Rat mammary gland nucleic acids and blood flow, measured by the xenon-133 clearance method, during hormone simulated and normal pregnancy and lactation

Joanne F. Richards
Iowa State University

Follow this and additional works at: http://lib.dr.iastate.edu/rtd
Part of the Zoology Commons

Recommended Citation
Richards, Joanne F., "Rat mammary gland nucleic acids and blood flow, measured by the xenon-133 clearance method, during hormone simulated and normal pregnancy and lactation" (1975). Retrospective Theses and Dissertations. 5632.
http://lib.dr.iastate.edu/rtd/5632

This Dissertation is brought to you for free and open access by Iowa State University Digital Repository. It has been accepted for inclusion in Retrospective Theses and Dissertations by an authorized administrator of Iowa State University Digital Repository. For more information, please contact digirep@iastate.edu.
INFORMATION TO USERS

This material was produced from a microfilm copy of the original document. While the most advanced technological means to photograph and reproduce this document have been used, the quality is heavily dependent upon the quality of the original submitted.

The following explanation of techniques is provided to help you understand markings or patterns which may appear on this reproduction.

1. The sign or "target" for pages apparently lacking from the document photographed is "Missing Page(s)". If it was possible to obtain the missing page(s) or section, they are spliced into the film along with adjacent pages. This may have necessitated cutting thru an image and duplicating adjacent pages to insure you complete continuity.

2. When an image on the film is obliterated with a large round black mark, it is an indication that the photographer suspected that the copy may have moved during exposure and thus cause a blurred image. You will find a good image of the page in the adjacent frame.

3. When a map, drawing or chart, etc., was part of the material being photographed the photographer followed a definite method in "sectioning" the material. It is customary to begin photoing at the upper left hand corner of a large sheet and to continue photoing from left to right in equal sections with a small overlap. If necessary, sectioning is continued again — beginning below the first row and continuing on until complete.

4. The majority of users indicate that the textual content is of greatest value, however, a somewhat higher quality reproduction could be made from "photographs" if essential to the understanding of the dissertation. Silver prints of "photographs" may be ordered at additional charge by writing the Order Department, giving the catalog number, title, author and specific pages you wish reproduced.

5. PLEASE NOTE: Some pages may have indistinct print. Filmed as received.

Xerox University Microfilms
300 North Zeeb Road
Ann Arbor, Michigan 48106
RICHARDS, Joanne F., 1944-
RAT MAMMARY GLAND NUCLEIC ACIDS AND BLOOD FLOW,
MEASURED BY THE XENON-133 CLEARANCE METHOD,
DURING HORMONE SIMULATED AND NORMAL PREGNANCY
AND LACTATION.

Iowa State University, Ph.D., 1975
Zoology

Xerox University Microfilms, Ann Arbor, Michigan 48106

THIS DISSERTATION HAS BEEN MICROFILMED EXACTLY AS RECEIVED.
Rat mammary gland nucleic acids and blood flow, measured by the xenon-133 clearance method, during hormone simulated and normal pregnancy and lactation

by

Joanne F. Richards

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of The Requirements for the Degree of DOCTOR OF PHILOSOPHY

Department: Zoology
Major: Zoology (Physiology)

Approved:

Signature was redacted for privacy.

In Charge of Maj or Work

Signature was redacted for privacy.

For the Major Department

Signature was redacted for privacy.

For the Graduate College

Iowa State University
Ames, Iowa

1975
### TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>iii</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>LITERATURE REVIEW</td>
<td>4</td>
</tr>
<tr>
<td>METHODS</td>
<td>44</td>
</tr>
<tr>
<td>RESULTS</td>
<td>54</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>76</td>
</tr>
<tr>
<td>SUMMARY</td>
<td>85</td>
</tr>
<tr>
<td>BIBLIOGRAPHY</td>
<td>87</td>
</tr>
<tr>
<td>APPENDIX I</td>
<td>101</td>
</tr>
<tr>
<td>APPENDIX II</td>
<td>104</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>107</td>
</tr>
</tbody>
</table>
LIST OF ABBREVIATIONS

B.W.  body weight
CBA  corticosterone binding activity
DFFT  dried fat free tissue weight
E  estradiol benzoate
EP20  ovariectomized virgin rats treated with E and P per day for 20 days
F  hydrocortisone acetate
FSH  follicle stimulating hormone
GH  growth hormone
HLS  ovariectomized, hypophysectomized virgin rats treated with E and P for 10 days followed by E, P, GH, PRL and F for 10 days and finally GH, PRL and F for an additional 3 days (simulated lactation)
HL?  ovariectomized, partially hypophysectomized virgin rats treated with E and P for 10 days followed by E, P, GH, PRL and F for 10 days and finally GH, PRL and F for an additional 3 days
HPS  ovariectomized, hypophysectomized virgin rats treated with E and P for 10 days followed by E, P, GH, PRL and F for 10 days (simulated pregnancy)
HPU  ovariectomized, sham-hypophysectomized virgin rats treated with E and P for 10 days followed by E, P, GH, PRL and F for 10 days
k  clearance constant (.693/T2)
L1, L3, L5  days 1, 3 and 5 of lactation
λ  mammary gland:blood partition coefficient
LH  luteinizing hormone
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>o.v.</td>
<td>ovarian venous blood</td>
</tr>
<tr>
<td>P</td>
<td>progesterone</td>
</tr>
<tr>
<td>P21</td>
<td>day 21 of pregnancy</td>
</tr>
<tr>
<td>PG</td>
<td>prostaglandin</td>
</tr>
<tr>
<td>PRL</td>
<td>prolaction</td>
</tr>
<tr>
<td>p.v.</td>
<td>peripheral venous blood</td>
</tr>
<tr>
<td>RCG</td>
<td>rat chorionic gonadotropin</td>
</tr>
<tr>
<td>T1/2</td>
<td>clearance rate</td>
</tr>
<tr>
<td>T4</td>
<td>thyroxin</td>
</tr>
</tbody>
</table>
INTRODUCTION

During pregnancy, mammary gland development may be divided into two phases: mammogenesis and lactogenesis. The proliferation of the lobulo-alveolar system which occurs gradually throughout pregnancy is defined as mammogenesis. The increase in cell number near term and the differentiation of these cells into competent secretory daughter cells is defined as lactogenesis. After parturition, lactation commences and the maintenance of this milk secretion is designated galactopoiesis.

Since the 1920's and 1930's many experiments have been performed to attempt to simulate the lobulo-alveolar growth that occurs during pregnancy and then to induce milk secretion in these developed glands to better understand the hormonal mechanisms of mammary gland proliferation and the initiation of lactation.

In classical experiments, Chen et al. (15) and Lyons et al. (81) demonstrated that ovariectomized, hypophysectomized, adrenalectomized rats given estrogen, progesterone, growth hormone, prolactin and hydrocortisone developed mammary glands similar to late pregnancy. They induced lactation in these same animals by removing estrogen-progesterone but continued stimulation with growth hormone, prolactin and hydrocortisone. The extent of lobulo-alveolar proliferation and lactational performance was evaluated by histological examination of whole mount tissues.
With the advent of a more quantitative measure of growth, DNA and RNA analysis, Moon et al. (94) and Griffith and Turner (46) observed that 1 µg estrogen and 3 mg progesterone was sufficient for the hormonal replacement of the ovary while Anderson and Turner (4) demonstrated that 250 µg hydrocortisone was adequate for adrenal gland substitution. The hormonal balance of the anterior pituitary, however, was more difficult to establish. Griffith and Turner (46) observed that 1-2 mg growth hormone and prolactin, along with estrogen-progesterone, stimulated maximal mammary gland growth in ovariectomized rats. But, their results are complicated by the fact that the anterior pituitary was present. Cole and Hopkins (19), using ovariectomized, hypophysectomized rats, asserted that estrogen, progesterone, growth hormone (40 µg) and prolactin (2 mg) developed mammary glands equivalent to late pregnancy and that the removal of estrogen-progesterone along with continued administration of growth hormone, prolactin plus hydrocortisone stimulated lactation. However, in analyzing their DNA and RNA data, the number of cells and the synthetic capability, in the ovariectomized, hypophysectomized simulated mammary glands were lower than the control group. Although they may have induced milk secretion in these mammary glands, the DNA and RNA values were indicative of late pregnancy rather than early lactation.

I repeated these experiments by injecting estrogen, progesterone, growth hormone, prolactin and hydrocortisone to stimulate full lobulo-alveolar development in ovariectomized, hypophysectomized Sprague-Dawley
rats. Milk secretion was induced by estrogen-progesterone withdrawal but continued treatment with growth hormone, prolactin and hydrocortisone. Suspecting that the five hormone complex might not be sufficient to stimulate full mammary gland growth, mammary gland blood flow as well as DNA and RNA was measured to determine whether this could be a limiting factor in mammary gland proliferation.

The xenon clearance method was selected to measure blood flow through the mammary gland because it permitted the injection of a tracer directly into the mammary gland thus avoiding the difficulty of isolating a single mammary gland blood vessel. Blood flow was calculated by measuring the xenon clearance rate and mammary gland:blood partition coefficient for each experimental group.

Because this research problem considers both the hormonal control of mammary gland proliferation and the importance of blood flow in this growth, the literature review is organized into two major sections. The first section concerns the development and proliferation of the mammary gland while the second section deals with mammary gland blood vessels and methods of measuring mammary gland blood flow.
LITERATURE REVIEW

Stages of Mammary Gland Development

The first recognizable structure concerned with the development of the mammary gland is a raised band of ectoderm which appears on either side of the midline of the embryo, the mammary band. Along this band appears a narrower ridge of ectoderm, the milk line or milk crest. The mammary band gradually disappears, the milk line diminishes in length and becomes fragmented into a series of thickenings or nodules of ectodermal cells. These nodules sink into the dermis and become mammary buds, the primordia of the future mammary glands. Each bud elongates, the base remains attached to the epidermis while the distal end penetrates into the mesenchyme so that a cord-like structure is formed, the primary mammary cord. The primary cord gives rise to two or three secondary buds which in turn elongate and branch. These branched cords develop a lumen and become ducts. In this manner, the duct system of the mammary gland is laid down before birth (107).

Most of the increase in mammary gland growth from birth to puberty is due to an increase in connective tissue and deposition of fat in the mammary gland; however, a certain amount of parenchymal growth does take place. After birth and until day 22, the rat mammary gland increases in area at the same rate as body surface area, isometric growth. At approximately 23 days of age, the time of weaning, the mammae come under the influence of ovarian hormones and the phase of isometry changes to one of positive allometry, the specific growth
rate of the glands becoming three times that of the body surface. This rapid growth period precedes puberty by 13-19 days (21).

In species with short estrous cycles, as in the rat, mammary gland growth is limited to an extension and branching of the duct system. A striking feature of these growing glands is the presence of club-shaped terminal end buds around their periphery. Small cyclic changes do occur in the proliferation of the duct system during the estrous cycle with a more active phase of duct growth in early estrus followed by regression towards diestrus.

During pregnancy the majority of mammary gland epithelial proliferation occurs. Further extension and branching of the duct system occurs, alveoli develop and the lobulo-alveolar system progressively takes over most of the space occupied by the stroma, so that the gland becomes a compact mass of lobules with alveoli separated from each other by septa of connective tissue.

In the nursing female rat, shortly after the litter is weaned, there is a decrease in the amount of epithelial tissue, an increase in the stromal adipose tissue, accumulation of fat and protein droplets in the epithelial cells, and vacuolization of the cells. The reduction in epithelial tissue is caused by a decrease in cellular volume and localized degeneration of cells without rupture of the duct system. The myoepithelial cells do not undergo degeneration but play an important role in bridging the gap where necrotic cells have been eliminated to hold the surviving cells together. The stored secretory material is digested without the participation of lysosomes. However, lysosomes play an active role in decreasing the cellular volume of
surviving cells and in the degeneration of the dying cells. The necrotic cells become vacuolated and are either eliminated as colostrum bodies into the alveolar lumina or lysed and eliminated in the interstitial tissue. By the fifth day of involution, nuclei, mitochondria, fat droplets and other structures are released into the alveoli and ducts. By the fifteenth day of involution, only small ducts with intact cells remain (115).

Hormones of Pregnancy and Lactation

To simulate mammary gland development of pregnancy and lactation in ovariectomized, hypophysectomized animals using exogenous hormones, one must first understand the hormonal fluctuations during normal pregnancy and lactation. The hormonal changes during pregnancy and lactation, more specifically, parturition and lactogenesis, have stimulated much research over the past few decades. I have attempted to summarize these hormonal changes in the pregnant and lactating rat.

Hormones of pregnancy (Fig. 1)

During the first few days of pregnancy there are transitory fluctuations in many of the hormones involved in gestation. There is a steady fall in serum gonadotropins during the initial days of pregnancy although a significant increase in LH release is detected on day 3. This temporary rise in LH induces an estrogen surge later on day 4 approximately 24 hours prior to the time of ovo-implantation (148). This estrogen (E) rise prepares the uterus for implantation and may prime the luteal cells for the action of pituitary prolactin (PRL)
Fig. 1. Concentration of hormones in the rat during pregnancy
Following these first few days of pregnancy until day 19, estrogen secretion remains low. Progesterone is needed throughout pregnancy to maintain the functional integrity of the endometrium. The corpora lutea of pregnancy are the major source of progesterone in the rat. The regulation of corpus luteum function was extensively studied by Matthies (82, 83) and Rothchild and colleagues (53, 103, 112). It is well documented that removing the pituitary luteotrophic factors by hypophysectomy of rats before day 12 of pregnancy, terminates pregnancy. If, however, hypophysectomy is performed on or after day 12, the corpora lutea are maintained, progesterone secretion remains high and pregnancy proceeds to term (7, 18, 36, 82, 83). Matthies (82, 83) demonstrated that there is some trophoblastic component of the 12-day conceptus synthesizing a protein hormone which is released into the maternal circulation. This hormone, rat chorionic gonadotropin (RCG), is capable of acting on the corpora lutea to induce progesterone secretion. Therefore, it seems that maternal anterior pituitary prolactin is essential for corpora lutea maintenance and progesterone secretion during the first half of pregnancy while "placental prolactin" (RCG) is responsible for corpora lutea maintenance during the second half of pregnancy. In later studies, Rothchild and colleagues (95, 103, 112) examined more closely the luteotrophic factors responsible for corpus luteum maintenance during the first half of pregnancy by injecting prolactin and LH inhibitors on different days of gestation. They concluded that during the first 12 days of pregnancy, luteal progesterone secretion is maintained by two intricately timed controlling mechanisms:
pituitary prolactin from days 1 through 7, and a placental luteotrophin (RCG)-LH complex from days 8 through 12. Between the evening of day 7 and morning of day 8, RCG assumes the role of prolactin in assuring luteal progesterone secretion. RCG apparently requires the synergistic presence of LH to accomplish this end, but only until day 12. After day 12, RCG alone maintains the corpora lutea.

These conclusions by Matthies and Rothchild are supported by an examination of the changes in prolactin, RCG, LH and progesterone concentrations that occur during pregnancy.

Serum prolactin values are highly erratic during the first half of pregnancy but stabilize thereafter at very low levels until day 21. The large day-to-day fluctuation in prolactin may be explained by a semicircadian release of prolactin with peaks occurring late in the light and dark periods (13). These fluctuations are detected only during the first ten days of gestation (95, 96). Estrogen inhibits the release of prolactin inhibiting factor from the hypothalamus thereby allowing the release of prolactin from the anterior pituitary (1). Because estrogen release is elevated in the initial days of gestation this may explain the higher serum prolactin concentration during the first few days of pregnancy. The low level of serum prolactin during most of pregnancy has been attributed to the high amounts of progesterone present which may partially or completely block the prolactin stimulating action of estrogen (85). There is also the possibility that serum prolactin concentration is low because estrogen secretion is also low (145).
At about the time pituitary prolactin becomes dispensable for corpus luteum maintenance, serum prolactin has declined and rat chorionic gonadotropin (RCG) is detectable. It was recently observed that RCG is present in small amounts as early as day 5 of pregnancy and reaches its peak concentration by day 12 when it becomes the major luteotrophin (121). Shortly after RCG reaches its peak, there is an increase in progesterone release indicating that the primary action of RCG must be to enhance progesterone secretion.

Besides luteotrophic action, RCG also has luteolytic properties (36, 83). Several types of corpora lutea are present during the first half of pregnancy while only one type persists through the second half of gestation. Matthies (83) suggested that the declining release of maternal pituitary prolactin during mid-pregnancy causes some corpora lutea to become refractory to the stimulatory effects of luteotrophin. Therefore, when RCG reaches its peak at mid-pregnancy, it destroys all luteal tissue that has become refractory and enhances the secretory activity of the remaining corpora lutea.

Although Linkie and Niswender (74) were unable to detect any discrete changes in LH release between days 5 and 12 of gestation, Morishige et al. (96) found a conspicuous fall in serum LH between days 11 and 13, precisely the time when the pituitary becomes dispensable to the maintenance of pregnancy in the rat (7, 83). After the first few days of pregnancy, serum FSH is present in only basal concentration. The same explanation for the basal release of prolactin during most of gestation may also apply to the release of serum gonadotropins: either the large amount of progesterone present inhibits the
stimulatory effects of estrogen or the low level of estrogen is insufficient to stimulate the release of gonadotropins.

Corticosterone binding activity (CBA) remains high and constant throughout pregnancy while the serum concentration of free corticosteroids remains low and constant until the last few days of gestation (37).

During the last few days of pregnancy, there is a dramatic change in serum hormone concentrations. Progesterone secretion declines markedly beginning on day 19. The endocrine factors responsible for this drop in progesterone are not known. Because the fall in progesterone occurs before the rise in prostaglandins (PG) or LH, the luteolytic properties of these factors are not responsible for the decline in corpora luteal activity. It is possible that the decline in progesterone secretion may not be due to a luteolytic factor but rather to the withdrawal of luteotrophic support, i.e., a disruption of placental function thereby causing a drop in RCG release (67).

The serum concentration of estrogen increases significantly a few days after the drop in progesterone. Coincidentally, prostaglandin concentration in the venous blood also increases. The function of the increase in estrogen secretion at the end of pregnancy may have a triple purpose: to stimulate the synthesis of PG by the uterus, to sensitize the uterus to the oxytocic properties of PG, and finally to trigger the LH release from the pituitary necessary for post-partum estrus and ovulation (67).
Although corticosteroid binding activity remains constant until parturition, the amount of free serum corticosterone increases during the last few days of pregnancy. The fetal adrenal gland during the last two days of gestation secretes as much corticosteroid as do adrenal glands of mature animals. Corticotrophic activity of the fetal pituitary reaches a maximum between day 15 and 20. Corticosterone from the fetus can cross the placenta by day 18 and may explain the increase in free corticosteroids at the end of pregnancy (37, 38).

Hormones of lactation (Fig. 2)

The decline in placental function at the end of pregnancy is associated with a decrease in RCG and a reduction in corpora luteal function. The increase in prolactin release resulting from this fall in progesterone accompanied by an increase in estrogen secretion, causes luteolysis of these now refractory corpora lutea (82, 83). The shift in the estrogen-progesterone ratio resulting in estrogen dominance also stimulates the release of LH from the pituitary (1). This temporary rise in LH induces post-partum estrus and ovulation. New corpora lutea, with a fresh supply of progesterone, form after ovulation and are maintained by the luteotrophic property of prolactin (82, 83).

Follicular quiescence and maintenance of corpora lutea during lactation are attributed to the ability of the suckling stimulus to suppress the secretion of gonadotropins from the pituitary on the one hand, and to induce the secretion of prolactin on the other (31). Amenomori et al. (1) observed that prolactin release is directly
Fig. 2. Concentration of hormones in the rat during lactation
proportional to the number of suckling pups. Also, the characteristic features in the secretory pattern of progesterone during lactation reflect the pattern of prolactin release from the pituitary (146). A high serum prolactin concentration is maintained for at least 8 days but by the 15th day post-partum, serum prolactin values decrease significantly. This decline is probably due to the less intense suckling stimulus because the pups are beginning to eat solid food found in the cage (1). Paralleling the release of prolactin, progesterone values remain high until approximately day 10 of lactation, then gradually decline to basal concentrations by about day 16 (131, 146).

There is a precipitous decline in CBA activity early in lactation paralleled by an increase in free serum corticosteroid concentration. The suckling stimulus may also be involved in maintaining this higher release of corticosteroid during lactation (37).

**Hormonal mechanisms controlling parturition**

In a recent review, Wagner et al. (143) proposed a hypothetical model for the hormonal control of parturition in farm animals. This model may also be applicable to the rat.

1. A stimulus to the fetal hypothalamus causes the fetal pituitary-adrenal axis to increase glucocorticoid secretion. This may result from the maturation of the appropriate hypothalamic centers making them responsive to stimuli that are already present (18, 60).
2. Increased prostaglandin production by the uterus follows the rise in fetal glucocorticoid production. The prostaglandins, through their oxytocic effect, increase myometrial activity in the uterus (67).

3. With the decline in placental function comes the decrease in RCG and a reduction in corpora luteal function culminating in a decrease in maternal serum progesterone (82, 83).

4. An increase in estrogen release occurs in late pregnancy. This is accompanied by increased prostaglandin production by the uterus (67). The shift in the estrogen-progesterone ratio resulting in estrogen dominance prepares the uterus for a stronger and more rhythmic response to the process of fetal expulsion. This altered ratio also increases the release of oxytocin from the posterior pituitary in response to neural stimuli from the reproductive tract and is important in the general relaxation of the birth canal and dilatation of the cervix and vagina.

5. Finally with the aid of the rhythmic contractions of the myometrium, the fetus is expelled from the uterus.

Because the rise in fetal adrenal corticosteroids precedes all the other hormonal changes in the maternal plasma just prior to parturition, it is postulated that the fetus is the trigger for parturition.

**Hormonal mechanisms of lactogenesis**

Theories for the hormonal inhibition of lactation during pregnancy and the initiation of lactation at parturition are presented by Meites and Turner (87), Folley (34) and Meites (85). The ovarian steroids play an
important role at different stages of pregnancy. The estrogen-progesterone equilibrium during most of pregnancy makes the mammary gland resistant to the stimulatory effects of lactogenic hormones. This estrogen-progesterone ratio inhibits the secretion of prolactin by the pituitary (85) and also probably the other constituents of the lactogenic hormone complex (34). At parturition the fall in plasma progesterone together with a temporary rise in estrogen secretion makes the mammary gland receptive to the pituitary hormones as well as stimulating their release.

One of the constituents of the lactogenic hormone complex may be lacking during pregnancy. Nandi and Bern (99) and Gala and Westphal (37, 38) have suggested that adrenal corticosteroids constitute the limiting factor in the initiation of milk secretion during pregnancy. Nandi and Bern (99) and Talwalker et al. (129) demonstrated that the administration of exogenous glucocorticoid, but not prolactin or growth hormone, induces premature milk secretion in pregnant mice. They observed that the amount of prolactin and growth hormone required for good lobulo-alveolar development and lactogenesis were the same. Therefore, they concluded that an increase in prolactin secretion at parturition is not necessary or sufficient for the initiation of lactation.

Free corticosteroid (biologically active) is in equilibrium with the fraction bound to plasma albumin and corticosteroid binding globulin (CBG). It is possible that the amount of biologically active
glucocorticoid present in the circulation during pregnancy is not sufficient to permit milk secretion. Pregnancy in the rat does not alter the total binding capacity or amount of CBG in the plasma (37); however, values for albumin-corticosteroid binding are significantly lower at the end of pregnancy resulting in an increase in free corticosteroid from the twentieth day of pregnancy (38), corresponding to the first signs of milk secretion. Because the administration of estrogens has been reported to potentiate the biological effects of corticoids (100), the increase in estrogen late in pregnancy may act in conjunction with the elevated free corticosteroid concentration to bring about the initiation of lactation.

An important consideration overlooked by Nandi and Bern (99) and Gala and Westphal (37, 38), however, is that the placenta, more specifically RCG, causes a general body resistance to the biochemical effects of glucocorticoids (24). Therefore, it is most likely that the decline of placental function at the end of pregnancy along with the shift in the estrogen-progesterone ratio increases the sensitivity of the mammary gland to glucocorticoids and prolactin. The altered estrogen-progesterone ratio along with increasing amounts of free glucocorticoids and prolactin all synergize to cause lactogenesis.

Although hormonal changes in the fetus may be considered the trigger for parturition, the fetal contribution to the circulating levels of maternal glucocorticoids is not essential for lactogenesis but sustained maternal adrenal activity is essential (8).
Simulation of Mammary Gland Development and Lactogenesis

In vivo model for mammogenesis and lactogenesis

The development of the mammary gland and initiation and continuation of lactation are under the control of several hormones as discussed previously. In the intact animal, the injection of any one hormone will usually result in a change in other hormonal concentrations. What is needed then is a model system in which the control of all pertinent concentrations of hormones is in the hands of the experimenter so that he can vary them one at a time. An ovariectomized, hypophysectomized or ovariectomized, hypophysectomized, adrenalectomized (triply operated) animal may serve as such a model.

The validity of these artificially developed glands as models depends upon the faithfulness with which they represent the normally developed mammary gland. Their suitability as models was initially judged qualitatively by their morphological characteristics, i.e., histological examination of whole mount tissues (81, 99).

Quantitative methods also were developed to study the experimentally induced changes in mammary gland structure and function, i.e., DNA and RNA analysis (63).

The use of DNA content as an index of cell number followed from the suggestion that the amount of DNA per somatic cell nucleus of a given species is constant (90, 140). This was later confirmed for the rat mammary gland during pregnancy and lactation (70, 133, 134). Kirkham and Turner (63) were the first to investigate the changes in
DNA content of the mammary gland during the lactational cycle. Subsequently, DNA values have been determined in the rat mammae at different stages of development. The results of some studies are summarized in Fig. 3.

From such results Griffith and Turner (44) concluded that 21% of the total mammary growth occurs during the first ten days of pregnancy, and 50% after twenty days. Following parturition, there is a wave of cell division (40) and a corresponding increase in DNA. By day 5 of lactation, growth of the mammary gland is 98% complete (44).

The major criticism against the use of DNA as an index of mammary gland proliferation has been that the mammary gland epithelial cells are surrounded by connective tissue and a fat pad. Is the increase in DNA of the mammary gland throughout pregnancy and early lactation indicative of an increase in the number of epithelial cells, an increase in the number of nuclei per cell, or an increase in the amount of connective and adipose tissue surrounding these parenchymal cells? Harkness and Harkness (51) reported little increase in collagen content of the mammary gland during the different stages of mammary gland development. Nicoll and Tucker (101) removed the mammary gland rudiment from one side of three week old mice. Some of these animals were mated while others remained nulliparous. They found that the virgin, gland-free fat pads and those of the lactators were the same, indicating that the population of the adipose stroma did not increase. The question of polyploidy in mammary epithelial cells was dismissed by Persson (104). Thus, the increase in mammary gland DNA during pregnancy and lactation
Fig. 3. DNA on a per 100 g body weight basis throughout pregnancy and lactation.

- Anderson and Turner (5)
- Griffith and Turner (43,44)
- Moon (92)
- Tucker and Reece (133,134)
represents a proliferation of epithelial cells.

RNA as an index of protein synthesis was substantiated by quantitative biochemical studies (10, 26). The amount of RNA and the value of the RNA/DNA ratio (the amount of RNA per cell) have been used widely to indicate the protein synthetic capability of a tissue. Figure 4 summarizes the results of some of these studies.

On the basis of such results, Greenbaum and Slater (40) divided mammary gland protein synthesis into two phases: that associated with the growth of the mammary gland during pregnancy, and that occurring after parturition for the maintenance of the mammary gland and the elaboration of milk proteins during lactation.

In vivo studies

Research efforts in mammary gland development during the 1920's and 1930's centered around three themes: first, that estrogen (estradiol benzoate, E) stimulated ductal growth; second, that progesterone (P), assuming estrogen priming, was responsible for lobulo-alveolar development; and third, that in order for estrogen and progesterone to have an effect on the mammary gland, a hormone(s) from the anterior pituitary was required (137, 138).

With the advent of DNA analysis, a quantitative technique, these presumptions were confirmed. Moon et al. (94), using ovariectomized rats, found that the daily administration of 1 μg E for 19 days stimulated some mammary gland growth while the injection of 1 μg E + 3 mg P for 19 days resulted in mammary gland development comparable
Fig. 4. RNA/DNA ratio throughout pregnancy and lactation
to that of 18 to 20 day pregnant animals. Increasing the amount of progesterone to 10 mg daily had little, if any, further beneficial effect on the growth of the mammary gland. Keeping the estrogen-progesterone ratio the same but doubling the amount of each hormone was also of no advantage (46). Hypophysectomized rats, given the same hormonal treatment, manifested a regression in mammary gland development indicating that the anterior pituitary is necessary for estrogen-progesterone stimulation of lobulo-alveolar growth (47).

There is a great deal of contradictory evidence concerning the role of thyroxine (T₄) and growth hormone (GH) in mammary gland development. Moon and Turner (93) injected 1 µg E + 3 mg P with 3 or 6 µg T₄ into ovariectomized rats for 19 days. The mean mammary gland DNA was similar to that of the estrogen-progesterone controls. However, when the concentrations of E and P were doubled, leaving the concentration of T₄ the same, the DNA was significantly increased. But, when this experiment was repeated, the higher levels of E and P had no additional benefit over the lower concentrations (66).

Following an examination of mammary gland development and pituitary prolactin (PRL) content in ovariectomized rats treated with E, P and T₄ with (0.0-2.0 µg) and without GH, Moon (91) observed a definite increase in mammary gland growth with the addition of GH, although there was no significant difference between the different concentrations of GH used. Moon concluded that GH stimulated mammary gland growth by enhancing the release of prolactin and that these two
hormones in conjunction with E and P brought about lobulo-alveolar
development. After performing pituitary autotransplants in rats,
Meites and Kragt (86) concluded that the role of $T_4$ may be to enhance
the release of GH which, in turn, stimulates the release of PRL,
thereby causing maintenance of body growth and indirectly the development
of the mammary gland.

Kumaresan and Turner (65) compared the effects of $T_4$ and GH in
estrogen-progesterone primed, ovariectomized rats to that of normal
pregnant animals. Their results, expressed as a percent increase
of DNA/100g B.W. above the appropriate control group, are summarized
below.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Pregnant day 20</th>
<th>Ovariectomized, $EP_{19}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>GH</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>$T_4$</td>
<td>26%↑</td>
<td>21%↑</td>
</tr>
<tr>
<td>GH+$T_4$</td>
<td>29%↑</td>
<td>28%↑</td>
</tr>
</tbody>
</table>

Although $T_4$ induced mammary gland development in the ovariectomized
rats, it had the same effect in the normal pregnant animals. This
agrees with earlier results of Griffith and Turner (45). Therefore,
if there is a synergism between E, P and $T_4$ in stimulating mammary
gland growth, it may not be of physiological importance. In contrast to
Moon's (91) results, the GH treatment, both separately and in combination
with $T_4$, had no significant effect on mammary gland growth. However,
GH did have an effect on somatic growth, either alone or in conjunction
with T₄, as indicated by an increase in body weight. Therefore, the
importance of T₄ and GH may be to maintain normal body growth and
function, and only indirectly affect mammary gland development.

Anderson and Turner (4) took the investigation of the hormone
requirements for mammary gland development one step further by
ovariectomizing and adrenalectomizing rats. Along with estrogen-
progesterone replacement, they found that between 100-250 μg hydro-
cortisone acetate (F) was optimum for mammary gland development. They
considered glucocorticoids necessary, but not adequate for mammary gland
development, since glucocorticoids alone did not stimulate growth.

The interpretation of the results from the preceding experiments
is complicated by the presence of the anterior pituitary. The anterior
pituitary hormones play an important part in the development of the
mammary gland and any exogenous administration of a hormone is likely
to alter the pituitary release of other hormones which may enhance or
mask the effects of the original treatment. Many investigators have
continued to experiment with mammary gland simulation of pregnancy
and lactation by using hypophysectomized rats.

In his earlier work, Lyons et al. (80) demonstrated that
ovariectomized, hypophysectomized rats given E, P and PRL consistently
lost weight. Despite this weight loss, the treatment caused proliferation
of mammary gland alveoli in the form of small lobules. By injecting
GH as well as E, P and PRL, the usual weight loss was prevented and
enhanced lobulo-alveolar growth occurred. Daily injections of E, P and
GH in the absence of PRL failed to cause lobulo-alveolar growth. This lends further support to the idea that the role of GH is to maintain body growth and function and only indirectly affects mammary growth. Lyons induced lactation in these same animals by removing E and P stimulation but continued with the administration of GH and PRL accompanied by ACTH. In a later study using triply operated rats, Lyons et al. (81) were able to induce milk secretion in competent mammary glands with PRL and glucocorticoids in the absence of GH again coming to the conclusion that GH was necessary for maintenance of body growth and only indirectly for mammary gland function.

A similar experiment was carried out on triply operated plus thyroidectomized rats to determine the influence of thyroxine on the mammary gland (15). Again, it was concluded that thyroxine is not essential for mammary gland growth or lactation.

All the above experimental results measured the extent of lobulo-alveolar development and lactational performance on the basis of histological examination of whole mount mammary glands. Cole and Hopkins (19) repeated Lyons' earlier experiments on ovariectomized, hypophysectomized rats but evaluated lobulo-alveolar development and lactation by DNA and RNA analysis as well as histological examination.
Their results are summarized below.

<table>
<thead>
<tr>
<th></th>
<th>DNA mg/100g tissue</th>
<th>RNA mg/100g tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>$P_21$</td>
<td>189 ± 69</td>
<td>110 ± 73</td>
</tr>
<tr>
<td>HPS</td>
<td>135 ± 45</td>
<td>91 ± 54</td>
</tr>
<tr>
<td>HLS</td>
<td>198 ± 59</td>
<td>105 ± 58</td>
</tr>
</tbody>
</table>

They concluded that the hormonal therapy of E, P, GH and PRL stimulated mammary gland growth equivalent to a normal 15 to 17 day pregnant rat, while continued treatment with GH, PRL and F produced lobulo-alveolar development of late pregnancy and stimulated milk secretion. However, they did not stimulate full lobulo-alveolar development of pregnancy in their simulated pregnant (HPS) group as is claimed by Lyons. Furthermore, although the DNA value for simulated lactation (HLS) is greater than HPS, indicating the wave of cell division associated with the onset of lactation, it is not significantly greater than the DNA of a normal animal in late pregnancy. Also, the synthetic capability (RNA) of the mammary glands of the HLS group simulates late pregnancy rather than early lactation.

In vitro studies

To better understand the hormonal requirements for lobulo-alveolar development, Ichinose and Nandi (56) cultured mammary gland tissue from a mouse that was pretreated with estrogen and progesterone. The incubation medium contained varied combinations of estrogen, progesterone, growth hormone, prolactin, aldosterone, and insulin. All media
containing insulin promoted epithelial maintenance, whereas media deficient in insulin, irrespective of the other hormones present, failed to do so. Media containing insulin but lacking either, or both, steroid and anterior pituitary hormones did not stimulate lobulo-alveolar development. But, when all six hormones were included in the medium, epithelial proliferation took place during the first few days of incubation followed by the organization of these cells into lobules. The lobulo-alveolar inducing activity of the medium containing the full complement of hormones confirms in vivo results that both steroid and anterior pituitary hormones are necessary for full lobulo-alveolar formation. Furthermore, the in vitro results imply that the synergism between these hormones must lie at the target tissue.

A more definitive explanation of the hormonal control of the initiation of milk secretion is possible from the results of mouse mammary gland organ cultures and studies of casein and lactose synthesis. Lockwood et al. (79) and Stockdale and Topper (126) monitored casein synthesis while Turkington and Hill (135) and Turkington et al. (136) measured lactose synthetase activity in mid-pregnant mammary gland explants cultured in media containing various combinations of insulin, hydrocortisone, and prolactin.

After 96 hours of incubation in insulin and hydrocortisone, mouse mammary gland cells do not produce casein unless prolactin is added. If mitosis is prevented by the addition of colchicine, prolactin no longer stimulates casein synthesis. Therefore, cell division is essential before functional differentiation is possible. From these and
similar results a sequence of events was proposed: insulin is involved in the initiation of DNA synthesis and is required for mitosis; prolactin causes cell differentiation after mitosis if hydrocortisone is present sometime after DNA synthesis but before mitosis occurs (79, 126).

Lactose synthetase, the enzyme concerned with lactose synthesis, is composed of two proteins: a galactosyltransferase and α-lactalbumin. During the latter half of pregnancy, galactosyltransferase activity of mouse mammary glands rises to nearly maximum values; however, α-lactalbumin values remain low and rise to a maximum after parturition. Prolactin induces the synthesis of both these proteins in culture; progesterone prevents the increase in activity of α-lactalbumin but does not prevent the induction of galactosyltransferase activity. Progesterone injections into mice one or two days before parturition prevents the marked rise in α-lactalbumin concentration after parturition seen in non-injected controls (135, 136). These results further substantiate the importance of the high levels of progesterone during pregnancy in inhibiting the initiation of lactation.

In summary, a possible scheme for the initiation of lactation is presented in Fig. 5.
Fig. 5. Proposed scheme for the initiation of lactation.
Mammary Gland Blood Vessels

Major arteries of rat mammary glands

The rat has six pairs of mammary glands: three thoracic, one abdominal and two inguinal. All of these glands receive their blood supply from the superficial blood vessels of the thoraco-abdominal wall (Fig. 6).

The thoracic glands are supplied by superficial blood vessels from two main arteries, the internal and external mammary arteries. These arteries arise from the subclavian artery and run downward along the thoracic wall. The internal mammary artery, which terminates as the superior epigastric artery, furnishes several small branches which pass between the ribs to the skin. The external mammary artery mostly supplies the larger muscles in the thoracic area.

The abdominal-inguinal mammary glands are usually supplied by vessels from the pudic-epigastric trunk which, in turn, arises from the common iliac artery. The pudic-epigastric trunk divides into many branches: inferior epigastric, superior and inferior external pudendal arteries and some branches that terminate directly in the mammary fat pad. The external pudendal artery also sends branches into the mammary fat pad. The superior epigastric artery springs from the femoral artery halfway down the thigh. It ends in a terminal anastomotic branch which perforates the abdominal wall and eventually anastomoses with the inferior epigastric artery, each sending small branches into the mammary gland.
Vessels to the thoracic glands

Vessels to the abdominal-inguinal glands

Fig. 6. Mammary gland blood vessels
If the pudic-epigastric trunk is missing, then the inferior epigastric and inferior external pudendal arteries arise from the hypogastric trunk, while the superior external pudendal arises from the external iliac artery (41, 77, 137).

**Mammary gland capillaries during mammary gland development**

The distribution of blood vessels in the mammary gland is the same as those of the mammary fat pad. As the gland grows during pregnancy, the capillaries in the adipose tissue are assimilated by the developing ducts and alveoli as they are formed (77). In the early days of pregnancy, the capillary nets are restricted to the ductal regions. With advancing pregnancy and the formation of lobules and alveoli, the vascularity of the gland increases. The prominent capillary plexuses are now found in the region of the lobules and are no longer associated with the major ducts. Further increase in the number of lobule-associated plexuses is observed until the fifteenth day of pregnancy. Beyond that time, and during lactation, the capillaries increase progressively in size but no new plexuses are formed. During post-lactational involution, there is a decrease in vascularity and the capillary nets are no longer seen around the lobules but have regressed to the major ducts (124).

The changes observed in the vascularity of the gland during pregnancy and lactation indicate that the capillary nets are found only in the proliferating (ductal system during early pregnancy) or actively secreting areas (lobulo-alveolar system during late pregnancy...
and early lactation). The increase in the number of capillary plexuses is correlated with the proliferation of the mammary epithelium whereas increase in secretory function is associated with dilatation of those capillaries already present (124).

**Blood Flow Measurement Techniques**

Many procedures are available for measuring blood flow through various organs of the body. Some of these procedures are discussed below with particular consideration to their application in measuring rat mammary gland blood flow.

**Electromagnetic flow meter**

When an electrical conductor (i.e. blood) moves across lines of force of a magnetic field, a potential difference is created in the conductor. This potential difference induces a current in the electrodes which also lie across the path of the conductor. If a) the field is uniform, b) the conductor moves in a plane at right angles to the magnetic field, and c) the length of the conductor extends perpendicular to both the field and direction of motion; the resulting potential difference is directly proportional to the field strength, the flow of the conductor and the length of the conductor within the field. If the field strength and length of the conductor within the field are constant, then the voltage developed is a linear function of the speed of the conductor (59). Such a technique was used to measure mammary gland blood flow in the goat by placing an electromagnetic flow
meter on the pudic artery (109). This method is impractical in the rat because it would be necessary to isolate a mammary artery and to find a sleeve of small enough diameter to attach electrodes to that artery once isolated.

**Thermodilution**

The principle of this method is that saline, cooler than blood, is injected at a known temperature and constant rate into a vein. A thermocouple or thermistor, lying in a blood vessel downstream measures the fall in temperature of the blood saline mixture. Knowing the temperature of the blood stream, the rate of flow can be calculated. Thermodilution is used to measure cardiac output and regional blood flow. Fegler (32) measured cardiac output in the goat by injecting cold saline into the right jugular vein and placing the thermistor into the aortic arch via the left carotid artery. Linzell (75) used this method to measure goat mammary gland blood flow by injecting cold saline into an exteriorized subcutaneous milk vein, recording the fall in blood temperature 10-25 cm downstream. Again, the difficulty in the rat is the isolation of an appropriate mammary vein.

**Fick method**

The time taken for an organ to become saturated with a freely diffusible substance is proportional to the rate it is delivered to the organ. If the amount of substance in the organ and the mean arterio-venous difference during uptake are known, then the flow rate is calculated by dividing the former by the latter. Several methods are
are based on this principle.

**Antipyrine method**  Reynolds et al. (109) measured goat mammary gland blood flow by injecting a known quantity of antipyrine into the milk duct system of the udder. They knew the quantity of antipyrine by measuring the amount remaining in the milk after 30 minutes. During the absorption period, they took blood samples from the carotid artery and subcutaneous abdominal vein to determine the arterio-venous difference. Knowing the rate of absorption of antipyrine at the time of sampling, they calculated the blood flow through the mammary gland.

**Clearance method**  An estimate of blood flow per unit volume of tissue can be made from the rate at which a freely diffusible indicator is cleared from the tissue; the faster the flow, the quicker the elimination (61). The Fick principle is simplified if xenon or krypton is used as the indicator because these are almost completely eliminated from the venous blood in one passage through the lung; therefore, it is not necessary to measure the arterial concentration (16, 105). This procedure was chosen to measure rat mammary gland blood flow and is discussed in greater detail beginning on page 38.

**Indicator fractionation**

The principle of the indicator fractionation method is that following a rapid injection into the right atrium, an indicator is uniformly distributed in the arterial blood and is delivered to the tissues in proportion to their blood supply. If the indicator is a freely diffusible substance, it passes rapidly from the circulation
into the tissue. Provided that it is extracted at an equal rate by all tissues, the proportion of the indicator in each tissue, soon after injection, represents the organ's share of the cardiac output. Therefore, if the circulation is stopped soon after injection and the tissues removed quickly, the distribution of the cardiac output is determined by measuring the amount of indicator in each organ. If the cardiac output is known, the blood flow to any organ can be calculated (50).

Sapirstein (114) applied this method to determine regional blood flow in the rat. He injected radioactive rubidium chloride ($^{86}$RbCl) into the femoral vein. From one group of animals, he removed blood samples from the carotid artery to determine the cardiac output. From another group he removed various organs and measured the uptake of radioactivity by each organ to determine the fractional distribution of the cardiac output to each organ. From the cardiac output and percent distribution, he computed the blood flow to each organ.

A difficulty with this method is that if the indicator used remains in the circulation after the first transit, as with rubidium, mathematical integration of the transit curves after correcting for recirculation is required. If, on the other hand, the indicator is retained in the tissue during the first transit, the integration is accomplished physically, and the need to determine transit time is eliminated. Thus, Minnesota Mining and Manufacturing Company developed radioactively labelled microspheres that are removed from the blood stream by becoming entrapped in the microcirculation of the organ. The limitation in
using microspheres to determine regional blood flow is related to both the diameter of the vessels and the size of the microspheres. Because capillary diameter may vary from organ to organ, the size of the microspheres chosen becomes important (88, 142).

Xenon Clearance Method

To determine blood flow by measuring the disappearance rate of a locally injected indicator, one must assume that there is no difference in the solubility of the indicator in blood or in tissues and that complete equilibrium is reached almost instantaneously between the tissue and its blood. This implies that diffusion is not a limiting factor and that blood flow through the tissue partakes in the equilibration, i.e., there are no arterio-venous shunts. On the basis of these assumptions plus the Fick principle, Kety (62) derived a generally applicable expression for inert gas uptake by a single tissue.

For an inert gas the Fick principle can be stated as follows: the amount of inert gas taken up by the tissue per unit time is equal to the quantity brought to the tissue by the arterial blood minus the quantity carried away in the venous blood:

\[
\frac{dQ_i}{dt} = F_i(C_a - C_v)
\]

\[
\text{Since}\quad C = \frac{Q_i}{V}
\]

Then

\[
\frac{dC_i}{dt} = \frac{1}{V_i} \frac{dQ_i}{dt} F_i(C_a - C_v)
\]

Where:

- \(i\) = tissue
- \(a\) = arterial
- \(v\) = venous
- \(Q\) = amount
- \(C\) = concentration
- \(F\) = flow
- \(V\) = volume
From the basic assumption that blood from a tissue is in equilibrium with the tissue itself, with respect to the inert gas, then at all times:

\[ C_i = \lambda C_v \quad (2) \]

Where \( \lambda \) is the partition coefficient, a correction factor for the difference in solubility of an inert gas in different tissues usually expressed as:

\[ \text{conc. of inert gas in tissue} \quad \text{conc. of inert gas in blood} \]

By substituting \( C_v \) of Equation 2 into Equation 1:

\[ \frac{dC_i}{dt} = -\frac{F_i}{V} (C_i - \lambda C_a) \]

Which, if \( C_a \) and therefore \( \lambda C_a \) are constant, then the expression for the desaturation of an inert gas from a tissue is:

\[ C_i = C_{i0} e^{-kt} \quad \text{where} \quad k_i = \frac{F_i}{V_i} \quad \text{or} \quad \frac{F_i}{V_i} = k \lambda \]

Where \( k \) is the clearance constant, \( C_i \) is the final concentration, and \( C_{i0} \) is the initial concentration.

In the past, sodium-24 \( (^{24}\text{Na}) \) and iodine-131 \( (^{131}\text{I}) \) were used to measure blood flow by the clearance method with considerable variability in results. The chemically inert gases krypton-85 \( (^{85}\text{Kr}) \) and xenon-133 \( (^{133}\text{Xe}) \) have certain advantages over sodium and iodine. They are chemically and physiologically inert; they are not normally found in the body; they are highly lipid soluble allowing them to pass freely through cell membranes \( (144) \); and they are rapidly excreted from the
body via the lungs (16). The long physical half-life of $^{85}$Kr (10.3 years) and $^{133}$Xe (5.27 days) made the use of these nuclides more convenient than $^{24}$Na (15 hours). Finally, the lower gamma ray energies of $^{85}$Kr (0.513 Mev) and $^{133}$Xe (0.081 Mev) are more suitable for external radiation detection than the high energy of $^{24}$Na (1.368 Mev) which is difficult to localize accurately in the body.

One important reason for choosing $^{133}$Xe as the indicator in measuring blood flow is that it comes out of solution very rapidly when exposed to air. This means that the clearance of xenon from the mixed venous blood is almost complete in one passage through the lungs and that the recirculation of the tracer via the arterial circulation is negligible. However, this same property increases the difficulty in measuring the partition coefficient of xenon in different tissues since the gas is lost to the atmosphere upon exposure of the tissue to air.

In spite of the fact that a great number of studies have involved the use of the xenon clearance method to measure blood flow (see Table 1), there have been very few reports of methods used to estimate the xenon partition coefficient. Some of the data reported is derived from in vitro work. Conn's (20) experiments were performed on dog tissues and those of Isbister et al. (57) and Veall and Mallett (139) on human whole brain, gray and white matter.

Andersen and Ladefoged (3) described an in vivo method for determining the partition coefficient in the rat and rabbit. They
Table 1. Summary of studies using xenon clearance method to measure blood flow.

<table>
<thead>
<tr>
<th>Tissue studied</th>
<th>Species</th>
<th>Injection</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney</td>
<td>Human</td>
<td>Renal artery</td>
<td>68</td>
</tr>
<tr>
<td>Spleen</td>
<td>Human</td>
<td>Splenic artery</td>
<td>9</td>
</tr>
<tr>
<td>Spleen</td>
<td>Guinea pig</td>
<td>Spleen</td>
<td>113</td>
</tr>
<tr>
<td>Liver</td>
<td>Dog</td>
<td>Hepatic artery/portal vein</td>
<td>108</td>
</tr>
<tr>
<td>Small gut mucosa</td>
<td>Dog</td>
<td>Lumen</td>
<td>48</td>
</tr>
<tr>
<td>Adipose</td>
<td>Human</td>
<td>Subcutaneous abdominal</td>
<td>72, 102</td>
</tr>
<tr>
<td>Cerebrum</td>
<td>Human</td>
<td>Cerebrum</td>
<td>30</td>
</tr>
<tr>
<td>Cerebrum</td>
<td>Human</td>
<td>Carotid artery</td>
<td>54</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>Rabbit</td>
<td>Hypothalamus</td>
<td>111</td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td>Human</td>
<td>Anterior tibial</td>
<td>49, 72</td>
</tr>
<tr>
<td>Myometrium</td>
<td>Human</td>
<td>Myometrium</td>
<td>58, 118</td>
</tr>
</tbody>
</table>

placed animals in an air-tight box containing an air-xenon mixture. After 24 hours the animals were exsanguinated and tissue and blood samples taken, all manipulations being carried out in the box from outside using air-tight gloves. Samples were placed in air-tight tubes containing concentrated hydrochloric acid to digest the tissue. After another 24 hours, the samples were removed from the chamber, counted in a well-scintillation counter and the tissue:blood partition coefficient computed.
Another in vivo method for measuring the partition coefficient is described by Rosendorff and Luff (111). They injected both Xe-133 and I-125-antipyrine (IAP) into the tissue. The simultaneous clearance of these two isomers was measured with an external scintillation counter. Because the two isotopes are being cleared by the same blood flow, the ratio of clearance rates ($T_{1/2}$) is related to the ratio of the partition coefficients ($\lambda$):

$$\frac{T_{1/2}(\text{Xe})}{T_{1/2}(\text{IAP})} = \frac{\lambda(\text{Xe})}{\lambda(\text{IAP})}$$

The non-volatile ($\lambda_{\text{IAP}}$) can be easily determined and the $\lambda_{\text{Xe}}$ calculated.

Rat Mammary Gland Blood Flow

Linzell and coworkers (14, 76) seem to be the only people publishing rat mammary gland blood flow data. They estimate blood flow in tissues by combining Fegler's (32) thermodilution method of measuring cardiac output with Sapirstein's (114) indicator fractionation technique of determining cardiac output distribution. A catheter coupled with a thermistor is placed into the right atrium via the right jugular vein, while a second catheter is attached to the left carotid artery. The cardiac output is measured by rapidly injecting saline at room temperature into the right atrium and recording the passage of cooled blood moving from the heart through the carotid as it passes the arterial thermistor. By injecting $^{86}\text{RbCl}$ through the catheter in the jugular vein, quickly killing the animal, removing the organs and counting their radioactivity, the percent of the original injected
radioactivity and cardiac output distribution is calculated. From these parameters, cardiac output and its distribution, Linzell and coworkers calculated blood flow in ml/min/100g tissue. Their results are summarized in Table 2.

Table 2. Mammary gland blood flow during late pregnancy and lactation

<table>
<thead>
<tr>
<th></th>
<th>Pregnancy(^a,b)</th>
<th>Lactation(^b)</th>
<th>Lactation(^b)</th>
<th>Lactation(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>day 22</td>
<td>day 1</td>
<td>day 2</td>
<td>day 12</td>
</tr>
<tr>
<td>Mammary gland</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wet wt./B.W. (%)</td>
<td>5.00 ± .30</td>
<td>5.25 ± .50</td>
<td>4.75 ± .15</td>
<td>7.63 ± .28</td>
</tr>
<tr>
<td>% Cardiac output</td>
<td>4.61 ± .45</td>
<td>7.25 ± .90</td>
<td>8.25 ± .30</td>
<td>9.96 ± .74</td>
</tr>
<tr>
<td>Blood flow (ml/min/100g)</td>
<td>33.90 ± 3.70</td>
<td>50.00 ± 3.30</td>
<td>68.00 ± 3.30</td>
<td>61.70 ± 6.00</td>
</tr>
</tbody>
</table>

\(^a\)Chatwin et al. (14).

\(^b\)Linzell (76).

If my values for rat mammary gland blood flow during late pregnancy and early lactation agree with Linzell's data, then I can be confident that the xenon clearance method is a valid technique for measuring rat mammary gland blood flow.
METHODS

Treatment of Animals

Sprague-Dawley-Rolfsmeyer rats were used in this study. Pregnant rats were obtained by placing five adult females and one adult male in a large cage for breeding. The presence of sperm in the vaginal smear was considered day 1 of pregnancy. Pregnant animals were housed individually and were either sacrificed on day 21 or permitted to continue to term and sacrificed on the first, third or fifth day of lactation. Litters were standardized to 8 pups per litter. Experimental rats were ovariectomized or ovariectomized and hypophysectomized and given the appropriate hormonal therapy to simulate late pregnancy or early lactation. The recovery time between each operation and the initiation of hormonal therapy was six days.

All animals were provided with Wayne Lab Blox and tap water or 5% glucose solution for hypophysectomized rats ad libitum. The temperature was controlled at $25^\circ C \pm 3^\circ$ and the lighting was regulated with light hours between 8:00 a.m. and 10:00 p.m.

Surgical Procedures

Bilateral ovariectomy was performed using the ventro-lateral approach. The rat was anaesthetized with ether, the body wall opened, the uterus ligated at the junction of the oviduct, ovaries removed, abdominal muscles sutured, and wound clips applied to the skin.
Hypophysectomy was performed by the intra-auricular method, using the Hoffman-Reiter Hypophysectomizer stereotaxic device. The ear canal was swabbed with 70% ethanol. The pinna was snipped above and below the ear canal to facilitate positioning of the ear bars. An 18 gauge, thin walled, wide beveled needle attached to a 10 ml syringe was inserted through the right ear canal and guided to a position above the sella turcica. The pituitary was removed by aspiration (147). Upon sacrifice, the sella turcica and any overlying tissue was fixed in 10% buffered formalin and stored in 70% ethanol for histological examination (55) as an indication of effectiveness of hypophysectomy (Appendix I).

Hormone Preparations

**Estrogen-Progesterone (EP)**

A stock estrogen solution of 1.0 mg of estradiol benzoate per 1.0 ml of carrier was prepared. The carrier was 5.0% benzyl alcohol and 95.0% Mazola Corn Oil. To prepare the estrogen-progesterone solution for injection, 1.0 ml of stock estrogen was diluted with 99 ml of carrier. Three grams of progesterone was dissolved in this mixture. To enhance dissolving, the mixture was continually stirred at 35°C for four hours. The quantity injected was 1 μg E + 3 mg P in 0.1 ml corn oil.

**Pituitary hormones: bovine prolactin (PRL) and bovine growth hormone (GH)**

Although these hormones are usually provided in bulk they are unstable in saline, therefore it is best to prepare them in small quantities, usually 50 mg at a time every three days or as needed.
Prolactin (NIH-P-B4) and growth hormone (NIH-GH-B17) was dissolved in less than 5 ml of .9% saline adjusted to pH of 9.0-9.5 to facilitate solubility, then filled to volume (5 ml). The injected concentration was 1 mg PRL or 1 mg GH in 0.1 ml of alkaline saline.

Hydrocortisone acetate (F)

The suspension medium was mixed by adding 1 drop of Tween 80 per 5 ml of saline adding benzyl alcohol as a preservative; 0.9 ml per 100 ml of saline. Hydrocortisone was placed in a tissue homogenizer with a small portion of the required volume of suspending medium and ground several times to ensure complete suspension (about 15-30 minutes). The suspension was then poured into a storage bottle. The tissue homogenizer was washed several times with the remaining suspending medium and the washings added to the storage bottle. The solution was shaken before each injection to maintain the suspension. The animal was given 250 μg F in 0.1 ml saline.

Treatment groups and symbols used to identify them are listed in Table 3. Subcutaneous injections of the hormones were given daily.

Mammary Gland Nucleic Acid Analysis

DNA and RNA analysis was performed on the six abdominal-inguinal rat mammary glands. The procedure is described in detail in Appendix II.
<table>
<thead>
<tr>
<th>Group symbol</th>
<th>Surgical procedure</th>
<th>Hormonal treatment</th>
<th>Day of sacrifice</th>
</tr>
</thead>
<tbody>
<tr>
<td>$EP_{20}$</td>
<td>Ovariectomy</td>
<td>$1\mu gE-3mgP \text{ (19d.)}^a$</td>
<td>21</td>
</tr>
<tr>
<td>$P_{21}$</td>
<td>None</td>
<td>None</td>
<td>21</td>
</tr>
<tr>
<td>HPS</td>
<td>Ovariectomy and hypophysectomy</td>
<td>$1\mu gE-3mgP \text{ (10d.)} + 1mgGH-1mgPRL-250\mu gF \text{ (10d.)}$</td>
<td>21</td>
</tr>
<tr>
<td>HPU</td>
<td>Ovariectomy and sham-hypophysectomy</td>
<td>$1\mu gE-3mgP \text{ (10d.)} + 1mgGH-1mgPRL-250\mu gF \text{ (10d.)}$</td>
<td>21</td>
</tr>
<tr>
<td>$L_1$</td>
<td>None</td>
<td>None</td>
<td>23</td>
</tr>
<tr>
<td>$L_3$</td>
<td>None</td>
<td>None</td>
<td>25</td>
</tr>
<tr>
<td>$L_{-5}$</td>
<td>None</td>
<td>None</td>
<td>27</td>
</tr>
<tr>
<td>HLS</td>
<td>Ovariectomy and hypophysectomy</td>
<td>$1\mu gE-3mgP \text{ (10d.)} + 1mgGH-1mgPRL-250\mu gF \text{ (10d.)} + 1mgGH-1mgPRL-250\mu gF \text{ (3d.)}$</td>
<td>24</td>
</tr>
</tbody>
</table>

$^a$Consecutive days injected.
Clearance rate ($T_{1/2}$)

Xenon-133 was purchased from Amersham-Searle Corporation. It was dissolved in isotonic saline and contained in a 1 ml air-free cartridge syringe, 10 mC/ml. An injection dose of 0.5 mC or 0.05 ml was sufficient to detect its elimination from the mammary gland. Xenon comes out of solution very rapidly when exposed to air. To prevent evaporation of xenon from the syringe between animal injections, the needle was forced through a rubber pharmaceutical stopper into a holding vial saturated with xenon. To prevent evaporation overnight, the needle was dipped into melted beeswax and allowed to cool forming a plug in and around the bevel of the needle. The beeswax could be removed without dulling the needle by briefly warming the tip in hot water.

All animals were anaesthetized with Nembutal (40 mg/kg B.W.). A mid-ventral and lateral incision was made. The flap of skin was pulled aside to expose the left abdominal-inguinal mammary glands. The xenon was injected directly into the first left abdominal-inguinal mammary gland close to the superficial epigastric artery. A 0.5 inch, 26 gauge needle was used and inserted at an oblique angle into the mammary gland to a depth of about 3 mm. The needle was withdrawn, the flap of skin closed with a wound clip, the area of the mammary gland was centered under the detector and the recording of the elimination rate began within 45 seconds of the initial injection (Fig. 7).
Fig. 7. Detection equipment for the measurement of the mammary gland xenon clearance rate
The elimination rate of the injected xenon was followed by an external scintillation detector with a 5 x 5 cm NaI(Tl) crystal. A rectangular lead collimator was used with an inner slit of 2 x 4 cm and a length of 11 cm from the crystal to the aperture. The rat was positioned such that the area of the mammary gland was centered under and was a total of 14 cm from the detector. The scintillation detector was coupled to a single channel analyzer to record the counting rate of the 81 KeV gamma radiation of xenon. The single channel analyzer was connected to a programmable printer lister. The clearance rate was continually recorded for 25 minutes. A counting interval of 12 seconds was chosen. A semilogarithmic plot of the counts per 12 second interval versus time was drawn and the slope ($T_{1/2}$) of the line determined.

**Partition coefficient ($\lambda$)**

To determine the partition coefficient, I modified the method reported by Andersen and Ladefoged (3). Five animals were housed individually in an air-tight environmental chamber containing atmospheric air and between 5-10 mC of xenon purchased from New England Nuclear (Fig. 8). All animals were provided with Wayne Lab Blox and tap water or 5% glucose solution for hypophysectomized rats *ad libitum*. The temperature in the chamber was 27°C and continuous lighting was maintained. For safety purposes, a negative pressure was maintained within the chamber at all times. Because the rats remained in the chamber for 24 hours to equilibrate with the xenon, the surplus carbon
Fig. 8. Air-tight chamber for the measurement of the mammary gland: blood partition coefficient for xenon
dioxide was absorbed by soda lime and water vapor with drierite. In order to both maintain a negative pressure and keep the animals alive within the chamber, the flow rate of oxygen (95% O₂, 5% CO₂) supplied was approximated from rat metabolic rates reported by Brody (11):

<table>
<thead>
<tr>
<th></th>
<th>Approximate oxygen consumption rate (ml/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virgin</td>
<td>3.8</td>
</tr>
<tr>
<td>Pregnant</td>
<td>5.3</td>
</tr>
<tr>
<td>Lactating</td>
<td>4.5</td>
</tr>
<tr>
<td>Pup</td>
<td>0.5</td>
</tr>
<tr>
<td>Hypophysectomized</td>
<td>3.5</td>
</tr>
</tbody>
</table>

To avoid loss of xenon from the samples to the air during manipulations, all procedures were carried out in the chamber from outside with the aid of air-tight gloves. To permit these manipulations, the pressure in the chamber was increased slightly with an external carbon dioxide source.

After living in the chamber for 24 hours, the animals were anaesthetized with Nembutal. Blood and liver samples weighing 2.0-3.0 g and mammary gland samples weighing 1.0-2.0 g were taken and placed in tared 0.5 dram glass vials. The vials were topped with xenon-equilibrated water to remove all air bubbles from the vials and tightly sealed with teflon coated caps to prevent evaporation. Glass instead of plastic vials were used to minimize the residual xenon contamination (127). A small sample of blood was withdrawn into heparinized capillary tubes to determine the hematocrit.

After a 24 hour period, the chamber was vented into a radioactive hood, the vials were removed and finally the radioactivity was measured in a well-scintillation counter with a 10 x 10 cm NaI(Tl) crystal and
well dimensions 1.9 cm in diameter and 6.5 cm deep. The detector was coupled to a single channel analyzer in coincidence with a 400 channel multichannel analyzer so everything falling within the window could be visually monitored. To determine the actual counts contributed by the tissue, the activity due to the water and the residual glass contamination in the vial were subtracted from the total counts. At least 10,000 net tissue counts were recorded.

The partition coefficient was obtained from counts/min/g tissue divided by counts/min/g blood, the sample values being the mean value of all that tissue type taken from an individual rat. The partition coefficients (g/g) were calculated from 50% hematocrit (3) according to:

$$
\lambda_{Hct_x} = \lambda_{Hct_{50}} \frac{0.0112 \cdot Hct_x + 1.02}{1.58}
$$

**Blood flow calculation**

The blood flow was calculated from the clearance constant (k) and the hematocrit corrected partition coefficient (62) according to:

$$
\text{Blood flow (ml/min/g tissue)} = k \lambda \\
\text{where } k = \frac{-693}{T_{1/2}}
$$

**Statistics**

The effect of each treatment on mammary gland DNA, RNA and blood flow was tested for statistical significance by Student's t test using a pooled variance.
RESULTS

For the purpose of clarity in reporting the results, the data are presented in the following sections:

1. Body weights and mammary gland weights
2. Mammary gland DNA
3. Mammary gland RNA
4. Mammary gland blood flow

The statistical comparisons reported for each parameter of mammary gland development are as follows:

1. Ovariectomized, estrogen-progesterone treated (EP$_{20}$) animals and ovariectomized, sham-hypophysectomized simulated pregnant (HPU) animals versus normal pregnant (P$_{21}$) controls, i.e.,

   \[ \text{EP}_{20} \text{ and HPU versus P}_{21} \]

2. Ovariectomized, hypophysectomized simulated pregnant (HPS) and lactating (HLS) animals versus normal pregnant (P$_{21}$) and lactating (L$_{1}$) controls, i.e.,

   \[ \text{HPS and HLS versus P}_{21} \text{ and L}_{1} \]

3. \[ \text{HPU versus HPS} \]

4. \[ \text{HLS and L}_{1} \text{ versus HPS and P}_{21} \]

After histological examination of the sella turcica to verify the completeness of hypophysectomy, a comparison was made between animals with microscopic amounts of pituitary present (HL?) and those with no
pituitary present (HLS) with respect to body weights and mammary gland dried fat-free tissue weights (Table 4), DNA parameters (Table 5) and RNA parameters (Table 6). There was no significant difference between these two groups in any parameter compared, therefore, the data from these two groups were combined into one group and designated as successfully hypophysectomized (HLS) animals.

Table 4. Effect of hormonal treatment on body weight and mammary gland DFFT in complete and partially hypophysectomized rats

<table>
<thead>
<tr>
<th>Group</th>
<th>n^a</th>
<th>B. W.^b(g) (mean)</th>
<th>DFFT^c(mg) (mean)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLS</td>
<td>7</td>
<td>212</td>
<td>450</td>
</tr>
<tr>
<td>HL?</td>
<td>9</td>
<td>224</td>
<td>576</td>
</tr>
</tbody>
</table>

^aNumber of animals per group.

^bBody weight.

^cDried fat-free tissue weight.
Table 5. Effect of hormonal treatment on mammary gland DNA in complete and partially hypophysectomized rats

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>µg DNA mg DFFT (mean+s.e.(^a))</th>
<th>Total mg DNA (mean+s.e.)</th>
<th>mg DNA 100g B.W. (mean+s.e.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLS</td>
<td>7</td>
<td>19.45 ± .37</td>
<td>8.80 ± 1.16</td>
<td>4.10 ± .38</td>
</tr>
<tr>
<td>HL?</td>
<td>9</td>
<td>18.48 ± .70</td>
<td>10.48 ± 0.79</td>
<td>4.66 ± .29</td>
</tr>
</tbody>
</table>

\(^a\)Standard error.

Table 6. Effect of hormonal treatment on mammary gland RNA in complete and partially hypophysectomized rats

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Total mg RNA (mean+s.e.)</th>
<th>mg RNA 100g B.W. (mean+s.e.)</th>
<th>RNA DNA (mean+s.e.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLS</td>
<td>7</td>
<td>19.96 ± 3.92</td>
<td>9.20 ± 1.52</td>
<td>2.18 ± .21</td>
</tr>
<tr>
<td>HL?</td>
<td>9</td>
<td>27.60 ± 3.40</td>
<td>12.22 ± 1.32</td>
<td>2.56 ± .13</td>
</tr>
</tbody>
</table>
Body Weight and Mammary Gland Weight

**Final body weight** (Table 7)

Final body weights of \( \text{EP}_{20} \) (256 g) and \( \text{HPU} \) (259 g) animals were similar to those of normal pregnant controls (252 g). Body weights of \( \text{HPS} \) (228 g) and \( \text{HLS} \) (219 g) were less (\( P<.05, P<.001 \), respectively) than those of normal pregnant and lactating controls (252 g, 256 g, respectively). The \( \text{HPU} \) and \( \text{HPS} \) animals were given the same hormonal treatment but the average body weight of the latter group (228 g) was significantly less (\( P<.01 \)) than the former (259 g). There was no significant difference observed in body weight of \( \text{HLS} \) (219 g) and \( \text{L}_{1} \) (256 g) animals when compared with their pregnant equivalence, \( \text{HPS} \) and \( \text{P}_{21} \), (228 g, 252 g, respectively).

**Mammary gland dried fat-free tissue weight** (Table 7)

No significant difference was observed between the mammary gland dried fat-free tissue weights (DFFT) of \( \text{EP}_{20} \) (578 mg) animals and their pregnant controls (676 mg). But, the DFFT of \( \text{HPU} \) (813 mg) was much greater (\( P<.001 \)) than that of the same controls. DFFT weights of \( \text{HPS} \) (348 mg) and \( \text{HLS} \) (521 mg) animals were significantly less (\( P<.001 \)) than those of their corresponding pregnant and lactating controls (676 mg, 875 mg, respectively). The mammary glands of the \( \text{HPU} \) (813 mg) animals responded to the hormonal replacement to a much greater extent (\( P<.001 \)) than the \( \text{HPS} \) (348 mg) animals. DFFT weights of \( \text{HLS} \) (521 mg) and \( \text{L}_{1} \) (875 mg) lactators were greater (\( P<.01 \)) than those of their pregnant counterparts, \( \text{HPS} \) and \( \text{P}_{21} \), (348 mg, 676 mg, respectively).
Table 7. Effect of treatments on body weight and mammary gland DFFT

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>B.W.(g) (mean)</th>
<th>DFFT(mg) (mean)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. EP\textsubscript{20}</td>
<td>10</td>
<td>256</td>
<td>578</td>
</tr>
<tr>
<td>2. P\textsubscript{21}</td>
<td>10</td>
<td>252</td>
<td>676</td>
</tr>
<tr>
<td>3. HPS</td>
<td>12</td>
<td>\textsuperscript{2-3} 228</td>
<td>\textsuperscript{2-3} 348</td>
</tr>
<tr>
<td>4. HPU</td>
<td>6</td>
<td>\textsuperscript{3-4} 259</td>
<td>\textsuperscript{3-4} 813</td>
</tr>
<tr>
<td>5. L\textsubscript{1}</td>
<td>12</td>
<td>256</td>
<td>\textsuperscript{2-5} 875</td>
</tr>
<tr>
<td>6. L\textsubscript{3}</td>
<td>13</td>
<td>276</td>
<td>871</td>
</tr>
<tr>
<td>7. L\textsubscript{5}</td>
<td>12</td>
<td>272</td>
<td>1006</td>
</tr>
<tr>
<td>8. HLS</td>
<td>16</td>
<td>\textsuperscript{5-8} 219</td>
<td>\textsuperscript{5-8} 521</td>
</tr>
</tbody>
</table>

\(\dagger\) denotes significance at the .05 level.
\(\S\) denotes significance at the .01 level.
\(\ast\) denotes significance at the .001 level.
Mammary Gland DNA

DNA per mg DFFT, total mg DNA, and mg DNA per 100 g body weight (Table 8, Figs. 9, 10, 11)

No significant differences were observed for any of the DNA parameters between EP20 and HPU animals and their normal pregnant controls. The number of cells per unit area (μg DNA/mg DFFT) and total number of cells (total DNA) of the HPS and HLS animals were significantly less (P<.01) than their corresponding normal pregnant and lactating controls. The mammary glands of the HPU animals were more sensitive to the hormonal therapy (P<.025) than the HPS animals for all the DNA parameters. Although a significantly smaller (P<.001) μg DNA/mg DFFT was demonstrated for HLS and L1 animals when compared with their pregnant counterparts, HPS and P21, there was no significant difference between comparisons of these same groups in relation to total DNA and mg DNA/100 g B. W.
Table 8. Effect of treatments on mammary gland DNA

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>( \text{ug DNA mg DFFT} ) (mean+s.e.)</th>
<th>Total mg DNA (mean+s.e.)</th>
<th>( \text{mg DNA 100g B.W.} ) (mean+s.e.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. EP20</td>
<td>10</td>
<td>32.97 ± 1.28</td>
<td>18.65 ± 1.05</td>
<td>7.27 ± 0.28</td>
</tr>
<tr>
<td>2. P21</td>
<td>10</td>
<td>29.97 ± 0.99</td>
<td>19.96 ± 1.23</td>
<td>7.99 ± 0.49</td>
</tr>
<tr>
<td>3. HPS</td>
<td>12</td>
<td>23.54 ± 0.97&lt;sup&gt;2-3&lt;/sup&gt;</td>
<td>8.20 ± 0.57&lt;sup&gt;2-3&lt;/sup&gt;</td>
<td>3.60 ± 0.24&lt;sup&gt;2-3&lt;/sup&gt;</td>
</tr>
<tr>
<td>4. HPU</td>
<td>6</td>
<td>28.10 ± 1.48&lt;sup&gt;3-4&lt;/sup&gt;</td>
<td>22.59 ± 0.99&lt;sup&gt;3-4&lt;/sup&gt;</td>
<td>8.72 ± 0.28&lt;sup&gt;3-4&lt;/sup&gt;</td>
</tr>
<tr>
<td>5. L1</td>
<td>12</td>
<td>22.20 ± 0.62&lt;sup&gt;2-5&lt;/sup&gt;</td>
<td>19.26 ± 0.68</td>
<td>7.54 ± 0.24</td>
</tr>
<tr>
<td>6. L3</td>
<td>13</td>
<td>25.54 ± 0.81</td>
<td>22.18 ± 0.86</td>
<td>8.04 ± 0.30</td>
</tr>
<tr>
<td>7. L5</td>
<td>12</td>
<td>24.52 ± 0.44</td>
<td>24.57 ± 0.94</td>
<td>9.06 ± 0.31</td>
</tr>
<tr>
<td>8. HLS</td>
<td>15</td>
<td>18.90 ± 0.43&lt;sup&gt;5-8&lt;/sup&gt;</td>
<td>9.75 ± 0.68&lt;sup&gt;5-8&lt;/sup&gt;</td>
<td>4.42 ± 0.24&lt;sup&gt;5-8&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

+ denotes significance at the .05 level.

§ denotes significance at the .01 level.

* denotes significance at the .001 level.
Fig. 9. DNA on a per mg DFFT basis
Fig. 10. Total DNA content of the six abdominal-inguinal mammary glands
Fig. 11. DNA on a per 100 g body weight basis
Mammary Gland RNA

Total mg RNA, mg RNA per 100 g body weight, and RNA/DNA ratio (Table 9, Figs. 12, 13, 14)

The mammary gland RNA parameters of the EP20 animals were not significantly different from their normal pregnant controls. However, these same parameters were significantly higher (P<.025) in HPU animals when compared to the same controls. Significantly lower values (P<.001) were observed for the RNA parameters of the HPS and HLS animals when compared with their respective normal pregnant and lactating controls. In comparing the RNA of HPU with HPS animals, the mammary glands of the former group were much more responsive (P<.001) to the hormonal replacement than the latter group. The RNA parameters of HLS and L1 animals were universally greater (P<.001) than their pregnant equivalence, HPS and P21.

Mammary Gland Blood Flow

A representative clearance curve for each group considered in this study, plotted in a semilogarithmic diagram, is seen in Fig. 15. In order to include all groups on the same plot, an arbitrary log scale is used. To be certain that these clearance curves represent blood flow carrying xenon away from the mammary gland, an animal was sacrificed prior to injection and the disappearance of the xenon was followed. The xenon remained within the mammary gland indicating that I was measuring a component of blood flow. A closer look at one of these curves (Fig. 16) reveals that it can be analyzed into two components, a fast
Table 9. Effect of treatments on mammary gland RNA

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Total RNA (mean+s.e.)</th>
<th>RNA 100g B.W. (mean+s.e.)</th>
<th>DNA (mean+s.e.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. EP₂₀</td>
<td>10</td>
<td>30.23 ± 3.10</td>
<td>11.66 ± 1.02</td>
<td>1.59 ± .11</td>
</tr>
<tr>
<td>2. P₂₁</td>
<td>10</td>
<td>36.74 ± 2.86</td>
<td>14.65 ± 1.02</td>
<td>1.84 ± .06</td>
</tr>
<tr>
<td>3. HPS</td>
<td>12</td>
<td>11.45 ± 0.92</td>
<td>5.06 ± 0.44</td>
<td>1.39 ± .05</td>
</tr>
<tr>
<td>4. HPU</td>
<td>6</td>
<td>50.07 ± 4.26</td>
<td>19.17 ± 1.17</td>
<td>2.19 ± .11</td>
</tr>
<tr>
<td>5. L₁</td>
<td>12</td>
<td>59.26 ± 3.45</td>
<td>23.06 ± 1.02</td>
<td>3.06 ± .10</td>
</tr>
<tr>
<td>6. L₃</td>
<td>13</td>
<td>63.36 ± 4.89</td>
<td>23.03 ± 1.83</td>
<td>2.84 ± .17</td>
</tr>
<tr>
<td>7. L₅</td>
<td>12</td>
<td>86.12 ± 4.34</td>
<td>31.94 ± 1.82</td>
<td>3.51 ± .14</td>
</tr>
<tr>
<td>8. HLS</td>
<td>16</td>
<td>24.26 ± 2.67</td>
<td>10.90 ± 1.04</td>
<td>2.39 ± .12</td>
</tr>
</tbody>
</table>

† denotes significance at the .05 level.
§ denotes significance at the .01 level.
* denotes significance at the .001 level.
Fig. 12. Total RNA content of the six abdominal-inguinal mammary glands
Fig. 13. RNA on a per 100 g body weight basis
Fig. 14. RNA/DNA ratio
Counts per 12 sec.
(arbitrary log scale)

Fig. 16. Two component system

Time in min.

$T_1 = 1.5 \text{ min}$

$T_2 = 16.60 \text{ min}$
(1.5 min) and a slow (16.60 min) component. The fast component represents the diffusion of xenon from the injection site into the tissue and the slow component represents a factor of blood flow, the clearance rate.

**Clearance rate, partition coefficient, and blood flow (Table 10, Figs. 17, 18, 19)**

Although there was no significant difference observed for the clearance rate, the partition coefficient and blood flow were significantly lower ($P<.01$) for $E_{gg}$ animals when compared with normal pregnant controls. The clearance rate and blood flow of HPU animals were similar to those of normal pregnant controls. A significantly slower clearance rate and a correspondingly slower blood flow ($P<.001$) were observed for HPS and HLS animals when compared with their normal pregnant and lactating controls. Again, the clearance rate and blood flow data indicate that the HPU animals are more sensitive ($P<.001$) to the hormonal replacement than the HPS animals. Although there was no significant difference observed between the HLS and HPS animals with respect to clearance rate and blood flow, there was a much faster (P<.001) clearance rate and blood flow for the normal lactating animals when compared with the normal pregnant animals.
Table 10. Effect of treatments on mammary gland blood flow

<table>
<thead>
<tr>
<th>Group</th>
<th>$T_e$ in min (mean±s.e.)</th>
<th>$\lambda$ (mean±s.e.)</th>
<th>B.F. ml/min/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. EP$_{20}$</td>
<td>17.06 ± 0.78(21)</td>
<td>6.10 ± .44(4)</td>
<td>.248 ± .011</td>
</tr>
<tr>
<td>2. P$_{21}$</td>
<td>15.58 ± 1.40(10)</td>
<td>8.30 ± .48(6)</td>
<td>.369 ± .033</td>
</tr>
<tr>
<td>3. HPS</td>
<td>32.72 ± 3.27(12)$^{*2-3}$</td>
<td>8.51 ± .37(7)</td>
<td>.180 ± .018</td>
</tr>
<tr>
<td>4. HPU</td>
<td>13.42 ± 1.56(6)$^{*3-4}$</td>
<td>d.51 ± .37(7)</td>
<td>.439 ± .051</td>
</tr>
<tr>
<td>5. L$_1$</td>
<td>7.68 ± 0.85(12)$^{*2-5}$</td>
<td>7.50 ± .32(4)</td>
<td>.677 ± .075</td>
</tr>
<tr>
<td>6. L$_3$</td>
<td>6.59 ± 0.95(12)</td>
<td>5.14 ± .39(7)</td>
<td>.540 ± .078</td>
</tr>
<tr>
<td>7. L$_5$</td>
<td>7.68 ± 0.80(12)</td>
<td>4.61 ± .17(6)</td>
<td>.416 ± .043</td>
</tr>
<tr>
<td>8. HLS</td>
<td>29.23 ± 2.94(16)$^{*5-8}$</td>
<td>8.34 ± .34(10)</td>
<td>.198 ± .023</td>
</tr>
</tbody>
</table>

(#{}) number animals per group

‡ denotes significance at the .05 level.
§ denotes significance at the .01 level.
* denotes significance at the .001 level.
Fig. 17. Clearance rate
Fig. 18. Partition coefficient
Blood Flow in ml/min/g

Fig. 19. Blood flow
DISCUSSION

It has been suggested that a complex of hormones consisting of estrogen, progesterone, growth hormone, prolactin and hydrocortisone, stimulates lobulo-alveolar proliferation in ovariectomized, hypophysectomized rats comparable to that found in late pregnancy. Furthermore, by removing estrogen and progesterone stimulation but continuing treatment with growth hormone, prolactin and hydrocortisone, lactation is induced (15, 19, 81). I repeated these experiments using quantitative indices of mammary gland growth, i.e., DNA-RNA analysis and blood flow measurements rather than the qualitative measure of histological examination of whole mount tissue.

The optimum concentration of each hormone used in this study was chosen from several previous studies reported in the literature:

1. 1µgE + 3mgP (94)
2. 1-2mgGH + 1-2mgMH (46)
3. 250µgF (4)

Although it has been suggested that 2µg:6mg is a better combination of estrogen and progesterone for synergism with other hormones than 1µg:3mg (46, 94), I wanted to duplicate the work of Chen et al. (15) and Cole and Hopkins (19), who used 1µg:4mg, and Lyons et al. (81), who used 1µg:2mg; therefore the combination of 1µg:3mg was chosen for this study.

An important result of this research is that total hypophysectomy is not necessary. A microscopic fragment of pituitary tissue may remain
and the animal still responds as if completely hypophysectomized. However, if visual amounts of pituitary tissue remain, the animals respond as if sham-operated.

Mammary Gland DNA and RNA

Since no significant difference was observed, with reference to any of the DNA or RNA parameters, between ovariectomized, estrogen-progesterone (EP20) treated animals and their pregnant controls, I concluded that the estrogen-progesterone treatment was optimum for mammary gland proliferation comparable to late pregnancy. However, this same degree of growth was not stimulated with the five hormone complex administered to ovariectomized, hypophysectomized rats, although some development of the mammary gland was achieved. The DNA values of the equivalent sham-hypophysectomized animals did demonstrate successful stimulation of mammary gland growth similar to late pregnancy, while the synthetic capability of the mammary gland was even greater than the controls. Therefore, I concluded that the pituitary replacement therapy was not adequate for mammary gland growth equivalent to late pregnancy. From a similar comparison, with respect to DNA and RNA data, between simulated lactating (HLS) animals and their normal lactating controls, the same conclusion can be drawn: that the pituitary replacement therapy was inadequate for mammary gland proliferation similar to early lactation. It was demonstrated, however, that the DNA and RNA values of the simulated lactating (HLS) animals
when compared with the simulated pregnant (HPS) rats paralleled a similar comparison between the normal lactating and pregnant animals, as indicated below.

<table>
<thead>
<tr>
<th></th>
<th>ug DNA DFFT</th>
<th>Total DNA</th>
<th>Total RNA</th>
<th>RNA DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>P21 vs L1</td>
<td>26%*</td>
<td>N.S.</td>
<td>38%*</td>
<td>40%+</td>
</tr>
<tr>
<td>HPS vs HLS</td>
<td>22%*</td>
<td>N.S.</td>
<td>53%*</td>
<td>42%+</td>
</tr>
</tbody>
</table>

where a decrease in ug DNA/DFFT indicates an increase in cell size and total DNA represents cell number.

The DNA and RNA absolute values of the simulated (HPS, HLS) animals were less than their normal controls (P21, L1). However, the percent increase in cell size and synthetic capability of the normal lactating animals over the normal pregnant animals was the same as the percent increase in DNA and RNA of the simulated lactating animals over the simulated pregnant animals. Therefore, the cessation of estrogen and progesterone administration did imitate the events occurring during parturition.

Because mammary growth was stimulated to some degree in HPS rats when compared with virgin animals (110) and the HLS animals did secrete milk, it is possible that qualitatively, by histological examination, there is no difference between the simulated and normal animals although quantitatively there is a definite difference.

One criticism that might explain the disagreement between the quantitative and qualitative results might be that Chen, Lyons and Cole all used Long Evans rats while I used Sprague-Dawley animals.
However, mammary gland DNA and RNA studies in different rat strains (51, 92, 120, 132) have demonstrated no difference between strains. Suspecting that the five hormone complex might not be adequate to stimulate mammary gland proliferation equivalent to late pregnancy, blood flow was measured to determine if it could be a limiting factor in mammary gland development.

**Blood Flow**

Of all the methods available for measuring rat mammary gland blood flow, the least complicated appeared to be the clearance method proposed by Kety (62). This technique permitted the injection of a tracer, i.e., Xenon-133, directly into the mammary gland thus avoiding the difficulty of isolating a single mammary gland blood vessel.

To determine blood flow by measuring the disappearance rate \( T_{1/2} \) of a locally injected indicator, two assumptions must be met. The first assumption, that there is no difference in the solubility of the indicator in blood and in tissue, is met by the determination of a correction factor, the partition coefficient \( \lambda \). The second assumption, that there are no arterio-venous shunts through the tissue, is dismissed by Linzell's (77) description of the mammary gland capillary network. With these assumptions fulfilled:

\[
\text{Blood flow} = \frac{-0.693}{T_{1/2}} \lambda = k\lambda
\]

where there is no recirculation of the indicator through the tissue as is the case with Xenon-133.
Although the clearance rates were not difficult to measure, there was tremendous variability in the results. Even so, the same trends were noticed when comparing treatments with respect to clearance rates as were apparent with the DNA and RNA data. The clearance rate of the ovariectomized, estrogen-progesterone treated (EP<sub>20</sub>) animals was similar to late pregnancy indicating that the estrogen-progesterone treatment was a sufficient substitute for the ovaries. The clearance rates of the simulated pregnant and lactating (HPS, HLS) animals were slower than their normal pregnant and lactating controls while the sham-hypophysectomized simulated pregnant (HPU) group returned to the control levels lending further support to the view that the pituitary hormonal replacement was insufficient.

The partition coefficient was not as easily obtained as the clearance rate. Conn (20) and Andersen and Ladefoged (3) independently measured the partition coefficient for xenon in different tissues of the dog, rabbit and rat but not in the rat mammary gland. For most tissues, they found a partition coefficient of 0.7 and this figure is used by most investigators measuring blood flow by the xenon clearance method. However, adipose tissue had a value of 10.0 indicating a much higher solubility of xenon in lipid. This complicated matters because as the mammary gland develops, it absorbs the surrounding fat pad. This means that there is a continual change in the fat content of the mammary gland throughout its development. Therefore, whenever the clearance rate is determined the partition coefficient must be measured in order to calculate the blood flow for that stage of development.
Because I substantially modified Andersen and Ladefoged's (3) procedure for determining the partition coefficient, I measured the xenon solubility in the liver as well as in the mammary gland to verify my technique. My value for the liver partition coefficient was 0.9. It has been demonstrated that there is an inverse linear relationship between xenon solubility and temperature (69). Andersen and Ladefoged (3) measured partition coefficients at 37°C while my measurements were made at 27°C. After making the appropriate correction for temperature difference, our liver partition coefficients were in agreement. Therefore, I am confident that the mammary gland partition coefficients for the different stages of growth are at least a good first approximation, but the number of samples was small and the variability was great.

After calculating the blood flow from the clearance rates and partition coefficients, it was again apparent that there was a deficiency in the hormonal replacement of the anterior pituitary. The blood flow in the simulated pregnant and lactating (HPS, HLS) groups were substantially slower than in their corresponding normal pregnant and lactating controls, whereas, the blood flow of the sham-hypophysectomized simulated pregnant (HPU) animals was not significantly different from the pregnant controls. Although there was a significant difference in blood flow between the ovariectomized, estrogen-progesterone treated (EP₂₀) animals when compared with the normal pregnant rats, this may be because of a possible underestimation of the partition coefficient due to the small number of determinations and large variability in the data.
An examination of the data revealed a dramatic increase in blood flow from late pregnancy to early lactation. This agrees with similar data reported by Chatwin et al. (14) and Linzell (76) (see Table 2). Although we used very different methods in measuring mammary gland blood flow, our values do agree and this lends support to the validity of the results from the simulated animals.

An unexpected result of this research was the decrease in blood flow noted from day 1 through day 5 of lactation. Since there was no difference in mammary gland DNA or RNA from day 1 to day 3 and only an increase in the synthetic capability of the mammary gland from day 3 to day 5 but not in proliferation, I would have expected no change in the blood flow. The clearance rates of these animals were constant but the partition coefficients decreased. As a result, the blood flow also decreased. It is probable that the partition coefficients are a good first estimation but further measurements might reveal that there is no difference in these partition coefficients and therefore no actual difference in blood flow from day 1 through day 5 of lactation.

The results of this study demonstrate that the estrogen-progesterone replacement of the ovary is satisfactory but the prevalent hormonal replacement of the anterior pituitary, i.e., growth hormone, prolactin and hydrocortisone, is by no means adequate to stimulate mammary gland development equivalent to late pregnancy or early lactation. Something is missing in this therapy that does not imitate a relationship that must exist between the anterior pituitary, blood flow and mammary gland proliferation. Various possibilities are mentioned below.
1. Blood flow may be a limiting factor in mammary gland development. Because the concentration of the hormones injected daily was greater than the daily secretion of these hormones from the anterior pituitary, it is possible that an inadequate blood supply could prevent these hormones from reaching the mammary gland thereby retarding growth. An experiment contemplated for the future is to cause either general vasodilation, by injection of a drug such as hydralazine, or local vasodilation, by application of a heat source directly over the mammary gland, in ovariectomized, hypophysectomized simulated pregnant and lactating rats to see if we can increase the DNA and RNA content of the mammary glands to that of a normal late pregnant animal. This might demonstrate the limiting nature of blood flow.

2. Autoregulation may be involved. As the mammary gland proliferates and its metabolic activity increases, more carbon dioxide is produced causing local vasodilation. This would allow more nutrients and hormones to reach the mammary gland causing further growth.

3. Hormones may cause mammary parenchymal growth and coincidentally proliferation of the capillary network, since the parenchymal tissue is essential for capillary expansion. The hormones would then indirectly cause an increase in blood supply to the mammary gland and a feedback system would be activated.

4. Although the concentration of hormones injected daily may be greater than the daily secretion of these hormones from the anterior pituitary, when one considers the half-life of these hormones in the
circulation; growth hormone and prolactin being 20-30 minutes (25) and hydrocortisone is 60-90 minutes (39), it is possible that they may be removed from the system before they have their full stimulatory effect. Also, the vehicle used for administration of these hormones is saline. Saline, unlike corn oil the carrier for estrogen-progesterone, does not delay the absorption of hormones, therefore a constant level of these hormones in the circulation cannot be maintained. The timing of the hormone injections may also be important. Because prolactin has a semicircadian release with peaks at the end of the light and dark periods, it may be necessary to administer prolactin in the evening as well as in the morning.

5. Lastly, the number of hormone receptors in the mammary gland may be inadequate therefore, the mammary gland cannot respond to the hormonal therapy.
The present investigation was concerned with the induction of mammary gland proliferation equivalent to late pregnancy and early lactation in ovariectomized and ovariectomized, hypophysectomized Sprague-Dawley rats. To simulate mammary gland development of late pregnancy, ovariectomized rats were given daily subcutaneous injections of estrogen and progesterone for 20 days. The ovariectomized, hypophysectomized animals were injected with estrogen and progesterone for 10 days followed by estrogen, progesterone, growth hormone, prolactin and hydrocortisone for an additional 10 days to simulate a normal late pregnant mammary gland. Estrogen-progesterone stimulation was withdrawn while growth hormone, prolactin and hydrocortisone treatment continued for 3 more days to induce milk secretion.

The nucleic acid content of the six abdominal-inguinal mammary glands was estimated colorimetrically while blood flow through these glands was determined by the xenon clearance method. Both of these quantitative indices were used to assess the extent of mammary gland development.

The results reported here indicate the following:

1. Estradiol benzoate (1 µg) and progesterone (3 mg) replacement of the ovary is adequate to stimulate mammary gland development equivalent to late pregnancy.
2. Anterior pituitary hormones synergize with estrogen and progesterone to induce mammary gland development but the prevalent hormonal replacement of the anterior pituitary, growth hormone (1 mg), prolactin (1 mg), and hydrocortisone (250 µg), is not satisfactory in stimulating mammary gland growth of late pregnancy or early lactation.

3. Estrogen-progesterone withdrawal imitates the events that occur during parturition: an increase in synthetic capability of the mammary gland and initiation of lactation.

4. Blood flow may be a limiting factor in mammary gland development. It is possible that mammary gland proliferation and blood flow must increase simultaneously to permit further differentiation of the mammary gland:

- Stimulatory hormones reach the mammary gland.
- Blood flow increases through the mammary gland.
- Mammary gland proliferation occurs.

An important achievement presented in this manuscript is a much improved method for determining the mammary gland partition coefficient thus permitting the use of the xenon clearance method for measuring rat mammary gland blood flow.
BIBLIOGRAPHY


APPENDIX I

Histological Verification of Hypophysectomy

Dehydration

Each tissue was carried through a tertiary butyl alcohol (TBA) dehydration series. The tissue remained in each of the following solutions for one hour:

<table>
<thead>
<tr>
<th>Solution</th>
<th>Water (ml)</th>
<th>Ethanol (ml)</th>
<th>TBA (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>200</td>
<td>150 (95%)</td>
<td>50</td>
</tr>
<tr>
<td>2</td>
<td>120</td>
<td>200 (95%)</td>
<td>80</td>
</tr>
<tr>
<td>3</td>
<td>100</td>
<td>200 (95%)</td>
<td>100</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>200 (95%)</td>
<td>200</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>140 (95%)</td>
<td>200</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td>-</td>
<td>Pure</td>
</tr>
</tbody>
</table>

Infiltration and embedding

After dehydration, the tissue is ready for infiltration with paraplast, a mixture of paraffin and plastic polymers of regulated molecular weight. Each tissue was transferred through a series of paraplast solutions kept in a warming oven at 60°C. The tissue stayed in each solution for one hour.

<table>
<thead>
<tr>
<th>Solution</th>
<th>TBA</th>
<th>Paraplast</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>75%</td>
<td>25%</td>
</tr>
<tr>
<td>2</td>
<td>50%</td>
<td>50%</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>Pure</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>Pure</td>
</tr>
</tbody>
</table>

Following infiltration, the tissue was placed into a small mold containing fresh paraplast and allowed to cool. The embedded tissue was sectioned at 10 microns and placed on slides.
Solutions needed for staining

1. **Aqueous periodic acid**: 0.6 g periodic acid and 0.3 ml concentrated nitric acid is dissolved in 100 ml distilled water.

2. **Schiff's leuco-fuchsin**: 0.5-1.0 g basic fuchsin is dissolved in 85.0 ml distilled water. On the day of use, 1.9 g of sodium metabisulfite and 15.0 ml of 1N hydrochloric acid are added to the basic fuchsin solution. This is filtered through 200 g activated charcoal until the stain is water-white.

3. **Iron alum**: 5.0 g ferric ammonium sulfate is added to 100 ml of distilled water and is stored in a refrigerator.

4. **Harris' hematoxylin**: 1.0 g hematoxylin is dissolved in 10 ml ethyl alcohol. Dissolve 20 g potassium or ammonium alum in 200 ml water and bring to a boil. Add the former to the latter and boil for \(\frac{3}{4}\) minute. Add 0.5 g mercuric oxide and cool rapidly. Add a few drops of glacial acetic acid to keep away metallic luster.

5. **Orange G**: 2.0 g Orange G and 5.0 g phosphotungstic acid are dissolved in 100 ml distilled water. Mildly agitate overnight. Centrifuge and decant off the clear supernatant.

Staining procedure

The length of time the slides remain in each staining solution is only an approximation and depends on the final concentration of each stain. A trial run must be carried out each day to verify the timing.

1. Put slides in xylene, two changes, two minutes in each or until all the paraplast is removed.
2. Put through a descending alcohol series: 95%, 85%, 70%, 50%, and distilled water, about one minute in each solution.

3. Periodic acid...5 minutes.

4. Wash in running tap water...5 minutes.

5. Schiff's reagent...10 minutes.

6. Wash in running tap water...5 minutes and rinse in distilled water.

7. Iron alum...30 seconds.

8. Wash 3 times in distilled water.

9. Hematoxylin...dip once, rinse 5 times in distilled water. Check the slide and repeat if darker staining is necessary.

10. Orange G...15 seconds, wash in running tap water for 3 minutes. Check the slide and if further destaining is necessary keep in water for an additional few minutes.

11. Put through an ascending alcohol series: 70%, 85%, 95%, 100%, 100%...dip four times in each or until all water is removed from the surface of the slide.

12. Clear in xylene, 3 changes, and mount in clearmount.

Check the slides periodically under a microscope for staining intensity but be sure they do not dry completely. The stained elements should look brilliant because clearmount fades the color.
Preparation of dried fat-free tissue (DFFT)

Immediately after removal, the six abdominal-inguinal mammary glands are defatted in approximately 50 ml of chloroform:methanol (2:1 by volume) for two, twelve hour periods. The jars are kept under constant mild agitation. The fat-free mammary glands are then dehydrated in 50 ml of anhydrous ether for two additional twelve hour periods. After the final solvent is decanted, the tissues are air dried. After the DFFT is weighed, it is ground to a fine powder using a Wiley mill equipped with a number 20 sieve.

Nucleic acid extraction

The method used for nucleic acid extraction was that described by Schneider (116) and modified by Ferreri (33).

1. Twenty five mg of DFFT suspended in 5 ml of 5% trichloroacetic acid (TCA) in thick walled 15 ml centrifuge tubes is allowed to stand at room temperature until the DFFT becomes saturated (about two hours) and settles to the bottom of the tube with gentle tapping.

2. Samples are covered with glass marbles to prevent evaporation and placed in a hot water bath (90-95°C) for 15 minutes and then cooled in ice water.

3. Samples are centrifuged for 15 minutes and the supernatant is decanted into 12 ml volumetric centrifuge tubes.
4. A second extraction in 5 ml of hot TCA is made on the residue and the supernatant is combined with the decant from the first extraction.

5. The total volume is brought up to 10 ml by addition of cold 5% TCA, transferred to screw capped glass tubes and stored at 4°C.

**Colorimetric determination of DNA content**

The method used was that described by Burton (12) and modified by Ferreri (33).

1. Diphenylamine reagent is prepared on the day it is used. It consists of 1.5 g of reagent grade diphenylamine in 100 ml of A. R. glacial acetic acid and 1.5 ml of concentrated sulfuric acid. Just before use, 0.5 ml of aqueous acetaldehyde (16 mg/ml) is added to the reagent.

2. A stock solution of 1N perchloric acid was on hand: 9 ml of concentrated (70%) perchloric acid to 91 ml of distilled water.

3. One ml of nucleic acid extract or DNA standard is combined with 4 ml of diphenylamine reagent and 1 ml of 1N perchloric acid.

4. Three blanks are prepared each containing 1 ml of 5% TCA, 4 ml of diphenylamine reagent, and 1 ml of 1N perchloric acid.

5. Color is developed after 16-20 hours of incubation at room temperature in the dark, and absorbance is measured against the blank at 600 μm.

6. The concentration of DNA in the sample is calculated from the least squares estimate of the slope and Y intercept of the standard
Colorimetric determination of RNA

The method used was that described by Mejbaum and outlined by Schneider (117).

1. A stock solution of 0.5 g of FeCl\(_3\) per 100 ml of concentrated HCl is prepared. Immediately before use, 1 g of orcinol is added per 100 ml of this solution.

2. Five-tenths ml of nucleic acid or 0.5 ml of DNA or RNA standard is added to 2.0 ml of 5% TCA. To each tube, 2.5 ml of orcinol reagent is added.

3. Three blanks are prepared each containing 2.5 ml of 5% TCA and 2.5 ml of orcinol reagent.

4. The marble covered tubes are incubated in a hot water bath (95°C) for 20 minutes to develop the color, then plunged into ice water to halt the reaction. Absorbance is measured against the blank at 660 μm.

5. Standard curves (absorbance vs. concentration) and least squares estimations of the slopes and Y intercepts for both the RNA and DNA standards are made. Corrections for absorbance due to DNA are made for each sample using the concentrations of the samples as previously determined by the Burton procedure.
ACKNOWLEDGMENTS

I would like to thank Dr. David R. Griffith for his encouragement and supervision throughout the execution of this research problem. His enthusiasm in teaching and research is an inspiration to all of his graduate students. I am grateful to Dr. Lloyd L. Anderson for his awesome command of the literature pertaining to reproductive physiology which inspired me to wade through the unending collection of journals in our library. My gratitude is extended to Dr. Jerry W. Young for serving on my committee and for his excellent classroom instruction. I am indebted to Dr. Donald C. Beitz for allowing me to use his spectrophotometer and for his constructive comments when I needed them. I would like to express my appreciation to Dr. Yola N. Forbes for our many philosophical discussion over tea on teaching, research, and education, which have helped me immensely in forming my own attitudes.

This research problem could not have been accomplished without the assistance of Mr. Wayne A. Stensland, my consultant in the use of radioactive detection equipment, and to Dr. Adolf F. Voigt, his supervisor, for the use of that equipment. My health would not be the same had it not been for the scrupulous attention paid to me by Mr. Emery E. Sobottka and the Environmental Health and Safety Department.

Every project needs financial support and my thanks is extended to Dr. Normon L. Jacobson and the Graduate College for this financial assistance.
Lastly, I must thank my husband for putting up with sleepless nights, abandoned weekends and the general frustrations that surfaced over the past five years. His encouragement and support were and are infinite.