hours at room temperature, centrifuged and the ether fraction[4] removed.

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[1]Kindly provided by Mrs. Ann Tucker.

[2]See lipid analysis, p. 37.

[3]Ibid.

[4]Ibid.

The residue was extracted twice with 5 ml of 10% ice-cold trichloroacetic acid (TCA) which was removed by washing with 5 ml of ice-cold 95% ethanol saturated with sodium acetate.

The samples were digested with 2 ml of 1N KOH for 15 hours at 37 C. The digest was acidified with 0.3 ml of ice-cold 6N HCl and 5 ml of 10% perchloric acid (PCA). The samples were centrifuged and washed twice with 5 ml each of ice-cold 5% PCA. The combined acid supernatant fluids containing the RNA were adjusted to 20 ml and were analyzed for RNA-ribose by the colorimetric orcinol procedure of Mejbaum (1939) as described by LePage (1957). This procedure consists of mixing a 3 ml aliquot of the above supernatant fluids with 3 ml of a 1.0% solution of orcinol in 0.1% $FeCl_3 \cdot 6H_2O$ dissolved in concentrated HCl. The reaction mixture was boiled in a water bath for 30 minutes and the resulting color development was determined in a Beckman DB spectrophotometer at 670 mu. The RNA content was calculated from a standard curve obtained from highly purified yeast RNA (Worthington Biochemical Corp.).

The DNA was extracted from the residue remaining after cold PCA treatment with 5 ml of 5% PCA heated to 70 C for 15 minutes. After centrifugation, the residue was washed twice with 5 ml of ice-cold 5% PCA. Those DNA containing supernatant fluids were combined and adjusted to 20 ml with 5% PCA. The absorbancy of the

DNA-containing solution was read at 268 mu in a Beckman DB spectrophotometer. Highly polymerized DNA (Worthington Biochemical Corp.) was used as the standard.

Murine

Uterus. A 40-60 mg sample of the uterus was weighed out and lipid extracted with 10 ml of 95% alcohol[1] at room temperature for 24 hours. The uterine sample was further extracted with 10 ml each of methanol-chloroform[2] (2:1) and ether[3] and then further extraction of the nucleic acids was performed as in the bovine mammary tissue.

Mammary glands. Rats have 12 mammary glands, of which only the six posterior abdominal-inguinal glands were used in this study. Although the mammary parenchyma of a nonparous rat is embedded in a fatty pad of connective tissue, treatment of the gland with 95% alcohol for 24 hours or more helps to delineate the mammary parenchyma from the extraparenchymal connective tissue. Following this treatment the alcohol-treated glands were stretched gently and the extraparenchymal connective tissue, lymph nodes, nerves and large blood vessels were trimmed off. The trimmed mammary tissue was further extracted with 95% alcohol and then chloroform-methanol

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[1]See lipid analysis, p. 37.

[2]Ibid. [3]Ibid.

(2:1) for 24 hours each. The tissue was dried in an incubator at 37 C for 12 hours, weighed and pulverized to a fine powder in a micro Wiley mill (Arthur H. Thomas Co.). Duplicate samples of 20 mg each were extracted with 10 ml of ether for 24 hours, following which the nucleic acids were determined as described for bovine tissue beginning with the TCA extraction step.

(b) Protein

Protein content was determined in the bovine mammary glands. The assay was performed according to the procedures of Gornall, Bardawill and David (1949) which is based on the Biuret reaction--a reaction specific for the peptide bonds of proteins and peptides.

To 2 ml of the mammary gland homogenate (50 mg/ml) was added 2 ml of 2N potassium hydroxide (KOH) and the mixture was incubated at 37 C for 15 hours. One ml of this digest was added to 2 ml of Biuret reagent (Appendix III); the solution was mixed and allowed to stand at room temperature for 30 minutes. The optical density was measured at 540 mu in a Beckman DB spectrophotometer and the amount of protein was calculated from a standard curve derived from crystalline bovine serum albumin (Nutritional Biochemical Corporation).

(c) Collagen

Assay of collagen is based on the measurement of hydroxproline, an amino acid uniquely found in the

collagenous component of mammalian tissue. The assay procedure used is a modification of the procedure described by Prockop and Undenfriend (1960). Only in the bovine mammary glands was this component measured.

To a 2 or 1.5 ml sample of the mammary homogenate (containing 100 or 75 mg of mammary tissue, respectively) was added 3.5 ml of $HCl:H_2O$ (2.0:1.5) and the mixture was autoclaved at 15 lb pressure for 15-24 hours. The hydrolyzed contents were poured into graduated test tubes and the volume adjusted to 8 ml. To this was added 1 ml of a resin-charcoal preparation (Appendix IV); the solution was stirred on a Vortex mixer, poured into plastic centrifuge tubes and centrifuged at 17,000 rpm for 10 minutes.

One ml of the supernatant fluid was transferred into 100 ml culture tubes; one drop of 1% phenolphthalein in 95% ethyl alcohol was added and the pH adjusted to light pink first with 1N and then 0.1N KOH. The volume was adjusted to approximately 8 ml with distilled water and the solution was saturated with an excess of potassium chloride (KCl). If necessary, readjustment to a light pink color was made with 0.1N KOH. Two ml of borate buffer and 1 ml of 10% alanine solution (Appendix IV) were added and the reaction mixture was oxidized with 2 ml of a freshly prepared solution of 0.2M chloroamine T in 2-methoxyethanol for 20 minutes. The oxidation was stopped by the addition of 6 ml of a 3.6 M solution of

sodium thiosulfate in water. The mixture was again saturated, if necessary, with KCl.

Finally, 10 ml of toluene was added to the reaction mixture and stirred with a Vortex mixer. The resulting two phases were allowed to separate; the top phase was removed by suction and discarded while the bottom phase was boiled in a water bath for 30 minutes. After cooling, 10 ml of toluene was added again and mixed. Five ml of the top toluene phase was pipetted out and mixed immediately with 2 ml of sulfuric Ehrlich's reagent (Appendix IV). The color development was read within 15-60 minutes at 560 mu in a Beckman DB spectrophotometer. The amount of hydroxyproline was calculated from a standard curve derived from pure hydroxy-L-proline (Calbiochem).

(d) Lipid

The alcohol, methanol-chloroform and ether extracts of the tissues obtained in the process of extracting the nucleic acids were used to estimate the lipid content of the heifer mammary glands and the rat uterus. The three lipid-containing extracts were pooled into tared 50 ml beakers and allowed to evaporate for 24 hours at room temperature. Such treatment removed most of the ether and the remaining lipid solvents and moisture were evaporated at 65 C on a hot plate. The beakers containing

the lipid fractions were placed into a dessicator for at least 24 hours and then weighed on an analytic balance.

C. Bioassay of Pituitary Hormones

The anterior pituitaries of the heifers and the rats which had been stored at -20 C were thawed and homogenized in a Potter-Elvehjem homogenizer in 0.85% sodium chloride prepared in pyrogen-free distilled water. The heifer pituitaries were homogenized in 10 ml of saline and the volume of the homogenate was adjusted to a final concentration of 50 mg of pituitary per ml. In the case of rats, a single pituitary did not provide enough tissue for the assays. Thus rat pituitaries were pooled in groups of 4, 6 or 12 depending upon the amount of tissue available and then homogenized in the concentration of 5 mg per ml. The homogenates were centrifuged at 11,000 x g for 15 minutes and the supernatant fluids were used in the assays for LH and prolactin.

(a) Luteinizing Hormone

The levels of LH in the pituitaries of heifers and rats were measured by the ovarian ascorbic acid depletion method of Parlow (1961). The assay rats (Sprague-Dawley strain from Spartan Research Animals, Incorp., Haslett, Michigan) were injected with 50 IU of pregnant mare serum[1] (PMS) at 25 days of age and with 25 IU of

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[1]PMS, Equinex was obtained from the Ayerst Laboratories, New York, N. Y.

human chorionic gonadotropin[1] (HCG) 56 to 60 hours later. Five days after the HCG injection, the rats were injected intravenously with 0.5 ml of the test substance or standard preparation and 4 hours later one ovary was removed surgically for ascorbic acid determination (Appendix V). At the time of ovariectomy, each rat was injected subcutaneously with 30,000 IU of penicillin G. Two days later, the same rats were injected with 0.5 ml of another test substance and 4 hours later the ascorbic acid concentration was measured in the remaining ovary.

Each pituitary preparation was assayed at two dose levels with five rats at each dose level. The low and high dose levels of the rat pituitaries consisted of 0.2 and 0.8 mg of pituitary equivalent per rat. The LH potency was computed from a parallel line assay comparison with two dose levels--0.4 and 1.6 ug--of NIH-LH-B2[2] (Bliss, 1952). Twelve or thirteen pituitary preparations picked at random were assayed simultaneously. Calculation of the potencies and the criteria for the validity of the assay, such as the test for nonparallelism, lambda (λ), standard error and 95% confidence interval, were performed based on the several unknowns and standard preparation assayed at one time.

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[1]HCG was obtained from The Upjohn Company, Kalamazoo, Michigan.

[2]The standard LH was supplied by the Endocrine Study Section of the National Institutes of Health.

(b) Prolactin

Although several assays for prolactin are in use (Meites and Nicoll, 1966), the pigeon crop sac "micro" method of Reece and Turner (1937) is probably the most sensitive and widely used. Bioassays in general are notorious for their variability and the prolactin assay is no exception. So, unless the assay is designed in a manner which allows estimation of the variability associated with the potency estimate, the data obtained remain of questionable merit. Consequently, a four-point assay design, similar to the one for LH assay, was incorporated into the Reece and Turner (1937) method of prolactin assay.

The independence of the responses on the left and right side of the crop sac of the pigeon provides an opportunity to use the same bird twice. Indeed, there are several alternative ways, as shown in Figure 1, in which the assay could be designed with advantage, utilizing both the right and the left side of the crop sac.

(1) In this arrangement (Fig. 1a), the low (S_L) and the high (S_H) dose of the standard preparation are injected into the two crop sac areas of a bird. Similarly, the low (U_L) and the high (U_H) dose of the unknown are injected into the two crop sac areas of another bird. The potencies of several groups of birds receiving several unknowns are then calculated based on a comparison with the one group of birds receiving standard hormone.

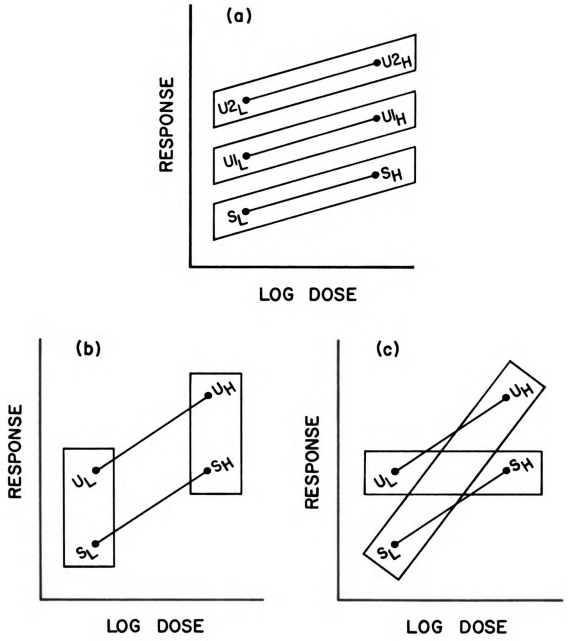

Figure 1.--Design of Prolactin Assay

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In a parallel line assay (Bliss, 1952), the potency of an unknown is, among other things, a function of (1) the difference between the unknown and the standard (Ta) and (2) the combined slope of the preparations (Tb). This relationship can be expressed as

$$M' = K \frac{(Ta)}{(Tb)} \qquad \text{Eq. 1}$$

where K is a constant derived from the number of animals used per point, log of the fold increase between the low and the high dose and the number of preparations assayed at a time.

$$Ta = (U_L+U_H) - (S_L+S_H) \qquad \text{Eq. 2a}$$

or $$(U_L-S_L) + (U_H+S_H) \qquad \text{Eq. 2b}$$

or $$(U_L-S_H) + (U_H-S_L) \qquad \text{Eq. 2c}$$

and $$Tb = (U_H-U_L) + (S_H-S_L) \qquad \text{Eq. 3a}$$

or $$(U_H-S_L) + (S_H-U_L) \qquad \text{Eq. 3b}$$

By injecting (U_H&U_L) and (S_H&S_L), each pair in the same pigeon, the pigeon variation in the estimate of slope (Eq. 3a) is reduced in this design. On the other hand, the variation in the estimate of Ta (Eq. 2b) is inflated because (U_L&S_L) and (U_H&S_H) are injected in different birds. But the major advantage of this design

lies in the fact that potencies of several unknowns assayed at a time can be computed from the same standard resulting in a considerable reduction in the number of assay animals required to complete an experiment.

(2) Of the two elements, Ta (difference between the unknown and the standard) and Tb (the slope factor), the contribution of Ta is usually of major consequence in the estimate of potency since, with standardized conditions of assay, the slope of the assay does not vary too much within a laboratory. Under such circumstances, Ta is the prime source of discrepancy in the evaluation of potency and therefore it may be desirable to improve the precision of the estimate of Ta.

The second design (Fig. 1b) makes an attempt in this direction. In this plan, the low dose of the unknown (U_L) and the standard (S_L) are injected in the same birds and the high dose of the unknown (U_H) and the standard (S_H) injected similarly in the others. Thus by combining (U_L&S_L) and (U_H&S_H) in the same pigeons, bird variation in the estimate of Ta (Eq. 2b) is reduced. The bird variation is, however, increased in the estimate of the slope (Eq. 3a) since (U_H&U_L) and (S_H&S_L) are injected in different birds. In addition, a separate standard is needed for each unknown and thus the number of assay animals required is almost doubled.

(3) On other occasions it may be desirable to share the gain in precision, offered by the use of the two sides of the crop sac, in the estimate of both Ta and Tb. The plan outlined in Figure 1c, in which (U_L&S_H) are injected in one bird and (S_L&U_H) injected together in the other, attempts to achieve this objective. By combining (U_L&S_H) and (U_H&S_L) and also (U_H&S_L) and (S_H&U_L) in the same birds the precision of the estimates of both Ta (Eq. 2c) and Tb (Eq. 3b), respectively, is improved. But as in the second method, a separate standard is required for each unknown and thus the number of assay pigeons needed is considerable.

(4) None of the above three methods, however, eliminates the pigeon variation completely: they only minimize it. The ideal case would be to remove the pigeon variation entirely, since the variation in the sensitivity of the birds is by far the largest source of variation in this assay. In order to do this, the following procedure may be appropriate: to inject the low and high dose of the unknowns and the standard on only one side of the crop sac and on the other, inject a known constant amount (somewhere in between the low and the high dose) of the standard hormone. Then dividing each of the individual responses with the constant response on the opposite side of the crop sac will correct for the bird variation. The data can then be used to calculate the potencies and related

information in the usual manner. Several groups of unknowns can be assayed on the basis of one standard run simultaneously like the scheme in method 1, but similar to methods 2 and 3, this procedure also requires larger numbers of pigeons in comparison with plan 1.

In the experiments of the present study, the procedure outlined in plan 1 was used for reasons of economy in the number of assay animals required. The pigeons (White King) of both sexes were obtained from Cascade Squab Farm, Grand Rapids, Michigan, at 5-8 weeks of age and housed in a room artificially illuminated between 6 a.m. and 8 p.m. They were fed mixed grain and water ad libitum. The breast feathers were plucked following the day of arrival. Two days later (usually beginning on Monday), the test materials were injected intradermally in 0.1 ml volume with a 1.0 ml tuberculin syringe and 27 gauge needle for 4 days. Twenty-four hours after the last injection, the pigeons were decapitated, crop sacs were removed and the area of the response was rated visually in terms of Reece-Turner (R-T) units. A response of the size of one nickel (diameter 2.1 cm) constituted one R-T unit. The scale ranged from 0.0 to 4.0 R-T units, increasing in multiples of 0.25.

Each pituitary preparation was assayed at two dose levels with eight pigeons at each dose level. The low and high dose levels of the heifer pituitaries consisted

of 1.0 and 4.0 mg and of the rat pituitaries of 0.5 and 2.0 mg of pituitary equivalents per pigeon. The prolactin potency of the pituitary gland was computed from a parallel line comparison with two dose levels, 1.0 and 4.0 ug, of NIH-P-S_6[1] (heifer) and NIH-P-S_5[2] (rats), respectively. In a given week, 8, 9 or 10 pituitary preparations and one standard hormone were assayed simultaneously. Calculation of potencies and statistical evaluation of the assay quality were performed similarly to those described for LH.

D. Histological Preparations

The pieces of the heifer mammary glands preserved in Heidenhain's fixative were sectioned at 6 μ and stained with Delafield's hematoxylin and eosin (H & E). The stained sections were examined under the microscope for the presence of secretions in the ducts and the alveoli.

The rat uteri preserved in Bouin's fluid were sectioned at 5 μ and stained with H & E stain. The sections were examined under the microscope and the endometrium, myometrium and the height of the epithelium under various stages of the estrous cycle were compared.

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[1]The standard prolactin was supplied by the Endocrine Study Section of the National Institutes of Health.

[2]Ibid.

E. Statistical Analyses

The statistical procedures used in the bioassays of LH and prolactin were those of Bliss (1952) for a parallel line log-dose factorial assay. Although the principles underlying the methods used stretch over the whole length of the book, pages 482 to 507 contain in particular the mechanics of computing the potency, lambda (λ), standard error of potency, 95% confidence limits of potency and the test for nonparallelism. The procedures used in the combination of independent assays was derived from a monograph published by Bliss (1956).

For the test of significance, all of the rat data were analyzed with the procedures for a hierarchical model (Li, 1964), whereas simple analysis of variance, regression analysis and response curve (Ostle, 1964) methods were used in the case of heifer data. Specific comparisons in either case, however, were made with the multiple range test of Duncan (1955) or orthogonal contrasts.

CHAPTER IV

RESULTS AND DISCUSSION

A. Age at First Estrus and Length of the Estrous Cycle

Bovine

Since the observation of estrus began when the heifers were about a year old, information regarding the age of these heifers at first estrus is not available. These heifers, however, were raised under management conditions very similar to those of the heifers of a study by Desjardins (1966). And Desjardins (1966) found the age of first estrus to range from 5.0 to 11.1 months with an average of 7.4 $\pm$ 0.3 months. Thus, to the extent the animals of the two studies were comparable, the age of first estrus in the animals of this study may be assumed to be approximately 7 to 8 months.

Data on the length of the estrus cycle is presented in Figure 2. The length of the estrous cycle averaged 20.6 $\pm$ 0.2 days. This value agrees closely with the values of 20.23 $\pm$ 0.05 and 20.5 $\pm$ 0.6 reported by Asdell, deAlba and Roberts (1949) and Desjardins (1966), respectively, for virgin heifers. The estrous cycle of parous

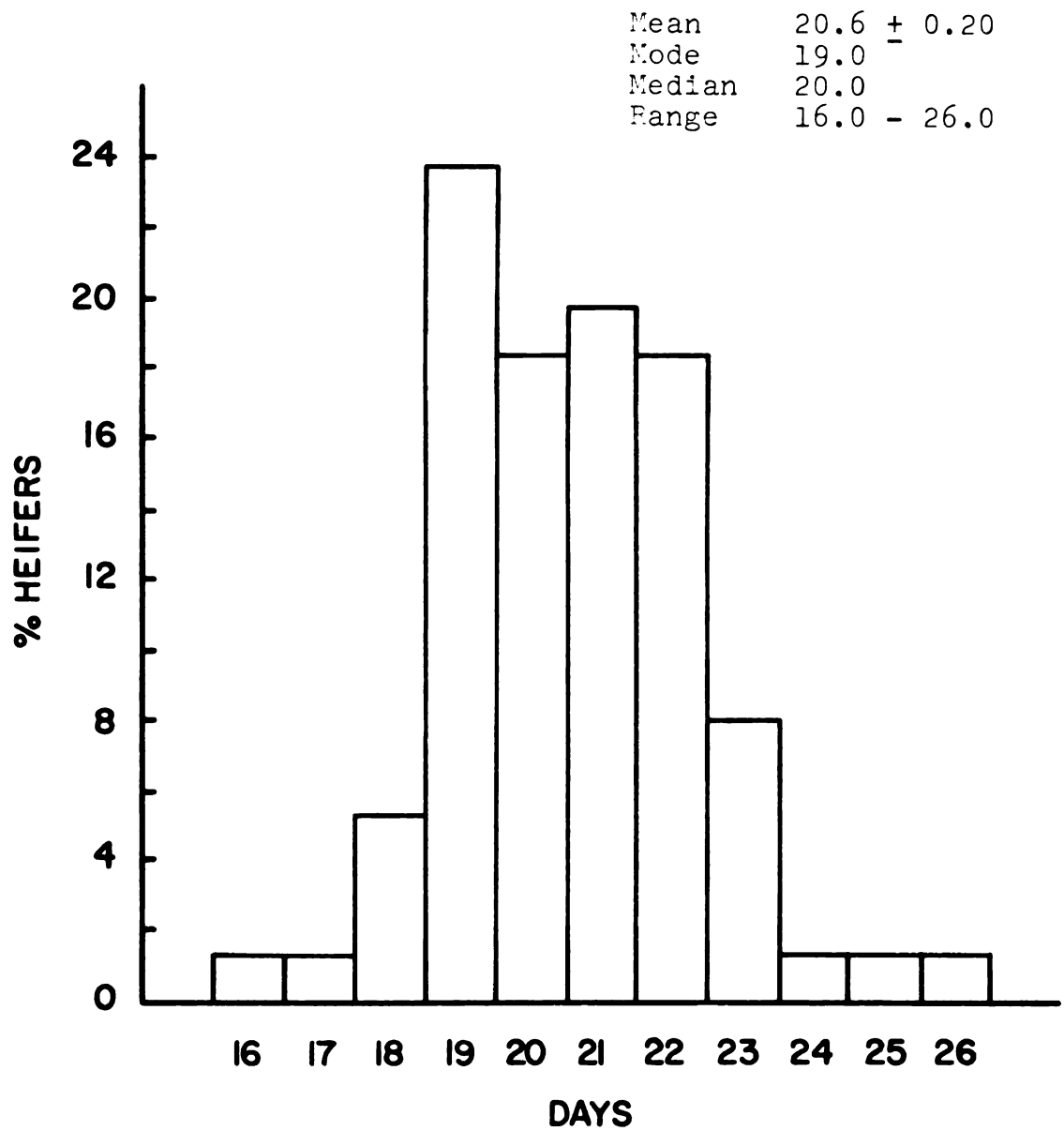

Figure 2.--Length of the estrous cycle of dairy heifers.

cows, however, averages a day longer--21.28 ± 0.06 (Asdell et al., 1949).

Murine

Figure 3 illustrates the distribution of age of the rats in this experiment at the time of vaginal opening. The average age of rats at vaginal opening was 36.7 ± 0.13 days with a range of 33 to 43 days. Vaginae of 19.2% of the rats opened at 36 days of age. These values are considerably lower than the mean length of 76.5 days with a range of 53 to 142 days reported originally by Long and Evans (1922). Ten years later, Freudenberger (1932) found the age of vaginal introitus in rats of the Long-Evans strain to be 57.2 days with a range of 39 to 101 days. And in 1939, Blunn reported vaginal opening to occur at an average age of 39 days (range 34-45 days) in the rats of the same strain--a finding very close to the results of this study. Thus, the age of rats at the time of vaginal opening has apparently undergone considerable change under the habitat of the laboratory. Indeed, the sexual activity of the rat and other animals is greatly influenced by environmental factors such as light and nutrition.

The opening of the vagina in rats is often thought to be associated with sexual maturity. In an experiment using copulatory response as an index of estrus, Blandau and Money (1943) discovered that vaginal opening occurred in most rats from 48 hours before to 12 hours after the onset

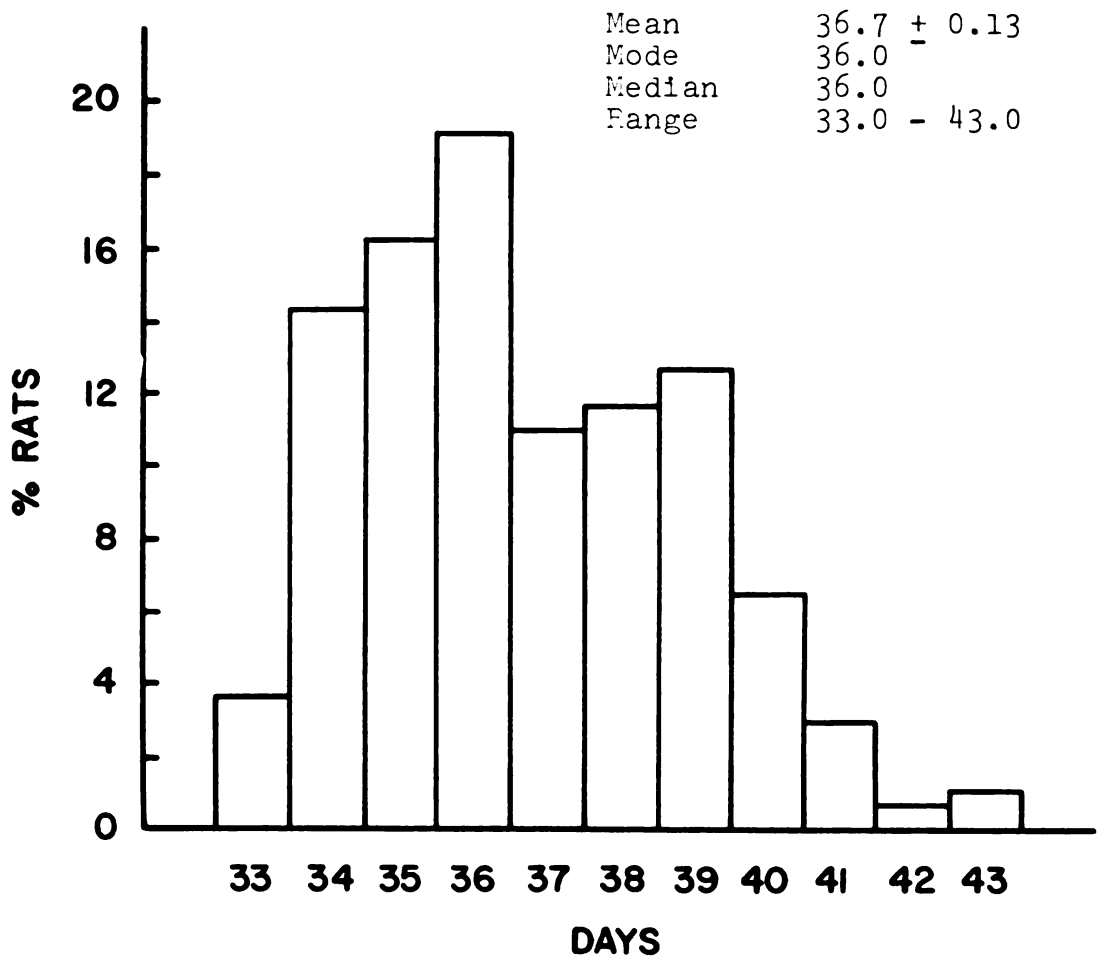

Figure 3.--Age of vaginal opening of rats.

of heat. Although the temporal relationship between vaginal opening and onset of first estrus was not strictly measured in the present study, in 58% of the rats vaginal opening coincided with the proestrus or estrus smear in the vagina. Thus vaginal opening may be highly correlated with attainment of sexual cyclicity in the rat.

Table 1 lists the length of four of the five estrous cycles studied in this experiment. Data on the fifth cycle is not included because the rats were killed on the first day of diestrus in the fifth cycle and therefore had no chance of undergoing the full cycle.

TABLE 1.--Length of the estrous cycle of pubertal rats.

Cycle No.	No. of Cycles	% Cycles of Various Length (Days)					Cycle Length* (Days)
		4	5	6	7	8	
1	117	6	53	31	9	1	5.5 ± 0.07
2	146	21	43	33	1	2	5.2 ± 0.07
3	100	36	50	12	2	0	4.8 ± 0.07
4	53	36	43	21	0	0	4.9 ± 0.04

*Mean ± standard error

The mean length of the first (5.5 days) and second (5.2 days) cycles was significantly greater ($P < 0.01$) than the third (4.8 days) and fourth (4.9 days) cycles. The data are in agreement with the findings of Long and

Evans (1922) in which they observed the first cycle to be distinctly longer than succeeding ones. Blandau and Money (1943) examined seven consecutive estrous cycles and found the first and second estrous cycles to be longer than the remaining cycles and the duration of the first heat period (9.8 hours) significantly shorter than the subsequent heat periods (average 13.9 hours).

The unusually long duration of the first and second cycles and short duration of the first estrus probably indicates that the attainment of cyclical reproductive rhythmicity is a gradual process and that the animal is still in the process of developing all the elements of this pehnomenon. But once the hormonal mechanisms are fully developed, the animal displays considerable consistency in the pattern of sexual behavior.

B. Uterine Development

The development of the uterus in the rat was studied with such parameters as weight, nucleic acid, lipid content and histology. All quantitative data have been expressed in terms of 100 g of body weight although the uncorrected values showed essentially the same trends.

Weight. Table 2 presents the wet weights of the rat uteri during proestrus, estrus, metestrus and diestrus of cycles one through five. On the average, the uterine weight declined from a maximum of 211 mg at proestrus to

TABLE 2.--Average uterine weight of rats during the first five estrous cycles.

Estrous Cycle No.	Stage of Estrous Cycle*				Average
	Proestrus	Estrus	Metestrus	Diestrus	
	mg/100 g BW**				
1	178 ± 13	153 ± 10	105 ± 4	110 ± 3	136
2	190 ± 13	204 ± 8	120 ± 7	135 ± 7	162
3	222 ± 10	180 ± 8	136 ± 5	145 ± 6	171
4	213 ± 8	189 ± 8	131 ± 3	138 ± 4	168
5	251 ± 8	188 ± 8	134 ± 5	143 ± 5	179
Average	211	183	125	134	

*Each group contained 12 rats.

**Mean ± standard error.

183 mg at estrus and finally to a minimum of 125 mg at metestrus--a decrease of 13 and 41%, respectively. Between metestrus and diestrus, however, the uterine weight increased about 7% and the increase continued between diestrus and the succeeding proestrus (57%) culminating in maximal values at proestrus. All but one of the first five cycles displayed this same pattern. The only deviation was in cycle 2 where the uterine weight was maximum at estrus. This result may have been due to sacrificing a large number of animals in that group in a very early stage of estrus.

Similar changes in uterine weight have been reported by other workers. Astwood (1939) observed maximal wet as well as dry weight of the uterus during proestrus and minimal weights on the first day of diestrus. The first day of diestrus of Astwood's experiment corresponds most nearly with metestrus of the present study. More recently, Schwartz (1964) has published uterine weight data on mature rats almost identical to the results of our experiment.

An average of the four stages within each cycle reflects the cumulative increase in a uterine parameter with the recurrent estrous cycles. Such averages (Table 2, last column) showed a cumulative increase in uterine weight of 19% between 1st and 2nd, 6% between 2nd and 3rd, -2% between 3rd and 4th and 6% between 4th and 5th estrous cycles. From these data it appeared that the size

of the uterus continued to increase although at a decreasing rate until the fifth estrous cycle.

Nucleic Acids. In each cycle, the uterine DNA (Table 3) was maximal at proestrus or estrus, decreased to a minimal value at metestrus and remained at the same level during diestrus. Looking at the combined averages of the five cycles, although the DNA content did not change ($P > 0.05$) between proestrus (0.88 mg) and estrus (0.85 mg) or between metestrus (0.70 mg) and diestrus (0.70 mg), it decreased 18% ($P < 0.01$) between estrus and metestrus suggesting a significant loss of uterine cells during the early luteal phase of the cycle. These losses in uterine DNA were, however, consistently regained between diestrus and the subsequent proestrus as reflected by a 26% increase ($P < 0.01$) in DNA between these periods.

Similarly to uterine weight, uterine DNA increased ($P < 0.01$) cumulatively between the first and second (23%) and the second and third (16%) cycles but no further cumulative increases occurred between the third and fifth cycles. The weight of the uterus continued to increase between the third and fifth cycles (Table 2). Thus it would appear that the small increases in uterine size after the third cycle may be due to hypertrophy. One can conclude from the cumulative DNA data that pubertal growth of the uterus is largely complete after the third estrous cycle.

TABLE 3.--Uterine DNA content of rats during the first five estrous cycles.

Estrous Cycle No.	Stage of Estrous Cycle*				Average
	Proestrus	Estrus	Metestrus	Diestrus	
	mg/100 g BW**				
1	0.66 ± 0.05	0.68 ± 0.05	0.58 ± 0.04	0.56 ± 0.02	0.62
2	0.77 ± 0.06	0.93 ± 0.05	0.69 ± 0.06	0.65 ± 0.05	0.76
3	1.10 ± 0.08	0.86 ± 0.07	0.76 ± 0.03	0.79 ± 0.04	0.88
4	0.98 ± 0.08	0.87 ± 0.02	0.70 ± 0.05	0.76 ± 0.04	0.83
5	0.89 ± 0.05	0.89 ± 0.04	0.75 ± 0.05	0.73 ± 0.05	0.82
Average	0.88	0.85	0.70	0.70	

*Each group contained 12 rats.

**Mean ± standard error.

The RNA content of the uterus (Table 4) fluctuated within stages of the cycle in a pattern similar to that of uterine weight: maximal at proestrus (1.50 mg), minimal at metestrus (0.68 mg) and a slight increase at diestrus (0.86 mg). On the other hand, the cumulative changes in the RNA content followed the pattern of uterine DNA: it increased 29% between first and second and 9% between second and third cycles with no increases thereafter.

It is noteworthy that the initial enhancement of the RNA during diestrus or initial decline during estrus always preceded similar changes in the DNA. This was expected since before a new cell can be formed, some of the constituents of that cell must first be synthesized.

The ratio of RNA to DNA is often calculated to illustrate the metabolic activity of the tissue on a per cell basis. The RNA:DNA ratios in the uterus are shown in Table 5. This ratio proved to be the most consistent and least variable of the uterine parameters measured in this study. The latter is illustrated by the relatively small standard errors of the means. Like uterine weight and RNA, the RNA:DNA ratio decreased progressively from a maximum of 1.70 at proestrus to a minimum of 1.00 at metestrus. And between metestrus and diestrus the RNA:DNA ratio increased 22%. But unlike uterine weight, RNA or DNA, the RNA:DNA ratio did not increase cumulatively

TABLE 4.--Uterine RNA content of rats during the first five estrous cycles.

Estrous Cycle No.	Stage of Estrous Cycle*				Average
	Proestrus	Estrus	Metestrus	Diestrus	
	mg/100 g BW**				
1	1.16 ± 0.10	0.92 ± 0.12	0.56 ± 0.06	0.68 ± 0.02	0.83
2	1.32 ± 0.12	1.42 ± 0.10	0.66 ± 0.06	0.82 ± 0.06	1.06
3	1.80 ± 0.22	1.16 ± 0.12	0.74 ± 0.04	0.98 ± 0.08	1.16
4	1.70 ± 0.12	1.32 ± 0.14	0.70 ± 0.04	0.88 ± 0.04	1.16
5	1.52 ± 0.08	1.22 ± 0.06	0.78 ± 0.04	0.90 ± 0.06	1.10
Average	1.50	1.22	0.68	0.86	

*Each group contained 12 rats.

**Mean ± standard error.

TABLE 5.--Uterine RNA:DNA ratio of rats during the first five estrous cycles.

Estrous Cycle No.	Stage of Estrous Cycle*				Average
	Proestrus	Estrus	Metestrus	Diestrus	
	Ratio**				
1	1.76 ± 0.08	1.32 ± 0.06	0.98 ± 0.02	1.20 ± 0.02	1.32
2	1.68 ± 0.10	1.52 ± 0.06	0.98 ± 0.02	1.26 ± 0.04	1.36
3	1.62 ± 0.08	1.34 ± 0.06	0.98 ± 0.02	1.24 ± 0.04	1.30
4	1.74 ± 0.04	1.54 ± 0.08	1.02 ± 0.04	1.18 ± 0.02	1.38
5	1.74 ± 0.06	1.38 ± 0.06	1.06 ± 0.02	1.24 ± 0.02	1.36
Average	1.70	1.42	1.00	1.22	

*Each group contained 12 rats.

**Mean ± standard error.

from the first through the fifth cycles ($P > 0.05$). This suggested a uniform rate of metabolic activity in the uterus during all the five estrous cycles studied.

Lipids. Variations in the lipid content of the uterus (Table 6) paralleled the changes in the uterine weight (Table 2). From a maximum of 37.1 mg at proestrus, the lipid content declined 13% at estrus and a further 26% at metestrus, then increased to 27.6 mg at diestrus. Cumulatively, it increased at a decreasing rate, the total increase between the first and fifth cycles being 27%. It is remarkable that analogous to uterine RNA, the decline in lipid content began initially during estrus--a time when the uterine cells (DNA) have not yet regressed. This close parallelism between lipids and RNA would imply that lipids in the uterus are quite intimately associated with uterine metabolism during the estrous cycle. Indeed, enhancement of lipid synthesis is one of the earliest detectable responses of the uterus to estrogen treatment (White, Handler and Smith, 1964). But the exact role lipids play in uterine metabolism is only speculative. They may furnish the constituents of the cell membrane in the formation of new cells. Whether they also serve as a source of energy in the growth and metabolism of the uterus or are involved in some other ways remains to be determined.

TABLE 6.--Uterine lipid content of rats during the first five estrous cycles.

Estrous Cycle No.	Stage of Estrous Cycle*				Average
	Proestrus	Estrus	Metestrus	Diestrus	
	mg/100 g BW**				
1	34.0 ± 4.8	27.8 ± 3.5	23.2 ± 2.9	20.4 ± 2.5	26.3
2	38.4 ± 5.2	36.2 ± 5.0	22.3 ± 3.8	28.4 ± 6.0	31.3
3	37.8 ± 4.1	33.6 ± 3.6	25.5 ± 3.5	31.9 ± 4.6	32.2
4	30.9 ± 1.8	30.3 ± 3.0	21.5 ± 1.9	28.2 ± 3.7	27.7
5	44.3 ± 2.5	33.9 ± 4.2	26.3 ± 4.3	29.1 ± 3.0	33.4
Average	37.1	32.4	23.8	27.6	

*Each group contained 12 rats.

**Mean ± standard error.

Morphology. Photomicrographs of the uterus during proestrus, estrus, metestrus and diestrus are shown in Figure 4(a-h). At proestrus, the uterine lumen was large due to the distention of the horns with uterine fluid and the epithelium lining the uterine cavity appeared cuboidal in shape. The endometrium contained numberous well developed uterine glands. At estrus, the uterine lumen shrank and the epithelium lining it changed to tall columnar in shape and revealed marked signs of degeneration such as loss of the basement membrane and vacuolar disintegration. The uterine glands were smaller than at proestrus. At metestrus and diestrus, the uterus remained small and avascular and possessed a slit-like lumen. The lumen was lined with simple columnar epithelium and the endometrium contained a few small uterine glands.

The structural appearance of the uterus concurred in general with the changes in the biochemical components of the uterus. The endometrium and uterine glands were considerably reduced during metestrus and diestrus as compared with proestrus and estrus. The DNA content of the uterus also decreased at this time signifying a loss of cells from the uterus. Thus, regression of the endometrium and the uterine glands may contribute partially to the loss of DNA between estrus and metestrus. However, the contribution of leukocytes and other uterine cellular elements may also be involved in these shifts in the uterine DNA.

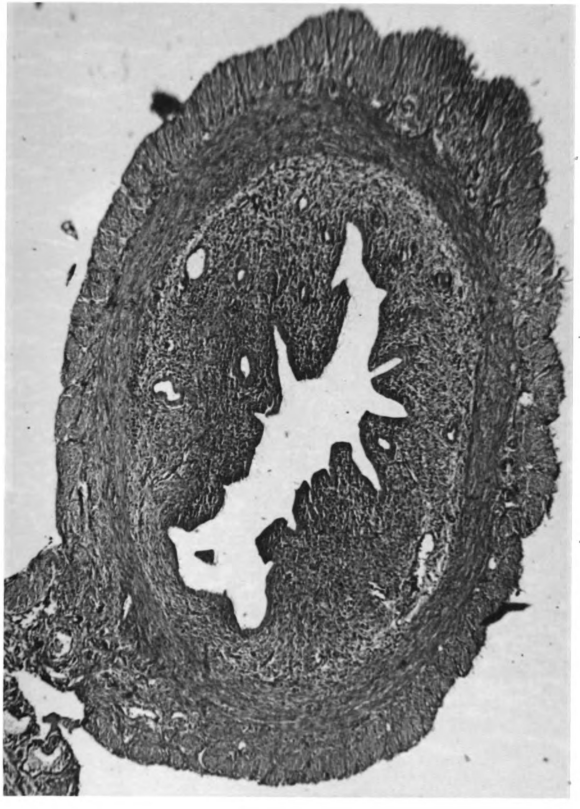

Figure 4a.--Photomicrograph of the rat uterus during proestrus. X87

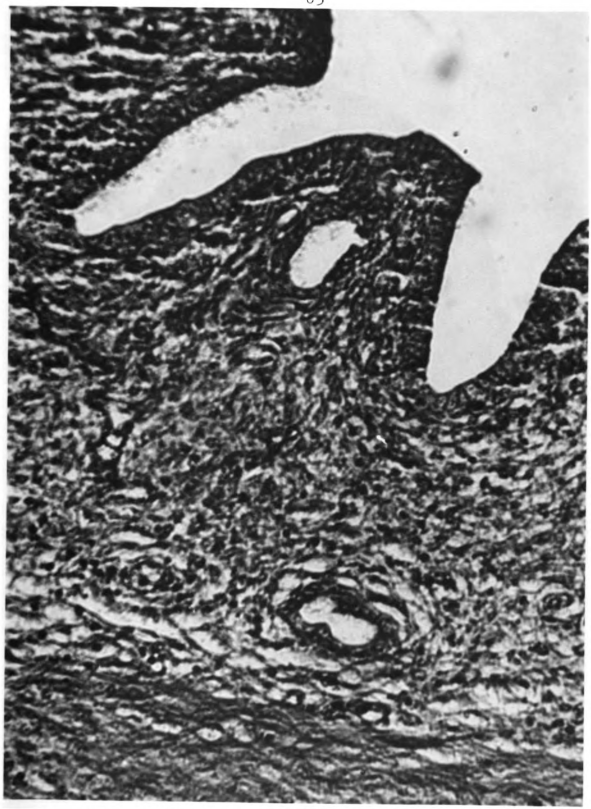

Figure 4b.--Photomicrograph of the rat uterus during proestrus. X435

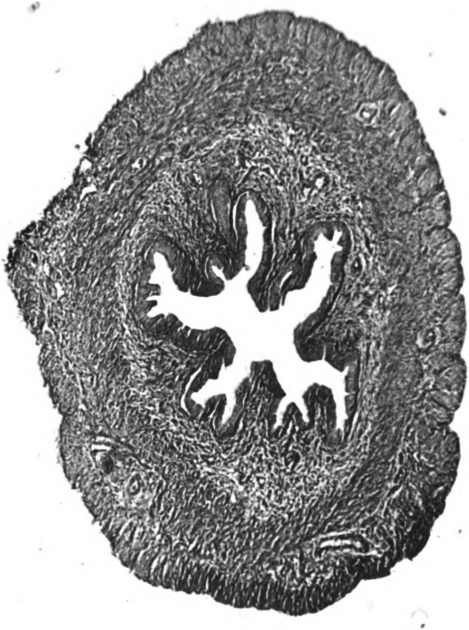

Figure 4c.--Photomicrograph of the rat uterus during estrus. X37

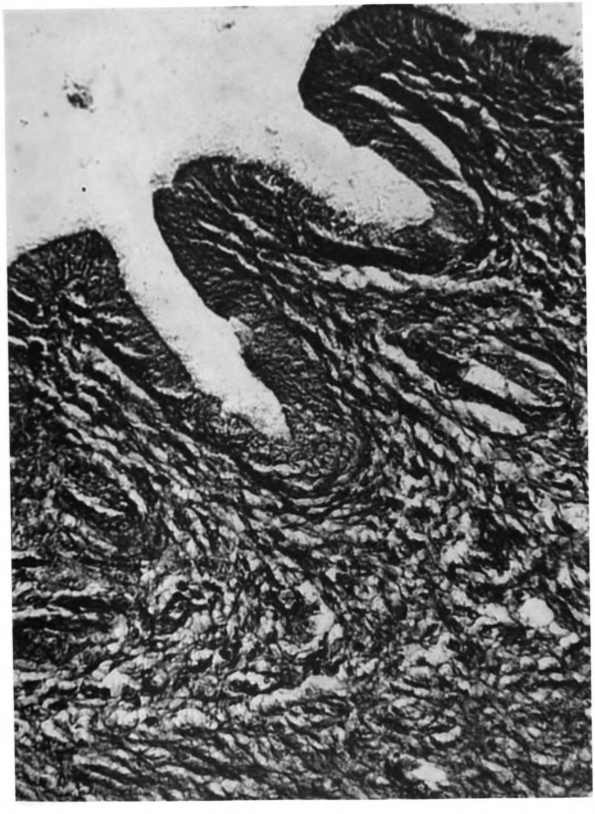

Figure 4d.--Photomicrograph of the rat uterus during estrus. X435.

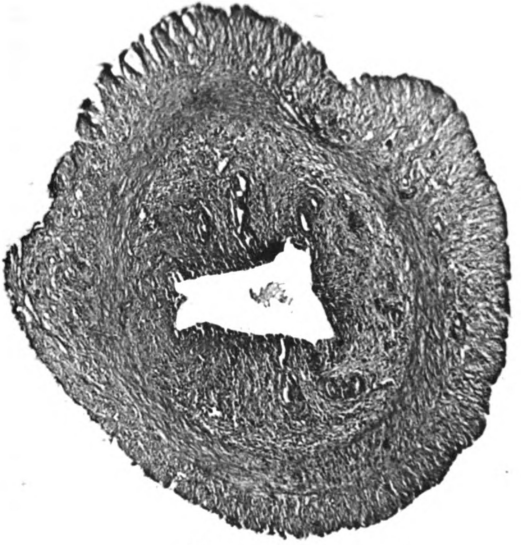

Figure 4e.--Photomicrograph of the rat uterus during metestrus. X87

Figure 4f.--Photomicrograph of the rat uterus during metestrus. X435

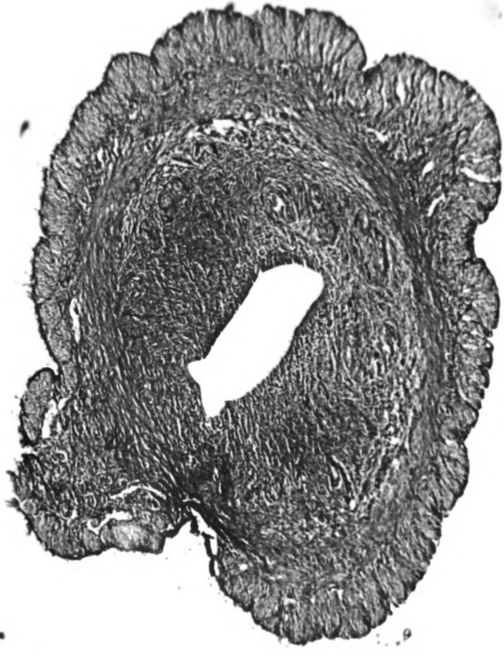

Figure 4g.--Photomicrograph of the rat uterus during diestrus. X87

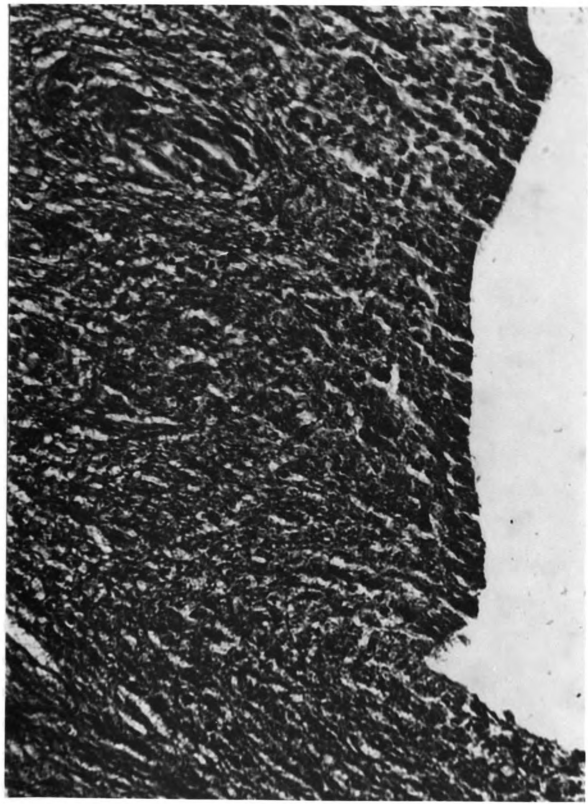

Figure 4h.--Photomicrograph of the rat uterus during diestrus. X435

C. Mammary Development

The influence of the estrous cycle on mammary development was studied both in dairy heifers and rats. In the case of heifers, mammary growth was assessed with parameters such as nucleic acids, protein, collagen, lipid and histological preparations whereas in the mammary glands of the rat only nucleic acids were determined.

Bovine

Changes in the mammary glands of heifers were studied at days 2, 4, 7, 11, 18, 20 and 0 (day of estrus) of the estrous cycle. Unlike the rat experiment, no effort was made to categorize the groups according to the number of estrous cycles experienced. That is, in a particular group, the animals may have undergone from one to several estrous cycles.

Nucleic Acids. The mammary gland weights and nucleic acid data of heifers are summarized in Table 7. In Table 8 the same data have been classified according to the conventional stages of the estrous cycle. The differences in any of the parameters among stages of the estrous cycle could not be found significant ($P > 0.05$) upon evaluation of the data by analysis of variance. But from the magnitude of the standard errors of the means (Table 7) this was not unexpected. In fact, calculations in the case of

TABLE 7.--Mammary nucleic acid content of dairy heifers during the estrous cycle.

Day of Estrous Cycle*	Weight	DNA	RNA	RNA:DNA
	g/100 lb BW**	mg/100 lb BW	mg/100 lb BW	ratio
2	50.8 ± 6.1	169.1 ± 7.2	142.6 ± 11.8	0.84 ± 0.06
4	42.9 ± 2.4	137.8 ± 22.7	105.1 ± 15.6	0.78 ± 0.04
7	38.8 ± 2.9	151.9 ± 17.7	133.6 ± 11.2	0.92 ± 0.08
11	37.0 ± 1.9	120.2 ± 15.6	87.2 ± 15.7	0.72 ± 0.06
18	41.3 ± 2.4	161.5 ± 19.4	111.7 ± 12.5	0.70 ± 0.06
20	31.8 ± 4.4	97.8 ± 13.6	70.7 ± 11.9	0.72 ± 0.06
Estrus	50.9 ± 12.6	213.7 ± 82.1	198.2 ± 68.8	0.96 ± 0.12

*Each group included 5 heifers. The age of all heifers averaged 16.2 months with a standard deviation of 0.11 month.

**Mean ± standard error.

TABLE 8.--Mammary nucleic acids, protein, hydroxyproline and lipid content of dairy heifers according to the conventional stages of the estrous cycle.

Mammary Parameters	Units	Stage of Estrous Cycle*			
		Proestrus	Estrus	Metestrus	Diestrus
Weight	g/100 lb BW	31.8	50.9	50.8	40.0
DNA	mg/100 lb BW	97.8	213.7	169.1	142.8
RNA	mg/100 lb BW	70.7	198.2	142.6	109.4
RNA:DNA	ratio	0.72	0.96	0.84	0.78
Protein	g/100 lb BW	9.6	22.6	21.8	14.5
Hydroxyproline	mg/100 lb BW	15.6	62.7	71.0	38.7
Lipid	g/100 lb BW	4.2	10.0	11.5	8.3

*Proestrus = day 20; Metestrus = day 2; Diestrus = days 4 to 18.

one parameter--DNA--showed that with the kind of variation encountered in this population, it would take about 50 heifers in each group to detect real differences of the order observed in this experiment at the level of $\alpha = 0.05$ and $\beta = 0.10$.

But despite the lack of statistical significance, physiological trends in the data were apparent. On day 20 of the cycle, which would represent late proestrus, the mammary DNA was at its lowest level--97.8 mg. But between day 20 and the day of estrus, mammary DNA increased to 213.7 mg--an increase of 118% which was statistically significant ($P < 0.05$) when analyzed with an individual degrees of freedom contrast. This increase was followed by a slight decrease (21% and 15%, respectively) during the following metestrus (day 2 of the cycle) and between metestrus and diestrus (day 4 to 18 of the cycle). If these differences are real, it would appear that at the time of estrus, there is a proliferation of mammary cells, but during subsequent metestrus and diestrus, some of these cells are lost. That this may be so is also evidenced from histological examination of the mammary glands (Fig. 5).

Mammary RNA followed a curve essentially similar to the DNA: low (70.7 mg) during proestrus, maximal (198.2 mg) during estrus but decreasing during metestrus (28%) and diestrus (23%). By the same token, the mammary RNA:DNA

ratio increased from a minimum of 0.72 during proestrus to a maximum of 0.96 at estrus followed by a decline during metestrus (0.84) and diestrus (0.78). Both these parameters indicated enhanced metabolic activity of the mammary gland during estrus which diminished progressively during the subsequent phases of the cycle.

Protein, Collagen and Lipid. The protein, hydroxyproline and lipid content of the mammary gland of heifers are shown in Table 9 and Table 8. Protein content increased from low values during proestrus (9.6 g) to peak values during estrus (22.6 g), remained at the same level during metestrus (21.8 g) but diminished during diestrus (14.5 g). Since RNA is related to protein synthesis, it was expected that protein content data would reflect changes in RNA content of the mammary gland. This was indeed so during proestrus and estrus as indicated by parallel increases in both parameters during those periods. But during metestrus, mammary RNA content declined 28% whereas the protein content remained at almost the same level. This suggested that although synthesis of mammary protein followed mammary RNA synthesis rather closely, its catabolism lagged substantially behind that of mammary RNA.

Hydroxyproline content of the mammary gland was low during proestrus (15.6 mg), increased rapidly during estrus (62.7 mg), remained at almost the same level during metestrus (71.0 mg) but declined during diestrus (38.7 mg).

TABLE 9.--Mammary protein, hydroxyproline and lipid content of dairy heifers during the estrous cycle.

Day of Estrous Cycle*	Protein	Hydroxyproline	Lipid
	g/100 lb BW**	mg/100 lb BW	g/100 lb BW
2	21.8 ± 3.6	71.0 ± 9.4	11.5 ± 1.8
4	17.6 ± 2.8	48.7 ± 7.7	12.4 ± 1.9
7	13.7 ± 2.3	37.4 ± 6.4	7.2 ± 0.4
11	11.7 ± 2.6	25.3 ± 5.6	6.4 ± 1.0
18	15.2 ± 2.2	43.5 ± 12.1	7.4 ± 0.5
20	9.6 ± 1.7	15.6 ± 2.4	4.2 ± 0.5
Estrus	22.6 ± 6.3	62.7 ± 42.0	10.0 ± 3.0

*Each group included 5 heifers. The age of all heifers averaged 16.2 months, with a standard deviation of 0.11 months.

**Mean ± standard error.

Changes in mammary collagen content paralleled the changes in protein content of the mammary gland (Table 8): both were highly stimulated during the estrogenic phase of the cycle. This finding was in contrast to the observation in the rat uterus where collagen and total nitrogen or non-collagen protein exhibited an inverse relationship during the estrous cycle (Morgan, 1963). These findings may suggest differences in the hormonal control of collagen synthesis between either the mammary gland and the uterus or between the heifer and the rat. Changes in the collagen content, however, were similar to the changes in the DNA and RNA content of the mammary gland (Table 8) except during metestrus when the nucleic acids declined but the collagen did not. Thus, it would appear that collagen synthesis in the heifer mammary gland, unlike the rat uterus, is stimulated concomitantly with the epithelial components of the organ, suggesting a common controlling mechanism.

The lipid content of the mammary gland, similarly to mammary protein and collagen content, increased from 4.2 g at proestrus to 10.0 g at estrus, remained at the same level during metestrus (11.5 g) and then declined 28% during diestrus (8.3 g). These changes were similar to the changes in the mammary DNA and RNA content except during metestrus when the nucleic acids declined but the lipid did not.

The parallelism among the nucleic acids, protein, collagen and lipid fluctuations suggested that both the

epithelial and connective tissue elements of heifer mammary glands were stimulated during the estrogenic phase of the cycle and that there was no severe competition between the two components at any stage of the estrous cycle.

Morphology. The photomicrographs of the mammary glands at estrus and diestrus are shown in Figure 5(a-d). The photomicrographs revealed that at estrus, the lumen of the alveolar ducts was filled with fluid and was lined with cuboidal epithelium. During diestrus, the lumen was small with no secretions and the epithelial cells lining it were columnar in shape. The structural differences in the mammary gland from metestrus to diestrus and proestrus, if any, could not be easily detected. The stimulatory appearance of the mammary glands during estrus agreed with the reports of Hammong (1927) and the results of the nucleic acid data.

Murine

Nucleic Acids. The DNA content of the rat mammary glands during proestrus, estrus, metestrus and diestrus of the first five estrous cycles is presented in Tables 10 and 11. On the average (Table 10), most of the increase ($P < 0.01$) in mammary DNA (8%) occurred between proestrus (1.41 mg) and estrus (1.52 mg) while the change between estrus (1.52 mg) and diestrus (1.50 mg) was not significant

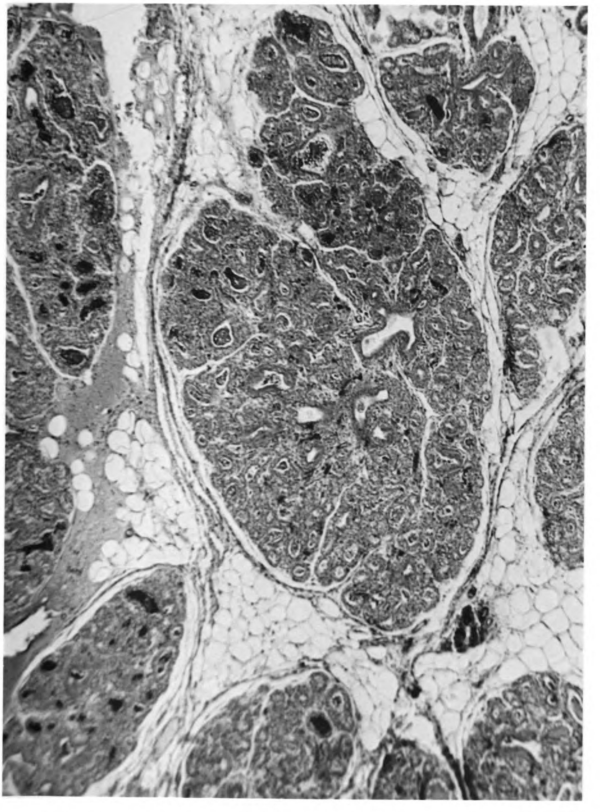

Figure 5a.--Photomicrograph of the heifer mammary gland on the day of estrus. X87

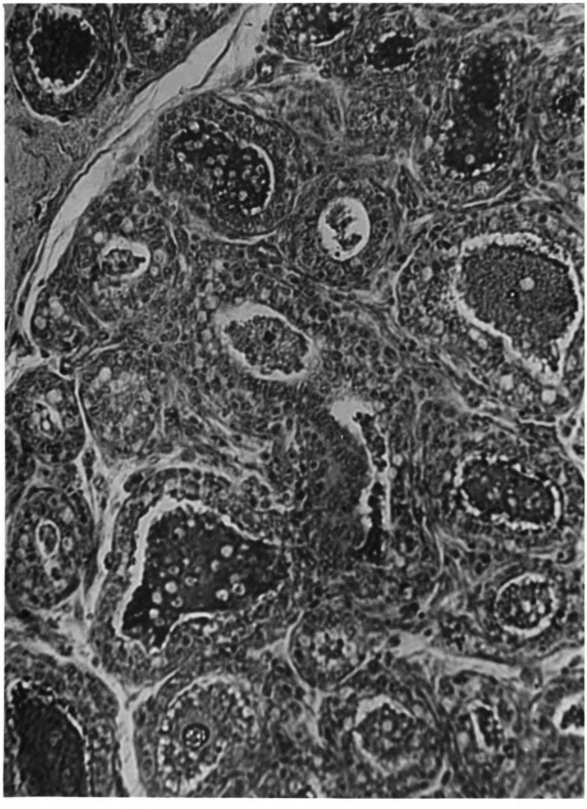

Figure 5b.--Photomicrograph of the heifer mammary gland on the day of estrus. X435

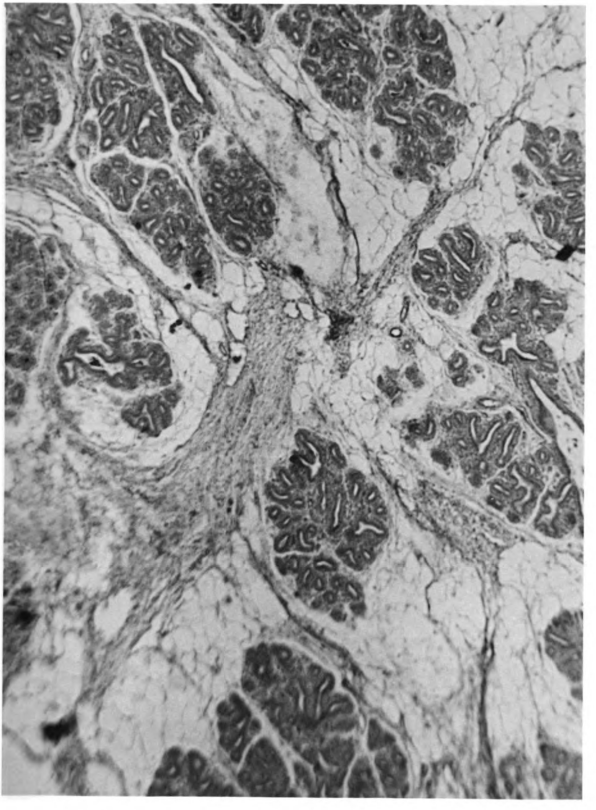

Figure 5c.--Photomicrograph of the heifer mammary gland on day 11 of the estrous cycle. X87

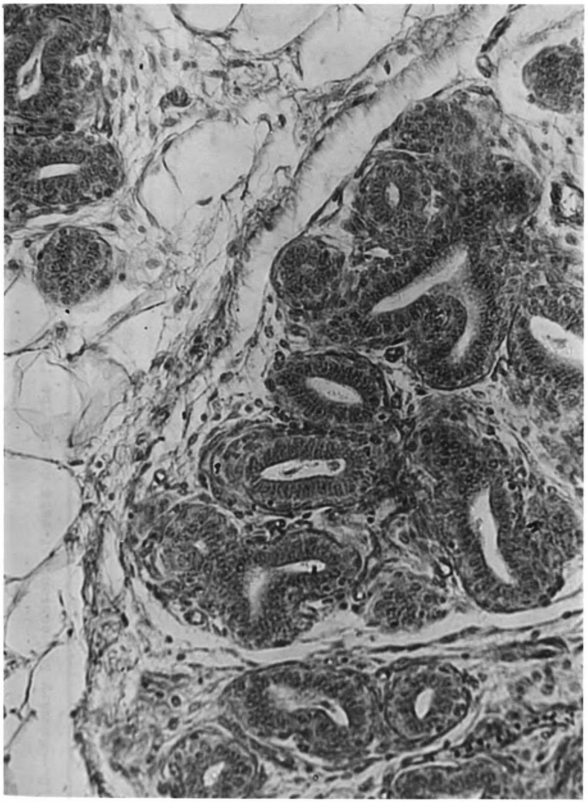

Figure 5d.--Photomicrograph of the heifer mammary gland on day 11 of the estrous cycle. X435

TABLE 10.--Mammary DNA content of rats during the first five estrous cycles.

Estrous Cycle No.	Stage of Estrous Cycle*				Average
	Proestrus	Estrus	Metestrus	Diestrus	
	mg/100 g BW**				
1	1.19 ± 0.05	1.29 ± 0.03	1.37 ± 0.06	1.37 ± 0.06	1.30
2	1.28 ± 0.03	1.36 ± 0.05	1.58 ± 0.09	1.48 ± 0.04	1.43
3	1.44 ± 0.03	1.66 ± 0.10	1.50 ± 0.03	1.56 ± 0.06	1.54
4	1.53 ± 0.04	1.61 ± 0.04	1.60 ± 0.06	1.57 ± 0.04	1.58
5	1.63 ± 0.05	1.68 ± 0.09	1.53 ± 0.06	1.54 ± 0.06	1.59
Average	1.41	1.52	1.52	1.50	

*Each group contained 12 rats.

**Mean ± standard error.

TABLE 11.--Mammary nucleic acid content of rats combined during the first two and the last three estrous cycles.

Nucleic Acid (mg/100 g BW)	Estrous Cycle No.	Stage of Estrous Cycle			
		Proestrus	Estrus	Metestrus	Diestrus
DNA	1 + 2	1.23	1.32	1.47	1.42
	3 + 4 + 5	1.53	1.65	1.54	1.56
RNA	1 + 2	1.64	1.97	2.06	1.92
	3 + 4 + 5	2.01	2.19	2.09	1.91
RNA:DNA	1 + 2	1.32	1.50	1.44	1.37
	3 + 4 + 5	1.30	1.33	1.36	1.23

($P > 0.05$). However, changes in mammary DNA content among the stages were not consistent from cycle to cycle. For example, in cycles 1 and 2, the increases in mammary DNA initiated during proestrus or estrus continued through metestrus, whereas in cycles 3, 4, and 5, the peak DNA content attained at estrus declined somewhat during metestrus. In this respect, there seemed to be a clear distinction between cycles 1 and 2 and cycles 3, 4 and 5.

Mammary DNA also increased cumulatively; 10% ($P < 0.01$) between first and second, 8% ($P < 0.01$) between second and third, 3% between third and fourth and less than 1% between fourth and fifth estrous cycles. From this it appeared that most of the pubertal mammary growth in the rat was completed by the fourth cycle.

If ammmary DNA does not increase cumulatively after the fourth cycle, there are two possibilities with regard to the phasic changes in mammary DNA in the subsequent cycles: (1) the over-all increase in mammary DNA observed between proestrus and estrus does not occur, or (2) if it does occur, it must be offset by a proportional decrease in DNA during the luteal phase. The data of the present experiments do not provide conclusive evidence either way but if the rat mammary glands grow similarly to the heifer mammary glands during the estrous cycle, the latter alternative is more probable. Indeed, an average of cycles 3, 4 and 5 (Table 11), which are more representative of a

mature cycle, does show such a trend: after an initial rise during proestrus, mammary DNA decreased during metestrus with no appreciable change during diestrus. Such an hypothesis would also explain the morphological regression observed in the mammary glands during the luteal phase of the cycle by earlier workers (Turner, 1939).

Although mammary DNA did not increase cumulatively after the fourth cycle, trimmed and defatted weight of the mammary glands (Appendix VIII) increased during this period. This suggested that growth of the connective tissue elements of the mammary glands may continue even after the bulk of the parenchymal growth had been achieved.

The over-all changes in mammary RNA content (Table 12) during proestrus (1.86 mg) and estrus (2.10 mg) were similar to the changes in mammary DNA--an initial rise ($P < 0.01$) of 13% during these two stages. At metestrus, the RNA content (2.10 mg) remained constant. But between metestrus and diestrus (1.92 mg), unlike mammary DNA, the RNA content decreased 9% ($P < 0.01$). An analysis in terms of the first two and the last three cycles (Table 11) revealed that although in the first two cycles RNA increased 5% between estrus and metestrus, it decreased 5% in the last three cycles during the same period. This decline of RNA during metestrus and the over-all decline during diestrus (Table 12) together with the decline in DNA during metestrus of the third, fourth and fifth cycles (Table 11)

TABLE 12.--Mammary RNA content of rats during the first five estrous cycles.

Estrous Cycle No.	Stage of Estrous Cycle*				Average
	Proestrus	Estrus	Metestrus	Diestrus	
	mg/100 g BW**				
1	1.60 ± 0.08	1.96 ± 0.06	2.08 ± 0.10	1.96 ± 0.08	1.90
2	1.68 ± 0.08	1.98 ± 0.08	2.04 ± 0.14	1.88 ± 0.08	1.92
3	1.92 ± 0.08	2.18 ± 0.10	2.02 ± 0.08	2.00 ± 0.12	2.02
4	1.98 ± 0.06	2.06 ± 0.10	2.14 ± 0.06	1.84 ± 0.06	2.00
5	2.14 ± 0.10	2.32 ± 0.14	2.10 ± 0.10	1.88 ± 0.06	2.10
Average	1.86	2.10	2.10	1.92	

*Each group contained 12 rats.

**Mean ± standard error.

suggested that the mammary glands of the rat involute during the luteal phase of the cycle as observed histologically by earlier workers (Turner, 1939). This involution may consist of lowered cellular activity (loss of RNA) as well as probably some atrophy of the mammary cells (loss of DNA).

Unlike uterine RNA:DNA ratio, mammary RNA:DNA ratio (Table 13) was neither less variable nor very consistent from cycle to cycle. However, on an over-all basis, it followed a pattern similar to the mammary RNA. The RNA:DNA ratio increased 8% ($P < 0.05$) between proestrus (1.30) and estrus (1.40), remained constant between estrus and metestrus (1.40) but decreased 9% ($P < 0.05$) between metestrus and diestrus (1.28). Significantly, the mammary RNA:DNA ratio was considerably higher during the first (1.46) and also somewhat higher during the second (1.36) cycles than during the third (1.32), fourth (1.28) and fifth (1.32) cycles. This fact provided another indication that the first and second estrous cycles may be different than the third, fourth and fifth cycles in yet another aspect: rate of metabolism. Having experienced the pinnacle of activity during the first and second cycles, the mammary gland, as it were, settles down to a more subdued rate of metabolism in the later cycles. The implication is that changes in the mammary glands during the first few cycles may not be quite representative of the changes that occur after puberty has been fully attained.

TABLE 13.--Mammary RNA:DNA ratio of rats during the first five estrous cycles.

Estrous Cycle No.	Stage of Estrous Cycle*				Average
	Proestrus	Estrus	Metestrus	Diestrus	
	Ratio**				
1	1.34 ± 0.04	1.54 ± 0.04	1.52 ± 0.02	1.46 ± 0.08	1.46
2	1.30 ± 0.04	1.46 ± 0.04	1.36 ± 0.02	1.28 ± 0.02	1.36
3	1.32 ± 0.04	1.34 ± 0.04	1.34 ± 0.02	1.28 ± 0.04	1.32
4	1.28 ± 0.01	1.28 ± 0.04	1.36 ± 0.04	1.18 ± 0.02	1.28
5	1.30 ± 0.02	1.38 ± 0.02	1.38 ± 0.04	1.22 ± 0.02	1.32
Average	1.30	1.40	1.40	1.28	

*Each group contained 12 rats.

**Mean ± standard error.

D. Pituitary and Ovarian Weights

The weights of the anterior pituitary gland of heifers during the estrous cycle are presented in Table 14. Due to large within animal variation, the differences among groups were not statistically significant ($P > 0.05$). There was some indication, however, that the anterior pituitary weights may be low during diestrus (day 4 to 11) and high from shortly before to shortly after estrus (day 18 to day 2).

TABLE 14.--Anterior pituitary weight of heifers during the estrous cycle.

Day of Cycle	No. of Animals	Anterior Pituitary* (g)
2	5	1.59 ± 0.18
4	5	1.33 ± 0.04
7	5	1.35 ± 0.07
11	5	1.36 ± 0.08
18	5	1.57 ± 0.08
20	5	1.41 ± 0.06
Estrus	5	1.43 ± 0.04

*Mean ± standard error.

The anterior pituitary weights of rats during the five estrous cycles are given in Table 15. The anterior pituitaries gained weight progressively from the first through the fourth cycle, increasing from 5.2 mg during

TABLE 15.--Anterior pituitary weight of rats during the first five estrous cycles.

Estrous Cycle No.	Stage of Estrous Cycle*				Average
	Proestrus	Estrus	Metestrus	Diestrus	
	mg**				
1	5.0 ± 0.19	4.7 ± 0.24	5.4 ± 0.21	5.8 ± 0.37	5.2
2	6.1 ± 0.14	6.7 ± 0.21	6.7 ± 0.14	6.9 ± 0.21	6.6
3	7.1 ± 0.29	7.8 ± 0.35	7.7 ± 0.29	7.1 ± 0.21	7.4
4	8.0 ± 0.26	8.5 ± 0.23	8.1 ± 0.17	7.9 ± 0.20	8.1
5	8.3 ± 0.33	8.3 ± 0.89	8.0 ± 0.26	7.8 ± 0.20	7.9
Average	6.7	7.2	7.2	7.1	

*Each group contained 12 rats.

*Mean ± standard error.

the first cycle to 8.1 mg during the fourth cycle. Among stages of the cycle, they appeared to gain weight through all the stages in the first and second estrous cycles. But in cycles 3, 4 and 5, they gained weight only during proestrus and estrus whereas they lost weight during metestrus and diestrus. The latter three cycles would indicate that there may be a cyclic fluctuation in pituitary weight according to the hormonal state of the cycle: high during estrogenic phase (proestrus and estrus) and low during the luteal phase (metestrus and diestrus).

The ovarian weights during the estrous cycle of the rat are listed in Table 16. The ovarian weight increased progressively throughout the five estrous cycles but most of the increase (7%) occurred between proestrus and estrus. The formation of new corpora lutea after ovulation on the morning of proestrus may have contributed to the pronounced ovarian weight increase at this time. At no stage of the cycle was the ovarian weight significantly reduced, suggesting that the reduction in ovarian weight due to regression of old corpora lutea is compensated for by the concurrent growth of new follicles.

E. Pituitary Luteinizing Hormone

The LH concentration of the rat pituitaries during the various stages of the first five estrous cycles is given in Table 17. Due to insufficient amount of tissue available from single pituitary, several pituitaries

TABLE 16.--Weight of the rat ovaries during the first five estrous cycles.

Estrous Cycle No.	Stage of Estrous Cycle*				Average
	Proestrus	Estrus	Metestrus	Diestrus	
	mg**				
1	31.5 ± 1.8	34.5 ± 1.9	39.9 ± 1.7	46.4 ± 2.4	38.1
2	46.5 ± 1.2	51.7 ± 2.7	53.2 ± 1.3	51.7 ± 1.5	50.8
3	57.4 ± 2.0	58.7 ± 1.8	61.1 ± 1.8	56.6 ± 2.1	58.4
4	61.2 ± 1.8	61.6 ± 2.2	64.2 ± 2.0	55.9 ± 1.7	63.2
5	62.5 ± 1.6	70.1 ± 2.9	63.0 ± 2.5	64.1 ± 1.5	64.9
Average	51.8	55.3	56.3	56.9	

*Each group contained 12 rats.

**Mean ± standard error.

TABLE 17.--Pituitary luteinizing hormone concentration of rats during the first five estrous cycles.

Estrous Cycle No.	Stage of Estrous Cycle*				Average	Comb Avg**	95% C.I.***
	Proestrus	Estrus	Metestrus	Diestrus			
	μg/mg						
1	0.55(1)	0.35(1)	0.23(1)	0.47(2)	0.41 ± 0.06	0.40	0.29 - 0.54
2	0.91(2)	0.23(2)	0.32(2)	0.30(2)	0.29 ± 0.12	0.37	0.25 - 0.56
3	0.79(2)	0.32(2)	0.35(2)	0.41(2)	0.47 ± 0.10	0.40	0.29 - 0.55
4	0.35(2)	0.34(3)	0.14(2)	0.27(3)	0.28 ± 0.06	0.23	0.14 - 0.38
5	0.71(2)	0.40(3)	0.34(2)	0.28(2)	0.43 ± 0.08	0.39	0.27 - 0.57
Average	0.67 ± 0.11	0.33 ± 0.05	0.28 ± 0.04	0.34 ± 0.04			
Comb Avg**	0.72	0.31	0.27	0.33			
95% C.I.***	0.43 - 1.29	0.22 - 0.43	0.20 - 0.38	0.26 - 0.42			

*Each value is the average of number of assays in each group indicated in parentheses.

**Values are weighted average combined by the procedure of Bliss (1956).

***95% combined confidence interval of the combined potency.

had to be pooled for the assay. The values in parentheses indicate the number of such pools assayed in each group. Since the LH content per pituitary (Appendix VI) displayed almost identical trends, it is not included in the text. A preliminary statistical analysis of the data by the hierarchical method showed no significant change ($P > 0.05$) in the average LH concentration from the first through fifth estrous cycle. Therefore, to test for differences among the stages of the estrous cycle, the data from all cycles were pooled and analyzed with a one-way analysis of variance. This procedure resulted in a considerable increase in the degrees of freedom for error mean square and thus an increase in the sensitivity of the test. The analysis disclosed highly significant ($P < 0.01$) differences in LH concentration among stages of the estrous cycle. From a maximum of 0.72 µg/mg at proestrus, the LH concentration declined 57% to 0.31 µg/mg at estrus, remained relatively constant at metestrus (0.27 µg/mg) but increased 22% at diestrus (0.33 µg/mg).

The correlation coefficients between average pituitary LH content and average uterine weight, uterine DNA and uterine RNA:DNA ratio were 0.59 ($df = 18$, $P < 0.01$), 0.44 ($df = 18$, $P < 0.05$) and 0.58 ($df = 18$, $P < 0.01$), respectively.

The interpretation of the pituitary content data of any hormone is very hazardous. High as well as low levels of hormone in the pituitary gland have been associated

with both high and low secretion rates of the hormone. And in many instances both interpretations have been found to be correct. But one basic feature seems to hold true in most of the cases: under very abrupt and acute conditions of physiological stimulation, release of the hormone in blood is marked with a depletion of the hormone in the pituitary whereas under conditions of gradual and chronic stimulation, enhanced secretion of the hormone in the blood is accompanied by increases in the pituitary content of the hormone as well. Sometimes, the response of a target organ, whose specific relationship with the particular hormone is known, also helps interpretation of pituitary content data.

The high concentration of LH in the pituitary observed on the day of proestrus in the present experiments was interpreted as indicating high levels of secretion of the hormone at proestrus. In fact, changes in the LH concentration or content of the pituitary gland paralleled closely ($r = 0.58$) the changes in the uterine RNA:DNA ratio (Fig. 6). In this connection, it is known that LH is required together with FSH to stimulate estrogen secretion from the growing follicles (Everett, 1961), and estrogen promotes RNA synthesis in the uterus (Telfer, 1953; Gorski and Nelson, 1965). Thus, I interpret these data as indicating that LH influenced estrogen secretion which in turn stimulated the uterine RNA:DNA ratio.

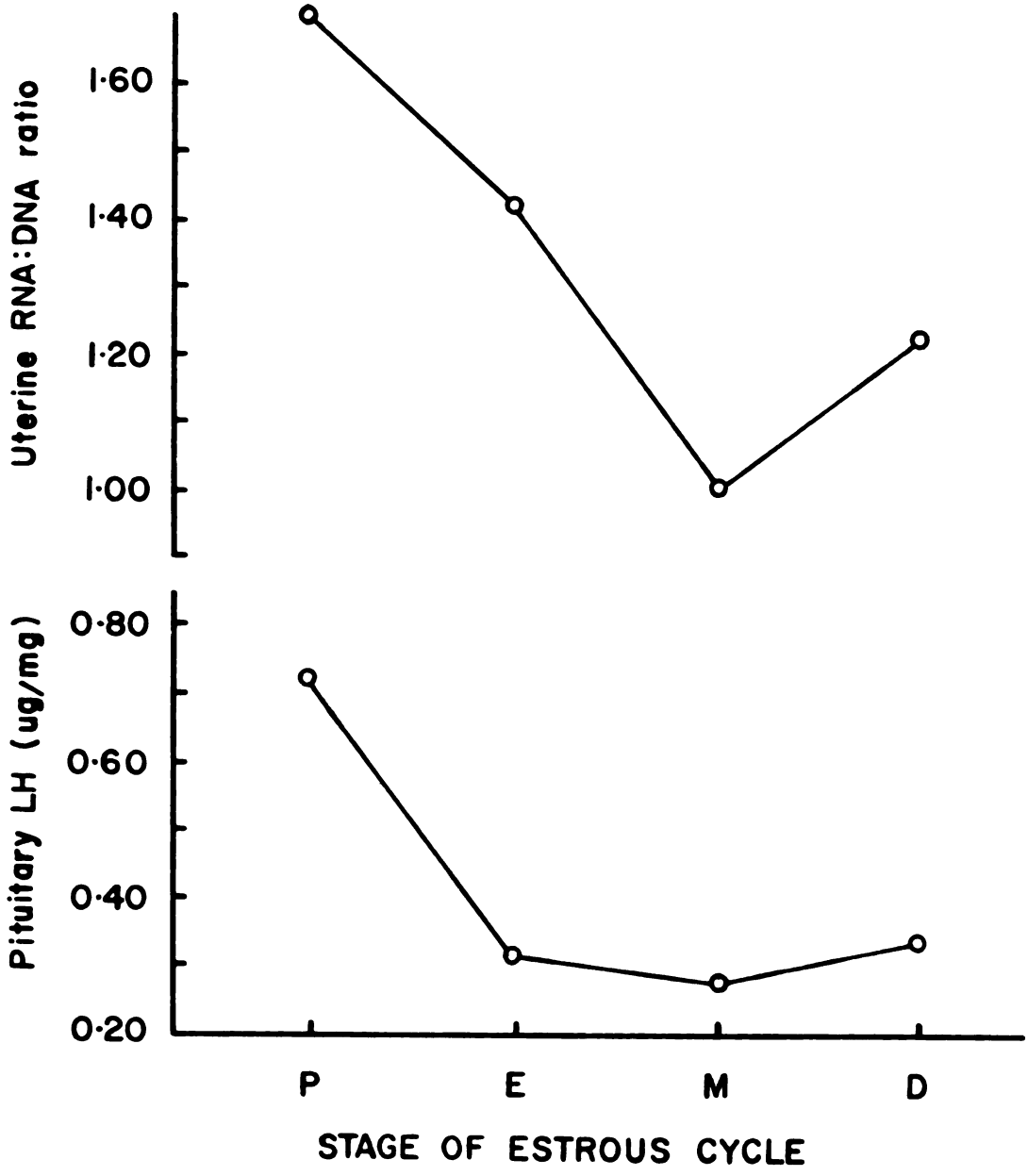

Figure 6.--Relationship between pituitary LH and uterine RNA:DNA ratio of rats during the estrous cycle.

During estrus, the LH concentration in the pituitary and the uterine RNA:DNA ratio, both declined. The drop in pituitary LH may have been caused by the feed-back action of estrogen or progesterone or both on LH, since estrogen in high amounts (McCann and Ramirez, 1964) and progesterone (McCann, 1962) both suppress LH secretion. On the other hand, the fall in the uterine RNA:DNA ratio may have occurred due to (1) a reduction in the blood titers of estrogen following ovulation on the morning of estrus or (2) inhibition of estrogen effect by progesterone (Velardo, 1959) which is secreted from the preovulatory follicles (Astwood, 1939) or (3) a combination of both factors.

Metestrus was the period of lowest uterine protein synthetic activity (RNA:DNA ratio) as well as lowest LH concentration in the pituitary. But during diestrus the uterine RNA:DNA ratio increased significantly. This enhancement of uterine metabolic activity may indicate stimulation of the uterus by estrogen at this time. Since it is known that there is a 6- to 24-hour lag period between initial estrogen treatment and rise in RNA content of the uterus (Telfer, 1953), it would appear that significant estrogen secretion by the growing follicles is probably initiated early in diestrus. This hypothesis is supported by the observed increase (22%) in the pituitary LH concentration between metestrus and diestrus. Although this

increase was not significant in the present study, Schwartz and Bartosik (1962) observed a similar increase in pituitary LH content at this time. The LRF content of the stalk median eminence (Ramirez and Sawyer, 1965; Chowers and McCann, 1965) is also elevated during diestrus which further supports the idea of increased LH and thus estrogen secretion during diestrus.

From the preceding discussion the relationship between LH and uterine metabolism during the rat estrous cycle may be summed up as follows: during metestrus, the LH and estrogen secretions are at basal levels and thus the uterus is quiescent. But low levels of estrogen during metestrus release the feedback inhibition of LH and therefore LH secretion increases during early diestrus. The LH, in synergism with FSH, stimulates estrogen secretion from the growing follicles. Since low levels of estrogen can stimulate LH release (Callantine, Humphrey and Nesset, 1966), the estrogen in turn stimulates more LH secretion culminating in the "ovulatory surge" of LH during proestrus. The estrogen is secreted maximally during proestrus which results in maximal uterine metabolic activity at this time and gradual suppression of LH during estrus. Then the estrogen secretion is either reduced after ovulation or its action on uterus is counteracted by progesterone which is also secreted at a high level during proestrus (Porter, Siiteri and Yates, 1967; Hashimoto and

Melampy, 1967) resulting in lowered uterine metabolic activity during estrus and metestrus. And this cycle of events is repeated over and over again.

F. Pituitary Prolactin

Bovine

The prolactin content of the heifer pituitaries during the various stages of the estrous cycle is given in Table 18 and Appendix VII. Pituitary gland from each heifer was assayed individually and the values in the table are the average of the five heifers in each group.

TABLE 18.--Pituitary prolactin of heifers during the estrous cycle.

Day of Estrous Cycle*	Prolactin**	
	Concentration	Content
	IU/mg	IU/pituitary
2	0.012 ± 0.004	19.39 ± 7.8
4	0.013 ± 0.003	17.53 ± 3.9
7	0.030 ± 0.018	39.51 ± 24.0
11	0.021 ± 0.013	28.94 ± 17.7
18	0.031 ± 0.012	51.01 ± 21.4
20	0.035 ± 0.007	50.02 ± 10.5
Estrus	0.045 ± 0.027	67.16 ± 42.2

*Each group included 5 heifers.

**Mean ± standard error.

The prolactin content per pituitary paralleled the prolactin concentration data (Table 18). As usual with the heifer parameters, the within animal variation in prolactin concentrations was very large. Therefore, the changes in prolactin concentration among stages of the estrous cycle were statistically not significant ($P > 0.05$) when analyzed with a one-way analysis of variance. However, evaluation of the data by regression analysis disclosed a significant slope at $\alpha = 0.08$ level of significance. Or, use of a t-test revealed significant differences between various comparisons such as day 2 vs day 20 of the cycle. Thus, even though the changes in prolactin concentration were of borderline statistical significance, some valid trends were still apparent. From a minimum of 0.012 IU/mg on day 2 of the estrous cycle, the prolactin concentration increased to a maximal value of 0.045 IU/mg on the day of estrus--a sizeable increase of 275%. These results agreed with the findings of Day et al. (1959) who, in the sheep, observed a significant linear increase in pituitary prolactin potency from day 2 to day 18-19 of the estrous cycle.

The relationship between pituitary prolactin concentration and mammary RNA:DNA ratio is shown in Figure 7. Between day 18 of the cycle and the day of estrus, mammary RNA:DNA ratio and pituitary prolactin concentration increased 37% and 45%, respectively. During this interval

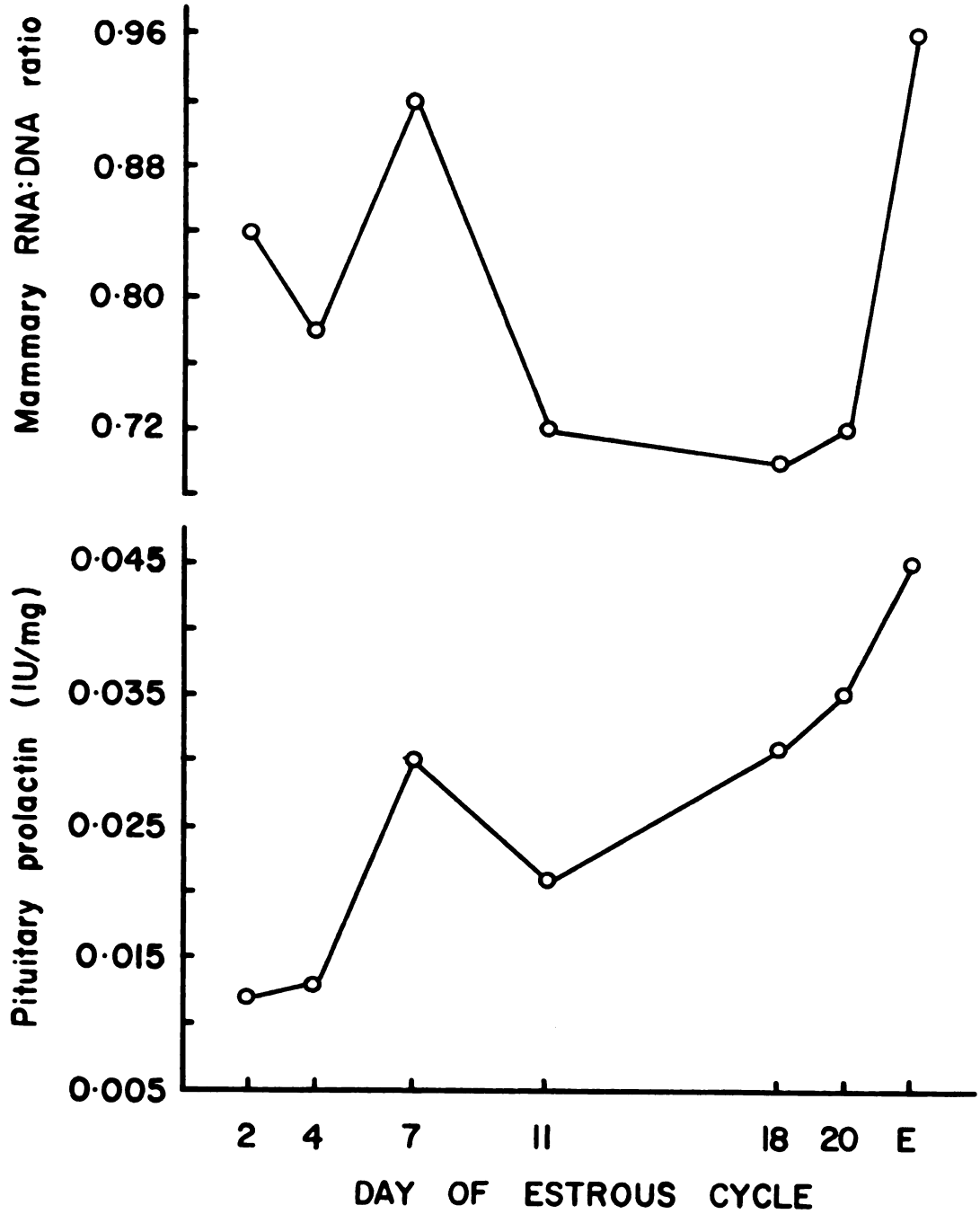

Figure 7.--Relationship between pituitary prolactin and mammary RNA:DNA ratio of heifers during the estrous cycle.

estrogen is also secreted at an enhanced level. It appears, therefore, that high level of prolactin in the pituitary gland during this period reflected elevated levels of secretion of the hormone which synergized with estrogen to stimulate mammary metabolism.

The drop in prolactin level of the pituitary between estrus and day 2 of the cycle probably indicated a very high secretion rate of the hormone at this time, so much so that synthesis could not keep up with the release of the hormone. And this high level of prolactin secretion probably maintained the mammary RNA:DNA ratio at a relatively high level at day 2 of the cycle (0.84 vs 0.96 at estrus) even though estrogen secretion is diminished by this time.

After day 2, synthesis may have caught up with release of prolactin once again because the pituitary concentration began to rise steadily. There was no appreciable change in either pituitary prolactin or mammary RNA:DNA ratio on day 4 of the cycle. The slight increase in prolactin content on day 7 of the cycle may be wholly due to chance but the parallel rise in mammary RNA:DNA ratio at the same time may not be so: it may actually reflect the intimate relationship between prolactin and mammary growth during the estrous cycle. Between days 7 and 18 of the cycle, despite the gradual rise in pituitary prolactin concentration, mammary metabolic activity declined and reached its lowest

level. During this period estrogen secretion is also at its lowest ebb. Therefore, it appears that the level of prolactin secreted during this interval could not by itself maintain mammary metabolism. Indeed, it is known that it takes very large amounts of prolactin to stimulate mammary growth in the absence of estrogen (Talwalker and Meites, 1961).

But stimulation of mammary growth is not the only function that prolactin performs: it is also said to be luteotrophic in rats and mice (Meites and Nicoll, 1966). Whether or not it has a similar role in the cow is controversial at the moment. Some researchers (Hansel, 1966) have produced evidence that LH is the luteotrophic hormone in the cow. But another view is that there may not be any one luteotrophic hormone but instead a "luteotrophic complex" in most species (Rothchild, 1966). In mice (Browning, Larke and White, 1962) LH and prolactin seem to be the members of that complex; in hamsters (Greenwald, 1967) prolactin and FSH. Whether prolactin together with LH constitutes a part of that complex in the bovine remains to be shown but the indications of the present data are suggestive.

A comparison of prolactin content with luteal progesterone and pituitary LH content (data from Hackett, Hafs and Armstrong, 1967) of the same heifers used in this experiment is shown in Figure 8. Changes in pituitary

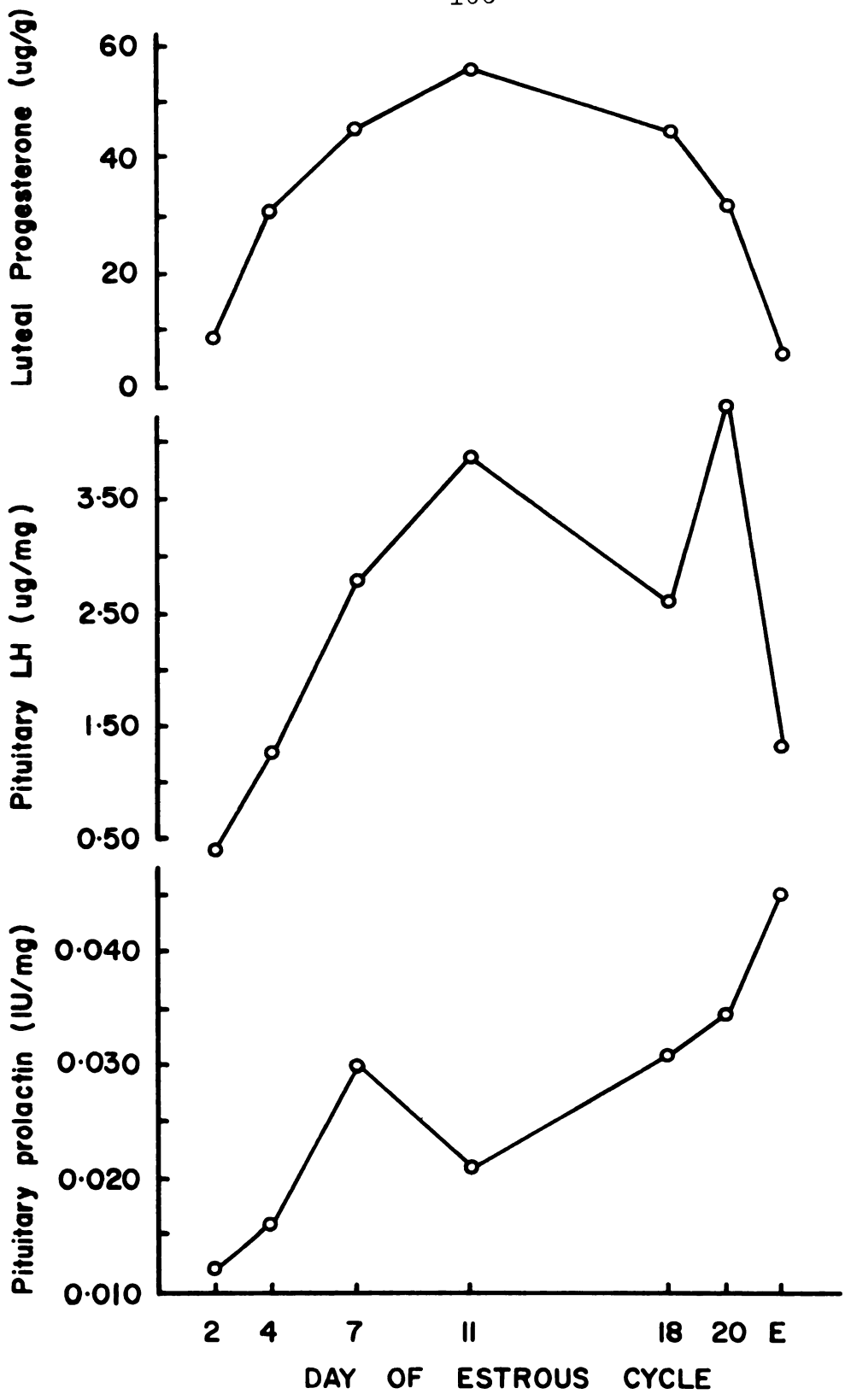

Figure 8.--Relationship between pituitary prolactin and LH and luteal progesterone of heifers during the estrous cycle.

prolactin content were very similar to the changes in the pituitary LH content except on the day of estrus when LH content decreased while prolactin content was still high. But both of these hormones paralleled the alterations in the progesterone content of the corpus luteum until day 18 of the cycle. After day 18, in contrast to prolactin and LH, the progesterone content of the corpus luteum declined abruptly. Just as the similarity between LH and progesterone secretion does not prove LH's role in corpus luteum function, neither does the parallelism between prolactin and progesterone. But a probable case for the involvement of prolactin in bovine luteal function is indicated at any rate. Indeed, the results of Snook, Brunner and Soatman (1967), in which they found that injections of antibovine LH serum reduced corpus luteum weight and total progesterone but did not alter progesterone concentration, may suggest that factors in addition to LH are involved. And one of those factors may very well be prolactin.

Additional evidence in the favor of prolactin participating in the function of the corpus luteum has also appeared recently. Progesterone secretion during the estrous cycle in stalk-sectioned cows (thereby presumably depriving the animal of LH but not prolactin) followed a curve identical to intact controls although at a reduced level (Henricks, Oxenreider, Anderson and Guthrie, 1967). In the sheep (Thibault, 1966), progesterone levels in the

stalk-sectioned animals were quite comparable to those observed in intact animals during the estrous cycle. But a final picture in this regard will have to await more investigation.

Murine

The prolactin concentration of the rat pituitaries during various stages of the first five estrous cycles is summarized in Table 19. The values in parentheses indicate the number of pituitary pools assayed in each group. Statistical analyses for the test of significance were performed identically to the LH data. Prolactin concentration among the five estrous cycles did not differ significantly from each other. But among stages of the cycle, pituitary prolactin concentration during proestrus (0.032 IU/mg) and metestrus (0.041 IU/mg) were significantly greater ($P < 0.025$) than during estrus (0.015 IU/mg) and diestrus (0.015 IU/mg). Changes in the prolactin content per pituitary (Appendix VIII) were similar to the changes in prolactin concentration.

The correlation coefficients of average pituitary prolactin content with average mammary DNA, RNA and RNA: DNA ratio were 0.09 ($df = 18$; $P > 0.05$), 0.01 ($df = 18$; $P > 0.05$) and -0.05 ($df = 19$; $P > 0.05$), respectively.

The maximal pituitary prolactin concentration observed during proestrus in the present study is in agreement with the reports of Reece and Turner (1937) who

TABLE 19.--Pituitary prolactin concentration of rats during the first five estrous cycles.

Estrous Cycle No.	Stage of Estrous Cycle*				Average	Comb Avg**	95% C.I.***
	Proestrus	Estrus	Metestrus	Diestrus			
	IU/mg						
1	0.092(2)	0.005(2)	0.076(2)	0.010(2)	0.046 ± 0.018	0.025	0.010 - 0.064
2	0.044(2)	0.010(2)	0.129(2)	0.017(2)	0.050 ± 0.027	0.027	0.012 - 0.063
3	0.029(2)	0.017(2)	0.073(2)	0.007(2)	0.032 ± 0.015	0.017	0.008 - 0.038
4	0.037(2)	0.044(3)	0.063(3)	0.020(3)	0.041 ± 0.011	0.030	0.017 - 0.052
5	0.069(2)	0.027(3)	0.023(3)	0.019(3)	0.031 ± 0.011	0.018	0.009 - 0.036
Average	0.054 ± 0.015	0.023 ± 0.008	0.068 ± 0.020	0.015 ± 0.003			
Comb Avg**	0.032	0.015	0.041	0.015			
95% C.I.***	0.015-0.070	0.008-0.028	0.024-0.068	0.011-0.020			

*Each value is the average number of assays in each group indicated in parentheses.

**Values are weighted average combined by the procedure of Bliss (1956).

***95% combined confidence interval of the combined potency.

noted an increase in prolactin concentration between diestrus and proestrus. However, they did not observe a decrease at estrus or an increase at metestrus as found in the present study. The findings of Everett (1945), who observed transient depletion of cholesterol in corpora lutea of rats during proestrus, also suggested an increase in prolactin secretion during proestrus. No direct evidence in the literature in support of elevated prolactin secretion during metestrus in rats is available to the best of my knowledge. But White and Browning (1962), based on observations of hyperemia of corpora lutea formed in intraocular ovarian grafts, suggested that prolactin, in mice, was released during metestrus. If the patterns of prolactin secretion during the estrous cycle in mice and rats are similar, the increased levels of pituitary prolactin observed during metestrus in the present study supported that contention.

Presumptive evidence that prolactin may be secreted at high levels during proestrus as well as metestrus is provided by the recent findings of Hashimoto and Melampy (1967). They observed two peaks of progesterone levels in the ovarian venous blood of the rat--one during proesterus, the other during early diestrus (comparable with metestrus of the present study)--suggesting prolactin stimulation of the corpora lutea during both of those periods. In fact, the prolactin data of the present

experiments combined with the progesterone data of Hashimoto and Melampy (1967) would suggest, as proposed by Everett (1961), that the corpora lutea of rats may be functional during the estrous cycle. And the periods of enhanced activity may include two phases of the cycle: proestrus and metestrus.

The relationship between pituitary prolactin concentration and mammary growth (DNA) is shown in Figure 9. During proestrus, pituitary prolactin concentration was high. At this time, estrogen secretion is also maximum (Astwood, 1939) as indicated by the RNA:DNA ratio of the uterus. These hormones probably contributed to the surge of mammary growth between proestrus and estrus. The lag between peak hormone secretion at proestrus and peak mammary cell proliferation at estrus was probably due to latent periods of these hormones with regard to their action on mammary tissue. This latent period may be also responsible for the low correlation between prolactin and mammary DNA ($r = 0.09$) and mammary RNA:DNA ratio ($r = 0.05$).

The lack of increase in mammary cell numbers at metestrus may be the result of reduction in prolactin activity at the preceding estrus. On the other hand, the absence of significant increases in mammary cell numbers during diestrus following the peak of prolactin activity during metestrus may be due to the low levels of estrogen secreted at this time. It is known that it takes very

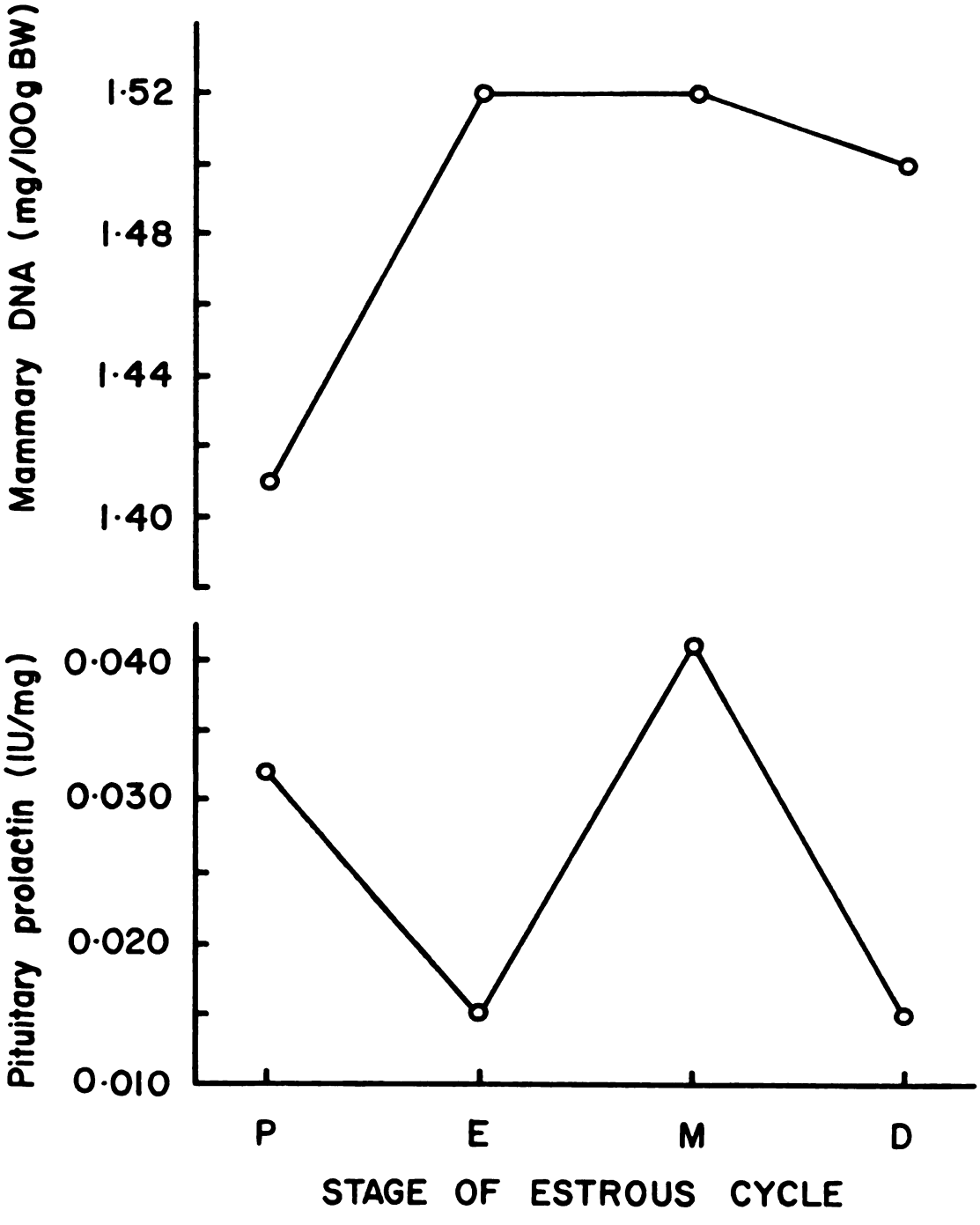

Figure 9.--Relationship between pituitary prolactin and mammary DNA of rats during the estrous cycle.

large amounts of prolactin (probably more than is secreted during the rat estrous cycle) to stimulate mammary growth in the absence of estrogen (Talwalker and Meites, 1961).

G. General Discussion

The results of this research provided a comparison of the physiology of mammary growth and prolactin secretion during the estrous cycle of two species--the heifer and the rat. Although there were some subtle differences, the similarities between the two were marked. In both of these species, the estrous cycle had a profound influence on mammary growth and metabolism. In both of these species, protein synthetic activity in the mammary tissue was significantly enhanced only during the estrogenic phase of the estrous cycle--that is, between proestrus and estrus. And in both species, mammary metabolic activity was considerably reduced during the progestational phase of the cycle as indicated by lowered RNA:DNA ratio during metestrus and diestrus. The lack of mammary growth during the luteal phase of the estrous cycle in the rat was not surprising since the rat possesses such a short luteal period. But in the heifer it indicated that the progesterone secreted during the estrous cycle was probably not enough to influence mammary growth to any appreciable extent. The conclusion is that the influence of progesterone in bovine mammary growth does not become consequential until the onset of pregnancy.

Before pregnancy, it is the estrogen in concert with prolactin that plays the main role.

The changes in the pituitary prolactin concentrations of the heifer were linear; in the rat there were two distinct peaks. But in both species prolactin concentration, in general, appeared to be elevated during the estrogenic phase of the cycle. Since estrogen, in moderate doses, can stimulate prolactin secretion from the anterior pituitary (Meites and Nicoll, 1965), the low levels of estrogen secreted during late diestrus may have stimulated prolactin secretion in both species during the early estrogenic phase of the cycle. But what caused a second peak of prolactin concentration in the rat during metestrus remains unexplained.

Although enhancement of prolactin secretion during the early estrogenic phase seems almost certain in both species, the role prolactin plays at this time besides stimulating mammary growth and corpus luteum function is not so clear. Desjardins, Kirton and Hafs (1967) observed rapid depletion of pituitary prolactin after copulation in the rabbit and suggested that prolactin may be involved in ovulation. This hypothesis together with the possibility that prolactin may be associated with the process of implantation needs to be investigated.

Mammary as well as uterine development has long been known to be influenced by the stages of the estrous cycle. But a study of their metabolism in the same animal revealed

some striking differences. Greatest metabolic activity (RNA:DNA ratio) of the mammary tissue was observed generally at estrus, a day later than that observed in the uterus. This delay of one day in the appearance of maximal protein synthetic activity between mammary and uterine tissue may reflect differences in the latent periods and/or optimal titers of the hormones necessary for mammary and uterine stimulation. In addition, mammary RNA:DNA ratio during the first and second estrous cycles was substantially higher than during the third, fourth and fifth cycles, whereas in the uterus the ratios during all cycles were virtually the same. Also mammary metabolism in the latter three cycles was at about the same level as the uterine metabolism in all cycles. There are two implications of this. Firstly, that mammary tissue is very sensitive to the hormones acting during the first and second cycles to which it becomes partially refractory during the later cycles. And secondly, that either this higher sensitivity to the hormones of the estrous cycle in the uterine tissue has occurred before the onset of the first estrus or that the refractoriness to these hormones never develops in the uterine tissue at all. Whatever may be the case, the above facts point to some important differences in the mechanism of mammary and uterine growth at the time of the initiation of estrous cycles.

Similarities in the mammary and uterine growth during the estrous cycle were also quite salient. Measured in

terms of DNA, the pubertal growth of both the mammary gland and uterus was largely completed by the fourth cycle. The mammary gland of the rat begins to grow at a notably accelerated rate (Sinha and Tucker, 1966; Cowie, 1949) at approximately 21 days of age. Similar information in the uterus is not available, but it may be assumed to commence growing at a faster rate at about the same age. Then, puberty in the rat, according to the definition of Donovan and van der Werff ten Bosch (1965; see review) and based on mammary and uterine development, would appear to begin at three weeks of age and be completed after the animal has undergone four estrous cycles.

The fact that uterine and mammary growth in the rat is completed by the fourth cycle has yet another more significant and practical implication. Does it take so few cycles to complete the pubertal mammary and reproductive growth in the bovine female as well? The data of the present experiments can not adequately answer this question. But Sinha and Tucker (1965) reported that mammary DNA in Holstein heifers plateaued at 9-10 months of age and the heifers in this study were approximately 16 months of age and contained no more DNA than the 9-10 month old heifers. The data of Desjardins (1966) indicated lack of significant increase in uterine DNA after 10 months of age. The heifers in Desjardins' (1966) study exhibited their first estrus at an average age of seven months. Thus it would appear that in the bovine too, relatively few cycles may be required

to complete the major portion of the pubertal mammary and reproductive growth. If that is the case, there may not be much advantage in terms of optimal mammary and uterine development to delay breeding of dairy heifers any longer than 10 months.

CHAPTER V

SUMMARY AND CONCLUSIONS

The relationship between the reproductive and mammary development and endocrine function of bovine and murine female during the estrous cycle was investigated in these experiments.

A. Estrous Cycle Parameters

1. The length of the estrous cycle of heifers averaged 20.6 ± 0.2 days.
2. The age of vaginal opening of rats averaged 36.7 ± 0.13 days. Vaginal opening coincided with proestrus or estrus smear in 58% of the rats. The mean length of the first (5.5 days) and second (5.2 days) cycles was significantly greater than the third (4.8 days) and fourth (4.9 days) cycles.

B. Uterine Development

3. Uterine weight of rats declined from a maximum of 211 mg/100 g BW at proestrus to 125 mg/100 g BW at metestrus but increased to 134 mg/100 g BW at diestrus. Cumulatively, the uterine weight increased progressively throughout the five estrous cycle.

4. Uterine DNA of rats did not change between proestrus (0.88 mg/100 g BW) and estrus (0.85 mg/100 g BW) or metestrus (0.70 mg/100 g BW) and diestrus (0.70 mg/100 g BW) but declined 18% between estrus and metestrus suggesting a significant loss of uterine cells during the early luteal phase of the cycle. Cumulatively, uterine DNA increased progressively from the first through the third estrous cycles but not thereafter.
5. Uterine RNA content of rats was maximum at proestrus (1.50 mg/100 g BW), minimum at metestrus (0.68 mg/100 g BW) but increased during diestrus (0.86 mg/100 g BW). Cumulatively, it increased progressively from the first through the third estrous cycles but not thereafter.
6. Uterine RNA:DNA ratio of rats decreased from a maximum of 1.70 at proestrous to a minimum of 1.00 at metestrus, then increased to 1.22 at diestrus. Cumulatively, it did not change from cycle to cycle.
7. Uterine lipid content decreased from a maximum of 37.1 mg/100 g BW at proestrus to 23.8 mg/100 g BW at metestrus, then increased to 27.6 mg/100 g BW at diestrus. Cumulatively, it increased at a decreasing rate throughout the five estrous cycles.

8. The histological appearance of the uterus reflected in general the biochemical changes in the uterus during the estrous cycle.

C. Mammary Development

9. Mammary DNA of heifers increased from 97.8 mg/100 lb BW at proestrus to 213.7 mg/100 lb BW at estrus then declined to 169.1 and 142.8 mg/100 lb BW during metestrus and diestrus, respectively, suggesting proliferation of mammary cells during estrogenic phase and loss of cells during the progestational phase of the estrous cycle.
10. Mammary RNA of heifers increased from 70.7 mg/100 lb BW at proestrus to 198.2 mg/100 lb BW at estrus, then declined to 142.6 and 109.4 mg/100 lb BW at metestrus and diestrus, respectively.
11. Mammary RNA:DNA ratio of heifers increased from 0.72 at proestrus to 0.96 at estrus, then declined to 0.84 and 0.78 at metestrus and diestrus, respectively.
12. Mammary protein content of heifers increased from a low of 9.6 g /100 lb BW at proestrus to 22.6 g/100 lb BW at estrus, remained at about the same level during metestrus (21.8 g/100 lb BW), then declined to 14.5 g/100 lb BW during diestrus.

13. Mammary lipid content of heifers increased from a low of 4.2 g/100 lb BW at proestrus to 10.0 g/100 lb BW at estrus, remained at about the same level during metestrus (11.5 g/100 lb BW), then declined to 8.3 g/100 lb BW during diestrus.
14. Mammary collagen content of heifers increased from a low of 15.6 mg/100 lb BW at proestrus to 62.7 mg/100 lb BW at estrus, increased slightly during metestrus (71.0 mg/100 lb BW), then declined to 38.7 mg/100 lb BW during diestrus.
15. The histological appearance of the mammary gland of heifers reflected in general the biochemical changes in the uterus during the estrous cycle.
16. Mammary DNA content of rats increased from 1.23 mg/100 g BW at proestrus to 1.32 and 1.47 mg/100 g BW at estrus and metestrus, respectively, and remained constant at diestrus (1.42 mg/100 g BW) in the first and second estrous cycles. In cycles 3, 4 and 5, however, mammary DNA increased between proestrus (1.53 mg/100 g BW) and estrus (1.65 mg/100 g BW) but declined at metestrus (1.54 mg/100 g BW) and remained constant at diestrus (1.56 mg/100 g BW). On the over-all basis, most of the increase in mammary DNA (8%) of rats occurred between proestrus

(1.41 mg/100 g BW) and estrus (1.52 mg/100 g BW), while the changes between estrus, metestrus (1.52 mg/100 g BW) and diestrus (1.50 mg/100 g BW) were not significant. Cumulatively, mammary DNA of rats increased between the first and the fourth estrous cycle but not thereafter.

17. Mammary RNA content of rats, on the over-all basis, increased 13% between proestrus (1.86 mg/100 g BW) and estrus (2.10 mg/100 g BW), remained constant at metestrus (2.10 mg/100 g BW) but declined 9% at diestrus (1.92 mg/100 g BW), suggesting stimulation of mammary growth during the estrogenic phase of the estrous cycle and involution during the progestational phase.

18. Mammary RNA:DNA ratio of rats on an over-all basis increased 8% between proestrus (1.30) and estrus (1.40), remained constant between estrus and metestrus (1.40) but decreased 9% between metestrus and diestrus (1.28). Mammary RNA:DNA ratio of rats was considerably higher during the first (1.46) and second (1.36) cycles than during the third (1.32), fourth (1.28), and fifth (1.32) cycles, suggesting enhanced mammary stimulation immediately following vaginal opening.

D. Endocrine Function

19. Weight of the anterior pituitary gland of heifers appeared to be high during the follicular phase and low during the luteal phase of the cycle.
20. The weight of the anterior pituitary gland of rats, although increased through all stages of the first and second cycles, was high during the follicular phase and low during the luteal phase of cycles 3, 4 and 5.
21. The weight of the ovaries of rats increased progressively throughout the five estrous cycles but most of the increase (7%) occurred between proestrus and estrus.
22. The luteinizing hormone concentration of rat pituitaries was maximal at proestrus (0.72 µg/mg) which declined to 0.31 µg/mg at estrus, remained relatively constant at metestrus (0.27 µg/mg) but increased 22% at diestrus (0.33 µg/mg). There appeared to be a close parallelism between uterine development and LH secretion during all phases of the estrous cycle of the rat.
23. The prolactin concentration of the anterior pituitary of heifers increased linearly from a minimum of 0.012 IU/mg on day 2 of the estrous cycle to 0.045 IU/mg on the day of estrus. There appeared to be a close parallelism between

mammary development and pituitary prolactin concentration during the estrogenic but not progestational phase of the estrous cycle of the heifer.

24. The prolactin concentration of the anterior pituitary gland of rats during proestrus (0.032 IU/mg) and metestrus (0.041 IU/mg) were significantly greater than during estrus (0.015 IU/mg) or diestrus (0.015 IU/mg). There appeared to be a close parallelism between mammary development and prolactin concentration during the estrogenic but not progestational phase of the estrous cycle of the rat.

25. It is concluded that the pubertal development of the reproductive and mammary apparatus of the heifer and the rat is completed within a few estrous cycles. In addition, although there are intrinsic differences in the mechanism of reproductive and mammary growth, both are heavily dependent upon the estrous cycle whose integrity is essential for the normal reproductive function of the female.

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APPENDICES

APPENDIX I

HEIDENHAIN'S FIXATIVE PROCEDURE AND COMPOSITION OF BOUIN'S FLUID

Heidenhain's Fixative

Reagents:

Water	90 ml
Potassium dichromate	1.8 g
Mercuric chloride	4.5 g
Glacial acetic acid	4.5 ml
Formaldehyde (40%)	10 ml

1. The fixative should be prepared immediately before use or as two solutions, one containing the acetic acid and formaldehyde and the other the remaining ingredients. Fixation should take place in the dark.
2. Tissue should be washed with 4% formaldehyde in the dark.
3. Soak tissue over night in Lugol's Iodine.
   Lugol's Iodine: mix 1 g potassium iodide with 0.5 g of iodine. Add 2-3 ml of water and shake until dissolved. Then dilute to 50 ml.
4. Transfer to several changes of 70% alcohol until no further color comes out.
5. Proceed with routine alcohol dehydration.

Bouin's Fluid

Reagents:

Picric acid (saturated aqueous solution)	75 ml
Formaldehyde (40%)	25 ml
Acetic acid (glacial)	4 ml

APPENDIX II

COMPOSITION OF THE RAT FEED

The rat feed was composed of the following ingredients:

Ground shelled corn (1/8 inch screen)	607.0 lb
Soybean oil meal, 50% protein	280.0 lb
Alfalfa meal, 17% protein	20.0 lb
Fishmeal, 65% protein	25.0 lb
Dried whey	25.0 lb
Pro-strep 20, 0.54% penicillin and 2.72% streptomycin	4.0 oz
Pro-Gen, 20% arsanilic acid	0.5 lb
Vitamin A, 10,000 units/g	364.0 g
Irradiated yeast, 9,000 units/g	38.0 g
Choline chloride	318.0 g
D, Ca Pantothenate	2.5 g
Riboflavin	1.5 g
Nicotinic Acid (niacin)	15.0 g
Vitamin B-12 (0.1% mannitol trituration)	3.0 g
DL alpha tocopherol acetate, 250 IU vitamin E/g	8.8 g
Menadione (vitamin K)	1.0 g
DL methionine	227.0 g
Limestone	16.0 lb
Dicalcium phosphate	17.5 lb
Iodized salt	5.0 lb
Manganous sulphate ($MnSO_4 \cdot H_2O$) 32.5% manganese	168.9 g
Ferrous sulfate ($FeSO_4 \cdot 7H_2O$) 20.9% iron	215.2 g

Calcium carbonate ($CaCO_3$) 40.4% calcium	83.8 g
Zinc carbonate, basic ($ZnCO_3$) 56.0% zinc	40.2 g
Cupric sulfate ($CuSO_4 \cdot 5H_2O$) 25.45% copper	12.9 g
Cobalt chloride ($CoCl_2 \cdot 6H_2O$) 24.77% cobalt	4.7 g
Potassium iodide (KI) 76.45% iodine	2.2 g

This ration gave the following analysis:

protein	21.2%
fat	3.9%
crude fiber	2.5%
productive net energy	902 C/lb

APPENDIX III

COMPOSITION OF SOLUTIONS FOR PROTEIN ANALYSIS

Biuret Reagent

Cupric sulfate ($CuSO_4 \cdot 5H_2O$)	1.5 g
Potassium sodium tartrate ($KNaC_4H_4O_6 \cdot 4H_2O$)	6 g

Dissolve in about 300 ml of water

Add slowly 300 ml of 10% sodium hydroxide solution to the above mixture and 1 g of potassium iodide and bring the volume to 1000 ml.

APPENDIX IV

COMPOSITION OF SOLUTIONS FOR HYDROXYPROLINE ANALYSIS

Resin-charcoal Preparation

Cation-exchange resin (AG 1-X8, 200-400 mesh, chloride form)	20 g
Norit A	10 g

Wash the mixture several times with 6N HCl in a course sintered-glass funnel. Dry with ethanol and ether to a fine powder.

Borate Buffer

Boric acid	61.84 g
Potassium chloride	225.0 g
Distilled water	800 ml

Adjust pH to 8.7 with 10N KOH. Make final volume to 1000 ml.

Alanine Solution

Alanine DL alpha	10 g
Distilled water	90 ml

Adjust pH to 8.7 with 10N KOH. Make final volume to 100 ml.

Ehrlich's Reagent

(a)	Sulfuric acid (concentrated)	27.4 ml
	Alcohol (absolute)	200 ml
	Add acid to alcohol slowly.	
(b)	p-Dimethyloaminobenzaldehyde	120 g
	Alcohol (absolute)	200 ml
	Mix in another beaker.	
(c)	Add (a) into (b) slowly while stirring.	

APPENDIX V

PROCEDURE FOR ASCORBIC ACID ANALYSIS OF OVARIES IN LUTEINIZING HORMONE BIOASSAY

Reagents

(a) Metaphosphoric acid solution: 2.5%

(b) 2,6-dichlorophenol indophenol solution: dissolve 20 mg of 2,6-dichlorobenzenoneindophenol (Eastman sodium) in distilled water and bring the volume to 500 ml.

(c) Sodium acetate solution: dissolve 22.65 g of sodium acetate·$3H_2O$ in 500 ml of distilled water, adjust pH to 7.0 with 0.6 ml of 6% acetic acid.

(d) Indophenol-acetate solution: mix equal volumes of (b) and (c).

Analytical Procedure

1. The trimmed ovary is homogenized with 2.5% metaphosphoric acid in the dilution of 10 mg/ml and filtered through Munktell's No. 00 filter paper.
2. 3 ml of the filtrate is added to 5 ml of the indophenol acetate solution and the color development is read between 20 and 60 seconds in a Beckman DB spectrophotometer at 515 mμ against a distilled water blank.
3. The amount of ascorbic acid in the ovaries is calculated from a standard curve obtained by using U.S.P. Reference Standard Ascorbic Acid and is expressed as mg ascorbic acid per g of ovarian tissue.

APPENDIX VI

PITUITARY LUTEINIZING HORMONE CONTENT OF RATS AND DATA FOR INDIVIDUAL ASSAYS

TABLE 1.--Pituitary luteinizing hormone content of rats during the first five estrous cycles.

Estrous Cycle No.	Stage of Estrous Cycle*				Average
	Proestrus	Estrus	Metestrus	Diestrus	
	μg/pituitary				
1	2.75(1)	1.65(1)	1.29(1)	2.75(2)	2.11
2	5.59(2)	1.51(2)	2.12(2)	2.03(2)	2.81
3	5.66(2)	2.39(2)	2.72(2)	2.89(2)	3.41
4	2.76(2)	2.91(3)	1.07(2)	2.11(3)	2.21
5	5.19(2)	3.34(3)	2.75(2)	2.19(2)	3.37
Average	4.63	2.56	2.06	2.37	

*Each value is an average of the number of assays indicated in parentheses.

TABLE 2.--Pituitary luteinizing hormone data of rats for individual assays.

Cycle No.	Stage of Cycle*	Pituitary Pool No.	LH Potency	Standard Error of Potency	95% Confidence Interval of Potency	Lambda
			µg/mg			
1	P	1	0.55	0.29	0.21 - 1.23	0.39
	E	1	0.35	0.21	0.12 - 0.83	0.42
	M	1	0.23	0.09	0.11 - 0.42	0.28
	D	1	0.38	0.17	0.17 - 0.75	0.32
		2	0.55	0.29	0.21 - 1.23	0.39
2	P	1	1.22	0.66	0.49 - 2.88	0.42
		2	0.60	0.25	0.28 - 1.18	0.32
	E	1	0.21	0.13	0.07 - 0.50	0.39
		2	0.24	0.14	0.08 - 0.55	0.39
	M	1	0.26	0.12	0.11 - 0.53	0.32
		2	0.38	0.17	0.17 - 0.76	0.32
	D	1	0.32	0.14	0.14 - 0.64	0.32
		2	0.27	0.16	0.09 - 0.62	0.39
3	P	1	1.10	0.38	0.60 - 1.96	0.28
		2	0.48	0.17	0.25 - 0.87	0.28
	E	1	0.21	0.10	0.09 - 0.44	0.32
		2	0.42	0.19	0.19 - 0.84	0.32
	M	1	0.30	0.14	0.13 - 0.60	0.32
		2	0.40	0.18	0.18 - 0.79	0.32
	D	1	0.50	0.18	0.26 - 0.90	0.28
		2	0.32	0.14	0.14 - 0.65	0.32
4	P	1	0.61	0.32	0.24 - 1.37	0.39
		2	0.08	0.06	0.02 - 0.21	0.39
	E	1	0.10	0.07	0.03 - 0.25	0.39
		2	0.44	0.19	0.20 - 0.86	0.32
		3	0.50	0.22	0.23 - 0.99	0.32
	M	1	0.19	0.13	0.06 - 0.47	0.42
		2	0.08	0.06	0.02 - 0.20	0.39
	D	1	0.13	0.09	0.04 - 0.32	0.39
		2	0.21	0.14	0.06 - 0.52	0.42
		3	0.47	0.25	0.18 - 1.50	0.39
5	P	1	0.69	0.39	0.26 - 1.62	0.42
		2	0.73	0.41	0.27 - 1.71	0.42
	E	1	0.72	0.41	0.27 - 1.70	0.42
		2	0.37	0.22	0.12 - 0.86	0.42
		3	0.14	0.09	0.04 - 0.35	0.39
	M	1	0.23	0.15	0.07 - 0.58	0.42
		2	0.45	0.27	0.16 - 1.07	0.42
	D	1	0.27	0.17	0.09 - 0.66	0.42
		2	0.28	0.18	0.09 - 0.68	0.42

*P = proestrus, E = estrus, M = metestrus, D = diestrus.

APPENDIX VII

PITUITARY PROLACTIN DATA FOR INDIVIDUAL HEIFERS

TABLE 1.--Pituitary prolactin data for individual heifers.

Day of Cycle	Animal No.	Body Weight	Age	Prolactin Potency	Standard Error of Potency	95% Confidence Interval of Potency	Lambda
		lb	mo.	µg/mg	µg/mg	µg/mg	
2	4A	902	16.6	0.32	0.23	0.09 - 0.88	0.63
	11	916	16.2	0.38	0.21	0.14 - 0.87	0.51
	61	872	16.2	0.31	0.18	0.11 - 0.73	0.54
	87	1030	17.4	1.21	0.81	0.42 - 3.62	0.63
	91	774	16.7	0.17	0.13	0.05 - 0.46	0.59
	Avg	898	16.6	0.48	----	---- - ----	----
	Comb Avg*	---	----	0.38	----	0.19 - 0.76	----
4	10	826	16.0	0.28	0.21	0.07 - 0.77	0.63
	71	776	16.2	0.94	0.49	0.40 - 2.23	0.51
	72	876	16.4	0.58	0.32	0.22 - 1.38	0.54
	83	792	15.9	0.30	0.20	0.09 - 0.76	0.59
	743	596	----	0.59	0.37	0.20 - 1.54	0.59
	Avg	773	16.1	0.54	----	---- - ----	----
	Comb Avg*	---	----	0.53	----	0.34 - 0.83	----
7	58	754	15.8	0.73	0.45	0.26 - 1.93	0.59
	60	866	15.9	0.31	0.18	0.11 - 0.73	0.54
	74	860	16.4	0.28	0.19	0.08 - 0.73	0.59
	78	844	16.4	4.09	3.13	1.48 - 15.79	0.63
	81	710	16.3	0.60	0.32	0.24 - 1.38	0.51
	Avg	807	16.1	1.20	----	---- - ----	----
	Comb Avg*	---	----	0.67	----	0.26 - 1.72	----
11	12	900	15.9	0.31	0.18	0.11 - 0.73	0.54
	27	850	15.9	0.26	0.16	0.09 - 0.63	0.54
	69	880	16.0	0.31	0.21	0.10 - 0.81	0.59
	76	906	16.4	2.91	2.11	1.06 - 10.28	0.63
	86	832	16.4	0.47	0.25	0.18 - 1.07	0.51
	Avg	874	16.1	0.85	----	---- - ----	----
	Comb Avg*	---	----	0.50	----	0.21 - 1.19	----
18	28	916	16.3	0.48	0.30	0.16 - 1.23	0.59
	41	800	15.9	2.63	1.53	1.11 - 7.30	0.54
	80	792	16.2	2.11	1.47	0.77 - 6.96	0.63
	84	914	16.5	0.84	0.44	0.35 - 1.96	0.51
	85	878	16.5	0.16	0.11	0.05 - 0.40	0.54
	Avg	860	16.3	1.24	----	---- - ----	----
	Comb Avg*	---	----	0.82	----	0.30 - 2.22	----
20	5	824	16.0	1.21	0.63	0.52 - 2.93	0.51
	29	860	16.3	1.77	0.94	0.77 - 4.49	0.51
	73	864	16.6	0.40	0.26	0.13 - 1.03	0.59
	75	934	16.5	1.77	0.99	0.74 - 4.61	0.54
	79	768	16.4	1.80	1.24	0.65 - 5.75	0.63
	Avg	850	16.4	1.39	----	---- - ----	----
	Comb Avg*	---	----	1.25	----	0.71 - 2.19	----
Estrus	16	808	16.3	0.36	0.21	0.13 - 0.85	0.54
	44	766	15.8	0.70	0.37	0.29 - 1.63	0.51
	77	756	16.1	6.12	5.13	2.18 - 27.03	0.63
	88	676	16.0	1.01	0.67	0.35 - 2.93	0.63
	90	705	16.5	0.81	0.50	0.29 - 2.14	0.59
	Avg	742	16.1	1.80	----	---- - ----	----
	Comb Avg*	----	----	1.00	----	0.39 - 2.52	----

*Values are weighted average combined by the procedure of Bliss (1956).

APPENDIX VIII

PITUITARY PROLACTIN CONTENT OF RATS AND DATA FOR INDIVIDUAL ASSAYS

TABLE 1.--Pituitary prolactin content of rats during the first five estrous cycles.

Estrous Cycle No.	Stage of Estrous Cycle*				Average
	Proestrus	Estrus	Metestrus	Diestrus	
	IU/pituitary				
1	0.454(2)	0.026(2)	0.424(2)	0.063(2)	0.242
2	0.274(2)	0.068(2)	0.886(2)	0.113(2)	0.335
3	0.206(2)	0.137(2)	0.549(2)	0.052(2)	0.236
4	0.288(2)	0.363(3)	0.506(3)	0.157(3)	0.329
5	0.517(2)	0.234(3)	0.183(3)	0.148(3)	0.270
Average	0.348	0.188	0.482	0.114	

*Each value is an average of the number of assays indicated in parentheses.

TABLE 2.--Pituitary prolactin data of rats for individual assays.

Cycle No.	Stage of Cycle*	Pituitary Pool No.	Prolactin Potency	Standard Error of Potency	95% Confidence Interval of Potency	Lambda
			µg/mg			
1	P	1	3.35	3.09	0.91 - 15.12	0.79
		2	7.49	9.44	1.76 - 66.20	0.90
	E	1	0.39	0.58	0.03 - 1.82	0.96
		2	0.25	0.38	0.02 - 1.10	0.90
	M	1	1.61	1.46	0.39 - 6.17	0.79
		2	7.39	7.46	2.12 - 42.74	0.78
	D	1	0.43	0.62	0.03 - 1.99	0.96
		2	0.80	0.93	0.11 - 3.46	0.90
2	P	1	3.21	2.94	0.87 - 14.31	0.79
		2	2.03	1.76	0.53 - 7.77	0.77
	E	1	0.21	0.37	0.01 - 1.00	0.96
		2	0.98	0.79	0.25 - 3.08	0.71
	M	1	13.88	16.33	3.83 -108.64	0.79
		2	1.32	1.16	0.32 - 4.74	0.77
	D	1	0.53	0.74	0.05 - 2.48	0.96
		2	1.45	1.59	0.26 - 6.89	0.90
3	P	1	3.08	2.81	0.83 - 13.54	0.79
		2	0.29	0.29	0.05 - 0.92	0.71
	E	1	1.38	1.65	0.21 - 7.30	0.96
		2	0.62	0.53	0.14 - 1.91	0.71
	M	1	7.60	7.85	2.15 - 45.57	0.79
		2	1.03	0.83	0.27 - 3.24	0.71
	D	1	0.41	0.61	0.03 - 1.92	0.96
		2	0.46	0.42	0.09 - 1.44	0.71
4	P	1	0.57	0.58	0.10 - 2.03	0.79
		2	3.72	3.32	1.06 - 16.43	0.77
	E	1	0.46	0.66	0.04 - 2.13	0.96
		2	1.90	1.64	0.49 - 7.16	0.77
		3	5.39	6.39	1.25 - 40.36	0.90
	M	1	2.18	1.96	0.56 - 8.83	0.79
		2	1.78	1.54	0.46 - 6.63	0.77
		3	7.09	8.84	1.67 - 60.88	0.90
	D	1	0.44	0.64	0.04 - 2.05	0.96
		2	2.32	2.01	0.63 - 9.12	0.77
		3	0.75	0.89	0.10 - 3.26	0.90
5	P	1	7.93	8.26	2.24 - 48.42	0.79
		2	0.24	0.25	0.04 - 0.80	0.71
	E	1	1.18	1.43	0.16 - 6.03	0.96
		2	0.15	0.07	0.02 - 0.51	0.71
		3	3.40	3.01	0.96 - 14.61	0.77
	M	1	1.24	0.98	0.34 - 3.97	0.71
		2	1.58	1.37	0.39 - 5.79	0.77
		3	1.23	1.37	0.20 - 5.66	0.90
	D	1	0.30	0.30	0.05 - 0.96	0.71
		2	1.19	1.05	0.28 - 4.20	0.77
		3	1.81	1.96	0.34 - 9.02	0.90

*P = proestrus, E = estrus, M = metestrus, D = diestrus.

TABLE 3.--Trimmed and defatted mammary gland weight of rats during the first five estrous cycles.

Estrous Cycle No.	Stage of Estrous Cycle				Average
	Proestrus	Estrus	Metestrus	Diestrus	
	mg/100 g BW				
1	43.8	44.6	49.4	53.2	48.2
2	49.6	54.7	58.5	59.2	55.5
3	61.3	71.7	60.4	61.9	63.8
4	66.3	68.5	68.3	65.8	67.2
5	71.1	74.5	70.8	70.2	61.6
Average	58.4	63.2	61.5	62.1	