

HISTOLOGY OF BOVINE MAMMARY TISSUE
DURING ADVANCED STAGES OF INDUCED LACTATION,

by

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Thesis submitted to the Graduate Faculty of the
Virginia Polytechnic Institute and State University
in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE
in
Dairy Science

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May, 1974
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ACKNOWLEDGMENTS

Sincere appreciation is extended to the following people:

for his sound guidance and objective criticism throughout the author's graduate program and preparation of this thesis.

for his departmental support in providing an excellent environment, facilities and opportunity for this research, as well as his evaluation of this research.

and for their suggestions and evaluation of this research.

for his assistance in the statistical analysis of these data.

for her assistance and patience in the typing of this manuscript.

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INTRODUCTION

Each year a large number of cows are culled due to a combination of two factors: reproductive failure coupled with declining lactation. Techniques to artificially return these cows to milk production by administering exogenous hormones have been sought since the early 1930's. Induced lactation would be beneficial to the dairy industry by: (1) bring heifers into milk production six to twelve months earlier, (2) return open mature cows to milk production, (3) extend the period for breeding subfertile cows that are nearly dry or dry, (4) reduce heifer and calf losses resulting from early calving and (5) bring 15 to 20 month old heifers into the milking herd for better nutritional, reproductive and breeding management.

Recently, a moderately successful, short term (21 da) technique for inducing lactation was introduced (Smith et al., 1971). Lactation yields obtained by this technique were extremely variable. The objectives of our study were: (1) to estimate the success and variability of a modified induced lactation procedure, (2) to histologically describe mammary tissue during late stages of such hormonally induced lactation, (3) to determine if this variability is expressed at the cellular level and (4) to compare such tissue morphologically with reported changes that occur in mammary tissue during pregnancy, at the time of parturition and during the onset of lactation.

REVIEW OF RELATED LITERATURE

Normal progression of mammary development, lactogenesis and full milk production in many species requires the coordinated secretions of the pancreas, pituitary, ovaries and adrenals or the exogenous replacement of these secretions. The role of these hormones in mammary development is reviewed first for several laboratory species and then for farm animals.

I. Mammary Development in Laboratory Animals In Vivo as Affected by Estrogens and Progestins

Exogenous administration of synthetic and natural ovarian steroids stimulate mammary development in ovariectomized rats, guinea pigs and rabbits. Duct growth has been demonstrated with exogenous administration of estradiol benzoate in the guinea pig (Benson et al., 1957) and rat (Moon et al., 1959) and estradiol-17 β in the rabbit (Chatterton, 1971). In addition to duct growth, Smith and Richterich (1958) reported that exogenous administration of estradiol benzoate induces formation of lobules in the guinea pig. These findings conflicts with the earlier results of Benson et al. (1957) who found no such development.

Progesterone in combination with estrogens is generally believed to be required for lobuloalveolar development. Lobuloalveolar development, as well as duct growth, resulted when a combination of estradiol benzoate or estradiol-17 β and progesterone were administered (Benson et al., 1957; Smith and Richterich, 1958; Moon et al., 1958; Chatterton, 1971) suggesting a syneristic response in mammary development. Benson et al. (1957) reported that no duct growth occurred in the guinea pig

with progesterone alone except when given in excess of 2400 mg/da. Smith and Richterich (1958) reported that no mammary growth occurred in the guinea pig with progesterone alone. Chatterton (1971), using ovariectomized estradiol-17 β primed rabbits treated with progesterone implants placed subcutaneously over one mammary gland, showed that mammary glands in contact with progesterone implants developed lobulo-alveolar structures. Control glands without progesterone implants were unstimulated except for ductal growth.

II. Mammary Development in Laboratory Animals In Vitro as Affected by Insulin, Hydrocortisone and Prolactin

Mills and Topper (1970) studied the effects of insulin, hydrocortisone and prolactin on explants from midpregnant mouse mammary glands. Before culture, explants were composed primarily of stroma, adipose tissue and small ducts (secondary and tertiary). The relatively few true alveoli found in untreated tissue had 8 cells per alveolar cross section. Explants cultured with insulin and hydrocortisone for 96 h had double the cells (16) per alveolar cross section and wider alveolar lumina. Alveolar epithelia were pseudostratified and contained lipid droplets. The alveolar lumen contained no secretory material. Insulin treated explants, after 96 h of culture, had 16 cells per alveolar cross section and were comparable to insulin-hydrocortisone treated explants except alveolar lumina were smaller and epithelia contained no lipid droplets. Explants cultured for 96 h with insulin, hydrocortisone and prolactin had more cells per alveoli (18) than insulin-hydrocortisone explants. Insulin, hydrocortisone and prolactin explants had greatly enlarged alveolar lumina filled

with secretory lipid and protein granules. The epithelia was no longer pseudostratified and contained numerous lipid droplets throughout the cytoplasm. Ultrastructurally, the untreated epithelia varied from primarily unspecialized cells having minimal cytoplasmic inclusions to a few isolated alveoli with cells having pronounced polarity (abundant rough endoplasmic reticulum [RER] in the basal cytoplasm and enlarged supernuclear Golgi apparatus) and secretory products. Epithelia from explants cultured in insulin alone had a limited amount of endoplasmic reticulum and poorly developed Golgi apparatus located lateral to the nuclei. Epithelia of insulin-hydrocortisone treated explants exhibited no polarity of organelles but had increased amounts of RER and a more extensively developed Golgi apparatus located lateral to the nuclei. Greatest ultrastructural development occurred in insulin-hydrocortisone primed explants subsequently cultured with prolactin. Prolactin caused the epithelial cell to enlarge and exhibit cytoplasmic polarity, i.e., large supernuclear Golgi apparatus, large accentually located nucleus and abundant basal RER. Insulin is the only hormone required in vitro for maintenance (Ichinose and Bern, 1966) and division (Topper, 1970) of mid-pregnant mammary epithelial cells, however, pharmacological levels of insulin are required and only one division cycle has been observed. Glucocorticoids appear to develop the membranes of the mammary cells. Mammary cells exposed to prolactin, in culture, apparently gain the ability to synthesize milk products. Thus, the addition of insulin, hydrocortisone and prolactin to mid-pregnant mammary explants brings about milk synthesis in 96 h.

III. Mammary Development in Rabbits In Vivo as Affected by Prolactin

Ultrastructural studies by Fiddler et al. (1971) and Tobon et al. (1973) showed prolactin induced differentiation of pseudopregnant rabbit mammary glands. Forty-eight h after intraductal injections of prolactin, epithelial cells had increased amounts of RER, Golgi material and Golgi vesicles containing electron dense granules (Fiddler et al., 1971). Tobon et al. (1973) obtained similar results at 120 h. Fiddler et al. (1971) showed that major protein and lactose biosynthesis did not occur until 72 h following prolactin treatment even though structural effects were apparent at 48 h.

IV. Mammary Development in Non-pregnant Farm Animals with Exogenous Hormones

A. Synthetic hormones used in treatment.

Sykes and Wrenn (1950) injected 1 mo-old heifer calves with diethylstilbestrol (3 mg/wk) for 4 wk after which calves were injected weekly with either: 1) diethylstilbestrol alone, 2) diethylstilbestrol and progesterone, 3) diethylstilbestrol and pituitary extract or 4) diethylstilbestrol, progesterone and pituitary extract. Weekly dose levels were: diethylstilbestrol 3 mg and progesterone 6 mg until 4 mo of age at which time dose levels were doubled. Ninety mg of pituitary extract was administered weekly. Half udders were removed at 5 and 9 mo of age for qualitative histological study. Immature alveoli forming irregular and indistinct lobules were found in diethylstilbestrol and diethylstilbestrol-progesterone treated heifers. Treatment including pituitary extract (3 and 4 above) produced well developed lobules

with mature appearing alveoli at 9 mo but not at 5 mo.

In 1951, Sykes and Wrenn treated heifers from 6 to 11 mo of age with various combinations of diethylstilbestrol, progesterone and pituitary extract. Weekly dose levels were diethylstilbestrol 6 mg, progesterone 240 mg and pituitary extract 90 mg. At 11 mo of age, diethylstilbestrol alone resulted in distended ducts and alveoli and indistinct lobule formation. Diethylstilbestrol-pituitary extract treatment resulted in improved lobule formation, but abnormal alveoli with thickened walls and papillae were frequently seen. Treatment with diethylstilbestrol-progesterone or diethylstilbestrol-progesterone-pituitary extract resulted in many areas of normal appearing lobular-alveolar development. Progesterone treatment reduced the frequency of abnormal alveoli in all cases.

Reineke et al. (1952) induced lactation in 5 dairy cows using implants containing 3-4 gms progesterone and 0.1-2.0 gms diethylstilbestrol. Implants were in place for 108 to 196 da. One cow came into lactation with its implant in place while the other 4 cows required removal of implants and milking stimuli to induce lactation. Initial milk production varied from a few kg/da to a maximum of 19.5 kg/da. The best 305 da lactation recorded from their technique was 5144 kg milk and 191 kg fat.

Cowie et al. (1952) induced mammary growth in 37 goats by using implants or subcutaneous injections of progesterone and hexoestrol or hexoestrol alone at various dose levels (progesterone 1.7 to 40 mg/da and hexoestrol .25 to 1.7 mg/da) for 59 to 140 da. Milk yields from

induced lactation were less than normal lactations. Lactational yields from hexoestrol treatment only were inversely related to duration of the hexoestrol treatment. Exogenous administration of hexoestrol (.25-1 mg/da) alone or hexoestrol (1 mg/da) in combination with progesterone (40 mg/da) resulted in abnormal alveolar development consisting of cystic alveoli, folded epithelium and immature lobules. A lesser ratio of hexoestrol (.25 mg/da) to progesterone (40-100 mg/da) resulted in a more normal lobular-alveolar development. Benson et al. (1955) reported similar results in the goat using hexoestrol alone (.025-.25 mg/da) or combination of hexoestrol (.5 mg/da) and progesterone (70 mg/da).

Hancock et al. (1954) induced lactation in one of each of seven pairs of twin heifers. The treated twin was non-gravid and the control twin delivered a calf. Udder development and initiation of lactation was accomplished using a 3 phase 150 da treatment. Progesterone and diethylstilbestrol were injected subcutaneously at a daily level of 50 mg and 50 µg the 1st 60 da, 75 mg and 75 µg the next 30 da and 100 mg and 100 µg the subsequent 60 da, respectively. During the last 30 da, the diethylstilbestrol injections were increased by 2 mg/da each 10 da until a level of 8 mg/da was obtained. Diethylstilbestrol injections continued until co-twins calved at which time regular twice daily milking was initiated. The length of time each heifer was treated with diethylstilbestrol alone was not given. Four of the 7 treated heifers produced approximately normal amounts of milk. Production of these 4 hormonally treated heifers ranged between 42 to 72% of their partur-

ient twins. Production from the remaining treated and parturient twin heifers were unsuccessful.

Turner et al. (1956) induced lactation in 8 dairy heifers by daily injections of a combination of estradiol benzoate (100 μ g) and progesterone (100 mg) for 180 da. The treatment period (180 da) was based on the theory that 1/2 to 2/3 of gestation is required for complete mammary development. Lactation was initiated by injecting 3 mg estradiol benzoate daily for 14 da after the completion of the 180 da estradiol benzoate-progesterone treatment. Average maximum daily production ranged from 5.6 to 15.2 kg fat corrected milk. Six induced heifers produced 66 to 137% of the milk their paternal and maternal half sisters produced. In another part of the same study, two pairs of twin heifers were used. Induced twins produced 85 and 93% of the milk produced by parturient co-twins. Production in a freemartin treated as above was non-significant. These early workers using synthetic hormones for induced lactation had lengthy impractical treatment periods, modest success and erratic milk yields.

B. Natural hormones used in treatment.

Sud, Tucker and Meites (1968) studied the estrogen and progesterone requirements for udder development of 30 ovariectomized heifers. Estradiol-17 β (E) and progesterone (P) were injected three times weekly for 20 wk as follows: (A) 200 mg P, 200 μ g E; (B) 200 mg P, 400 μ g E; (C) 200 mg P, 800 μ g E; (D) 50 mg P, 400 μ g E; (E) 100 mg P, 400 μ g E; (F) 400 mg P, 400 μ g E. Treatments C and E above were found to produce optimal mammary development with tightly packed alveolar cells

similar to 5 mo pregnant controls. Mammary weight and total DNA and RNA was greater in treatment C. Poorest histological development occurred with treatment D.

Administration of .1 mg estradiol-17 β and .25 mg progesterone/kg body wt/da for 7 da resulted in colostrum formation (Smith et al., 1971) and initiated lactation in 7 of 10 cows (Smith and Schanbacher, 1973). These hormone levels are lower with a smaller ratio than the optimum treatment reported by Sud et al. (1968). Milking was initiated twice daily when the gland became distended with fluid. Average time from first treatment until first milk withdrawal was 21 da (Smith et al., 1971) and 19.3 da (Smith and Schanbacher, 1973).

Smith and Schanbacher (1973) reported induced lactations were characterized by a daily increase in milk yield of $1.32 \pm .15$ kg per da during the first ten days of milking. Peak lactation occurred approximately 30 to 50 da after first milking. Mean 305 da production for induced lactations was $5,069 \pm 462$ kg (4% fat-corrected milk). When lactation was induced, it averaged 82% (63 to 106%) of the milk and 90% of the fat production when compared to the best previous lactation initiated by calving. Considering all cows treated, their induction procedure was 70% successful.

Smith, Redman and Schanbacher (1973) initiated lactation in 38 of 48 cows by injecting progesterone and estradiol-17 β . Mean milk yield was 4,074 kg/10 mo with a maximum production of 6,101 kg/10 mo. Time interval between estrus and initiation of hormone treatment was an important factor in the success rate, 5 of 9 cows determined to be 0-7

da post estrus (per rectal palpation) exceeded the mean milk yield where as only 2 of 9 cows determined to be 8-18 da post estrus exceeded the mean milk yield. They, also, found that if ovarian size regressed due to treatment the success rate was greater. Ovaries of 26 cows which lactated were devoid of corpora lutea and/or follicles 21 da after the first injection. Presence of corpora lutea or follicles were questionable 21 da after the first injection in 9 of 10 cows which lactated. Corpora lutea or follicles were present 21 da after the first injection in 9 of 10 cows which failed to lactate.

Erb et al. (1973) induced lactation in 5 cows using the procedure reported by Smith et al. (1971). Milk yields increased gradually and maximum daily production of 10 to 30 kg milk/da was obtained 60 da after the last treatment. During and 2-4 wk following treatment vulvas were enlarged, uteri were turgid and mucus was discharged. Ovarian size regressed due to treatment.

Plasma estrogen and progesterone levels during and following the above treatment were reported by Monk et al. (1973). Plasma progesterone was elevated from .9 $\mu\text{g/ml}$ on da 0 to 6.11 $\mu\text{g/ml}$ on da 7 and decreased to 38 $\mu\text{g/ml}$ on da 22 following last treatment. Plasma free estrogen was elevated from 38 pg/ml on da 0 to 1,100 pg/ml on da 7 and decreased to 82 pg/ml on da 22 following last treatment. During the injection period, plasma progesterone approximated luteal phase, while plasma free estrogen approximated 1 da prepartum levels.

Smith and co-workers using a 1:2.5 ratio of estrogen to progesterone were able to induce lactation for the first time with a short 1 wk injection regime. They found that time after estrus and corpus

luteum growth was an important factor in the failure of induced lactation. They were not able, with their treatment, to overcome the variability in milking response. However, their modest success with a short treatment period makes induced lactation more practical.

V. Mammary Development in Pregnant Farm Animals with Synthetic Adrenal Steroids

Tucker and Meites (1965) induced lactation in pregnant heifers by administering a powerful synthetic glucocorticoid, 9-fluoroprednisolone acetate, and twice daily milk withdrawal. Two heifers, pregnant 3 1/2 mo, were injected with 9-fluoroprednisolone acetate 10 mg/da for 7 da and 15 mg/da for the next 8 da. The resultant average daily production was .36 kg (maximum of .91 kg/da) during a 14 da milking period; control heifers did not respond to the milking stimuli alone. A third heifer, pregnant 5 1/2 mo, was injected with 15 mg/da of 9-fluoroprednisolone acetate for 7 da. Maximum daily production for this heifer was 10 kg. A third group of three heifers, pregnant 7 1/2 mo, was injected with 15 mg/da 9-fluoroprednisolone acetate for 6 da. Maximum average daily production for the treated heifers was 12.8 kg as compared to 8.5 kg for two control heifers. Their work established the roles of glucocorticoid and milking stimuli in intact pregnant cattle as a final inducer of mammary development. The drug did not express its role until late in pregnancy.

VI. Collagen and DNA Content of the Mammary Gland

Harkness and Harkness (1956) studied collagen and total DNA content of the rat mammary gland during pregnancy, lactation (da 12-24) and involution (3 wk). Collagen content has been used as a measure

of connective tissue and DNA a measure of cell numbers. Mammary collagen content, in their work, was constant during pregnancy and lactation. It increased slightly during involution. DNA content increased approximately 2.5 times during pregnancy and increased only slightly during lactation. After involution, total DNA content was slightly higher than virginal values. They concluded that pre-existing connective tissue provides physical support for the mammary gland during pregnancy and lactation. Tucker and Reece (1963) likewise have shown an increase in DNA content during pregnancy. DNA (mg/100 g body wt) content of the rat mammary gland, in their study, increased 184% from da 0 through da 20 of pregnancy.

Nicoll and Tucker (1965) determined the total mammary stromal and parenchymal DNA content in virgin and lactating mice. Stromal DNA content did not change between virgin and lactating mice. Parenchymal DNA accounted for 23 and 89% of the total DNA in virgin and lactating mice, respectively. They attributed the difference in total mammary DNA between virgin and lactating mice to proliferation of parenchymal cells.

Sinha and Tucker (1969) studied mammary nucleic acid and hydroxyproline (collagen) content of heifers from birth to age 12 mo and during the estrous cycle. Mammary DNA (mg/100 kg body wt) increased significantly from age 2 thru 9 mo. Between the 9th and 12th mo, mammary DNA did not change significantly. Hydroxyproline content followed a pattern essentially similar to mammary DNA from birth to age 12 mo. Mammary DNA values were similar at age 9 and 16.2 mo. During the

estrous cycle, hydroxyproline values decreased while the DNA values increased, suggesting that mammary cells proliferate at estrus. Hydroxyproline values at the onset of the estrous cycle were higher than reported for heifers between 9 and 12 mo.

Munford (1963b) found that total DNA was significantly correlated with the number of alveolar nuclei/histological section ($r = .83$) and total glandular tissue ($r = .94$) in mice and rats. Mammary DNA was 120.7 mg/gland in virgin gilts and 107.9, 115.0 and 580.4 mg/gland on da 25, 50 and 100 of pregnancy in the gilt, respectively (Hacker and Hill, 1972). Total mammary DNA increased 89 times between age 5 mo and 60 da of lactation in the bovine (Tucker et al., 1973). Considering 1) the positive correlations between histological indicators of mammary development and the increase in mammary DNA in the mouse and rat, 2) the work of Sud et al. (1968) reviewed above and 3) the observations that connective tissue is relatively constant in the adult rodent mammary gland, it is likely that a large portion of the DNA increase from virginal to lactational states in the gilt and heifer is development of mammary parenchyma.

VII. Quantitative Histological Techniques

Chalkley (1943) introduced a direct microscopic method for quantitatively determining the area of tissue components. This technique utilized a set of fixed points in one microscope ocular. Selection of tissue to be quantified was randomized by relocating the counting area while the specimen was out of focus. Tissue components under fixed points (contacts) were quantified at various focal planes. No

precautions were necessary to avoid repeated observations of the same area since point to point repetition is improbable. Unit area of different tissue components was calculated from the ratio of the summation of contacts for each tissue component.

Munford (1963a) evaluated rat and mouse mammary tissue by counting the number of nuclei per alveolar cross section, using a method similar to Chalkley's quantitative morphological technique. Alveolar cross sections were selected at random and nuclei were counted. Average number of nuclei per alveolar cross section remained relatively constant during late pregnancy and lactation in both rat and mouse mammary tissue. However, between da 20 of pregnancy and da 1 of lactation, the number of nuclei per alveolar cross section increased approximately 2 fold. Concurrent to the increase in nuclei per alveolar cross section, the alveolar diameter increased and the alveoli per unit area decreased. He measured the proportion of glandular tissue in the mammary gland and found it to increase during pregnancy and reach maximum values in mid-lactation. Munford (1964), reviewing international journals, summarized the work of several laboratories stating that an increase of nuclei per alveolar cross-section occurs about the time of parturition in the cow (Altman, 1945), goat (Naito et al., 1955) and guinea pig (Naito, 1958).

Saacke and Heald (1969) used Chalkley's technique to quantify epithelial cells based on lipid characteristics, Golgi development and general morphology. Tissue was obtained at slaughter from 9 zones in the mammary gland from heifers at various stages of their first lactation. Epithelia with lipid droplets of variable size and well developed

apically located Golgi predominated throughout lactation. Other epithelial cell types ranged from epithelia completely engorged with lipid to epithelia completely void of lipid. Epithelial cells within alveoli and generally within lobules were of the same type. Alveolar epithelia appeared synchronized in activity, including stage of lipid secretion.

Kinsella and Heald (1972) used Chalkley's technique to quantify mammary tissue from 2 prepartum cows. After characterizing 6,800 contact points, they found that 2 wk prepartum tissue had 30% more connective tissue per unit area than 2 da prepartum tissue. Two day prepartum mammary tissue had 30% more area assigned to alveolar lumen than did 2 wk prepartum mammary tissue. Epithelial, adipose and vascular tissue were similar in both cows.

Lactation response to Smith and co-workers (1971, 1973) induced lactation technique is variable. Tucker and Meites (1965) indicate that glucocorticoids are a factor of importance in initiation of lactation in pregnant cows, and the role of corticoids in mammary development in vitro has been described by several workers (Topper, 1970). The role of glucocorticoids in induced lactation, therefore, should be studied. Lactation yields, although important in application, are an indirect measure of mammary development and provided little insight for the understanding of basic factors controlling mammary development. Structural development of mammary secretory cells is under the control of several endogenous hormones and morphological techniques have been developed to directly quantify such histological development in laboratory species. Use of these histological techniques would provide a sequential des-

cription of mammary development during induced lactation. This quantitative information would be helpful in determining how induced lactation response is variable and provide a histological reference for the study of improved induced lactation techniques.

MATERIALS AND METHODS

Thirteen mature cycling nonlactating cows were hormonally induced to initiate lactogenesis. See appendix for life history data for all cows. During the experimental period, these animals were individually housed in boxstalls and fed a ration of silage, hay and concentrates. Water was available at all time.

A combination of estradiol-17 β (0.1 mg) and progesterone (0.25 mg/kg body wt/da) in 3 ml ethanol was administered twice daily (approximately equal intervals) for 7 da (Smith et al., 1971). Hormones were given by a subcutaneous injection just posterior to the scapula on the dorsal aspect of the rib cage. Day 1 of the treatment period was the 7th day after standing heat. No hormone treatment was given on da 8 thru 17. Dexamethasone (.028 mg/kg body wt) was administered by an intramuscular injection on da 18, 19 and 20 following the initiation of the estrogen-progesterone injections.

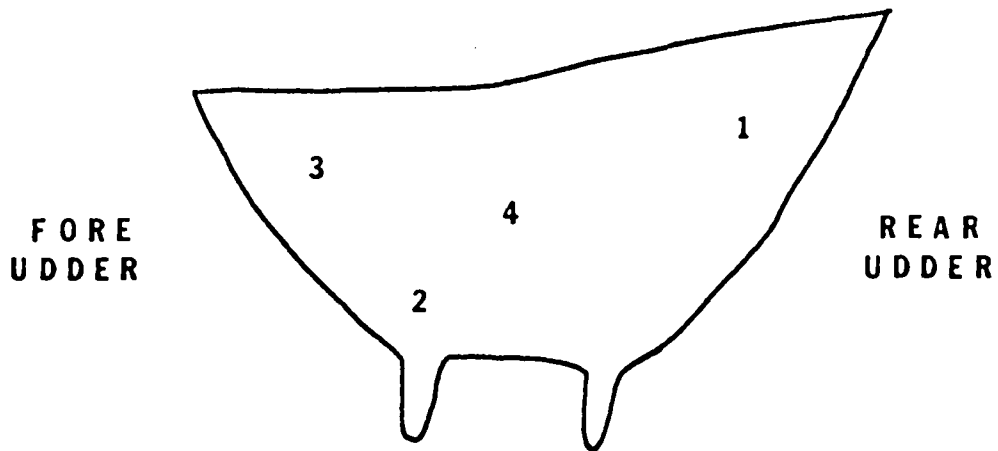
Estradiol-17 β (Δ 1, 3, 5 (10) estratien-3, 17 β -diol) and the progesterone (Δ^4 -pregnen-3, 20-dion) used in this study were obtained from Sigma Chemical Company, St. Louis, Mo. The dexamethasone (Azium) was manufactured by Schering Corporation, Bloomfield, N.J.

Complete lactation yields were recorded for four induced cows. Four heifers with no previous lactation and five cows with one or more complete lactation were used in the tissue study. Tissue samples were obtained by biopsy on da 18, 21 and 23 following the initiation of the estrogen-progesterone injections. There were three cows assigned to each day. Cows biopsied on da 21 were milked once prior to sampling.

Twice daily milking was initiated on da 21 for cows biopsied on da 23. Cows biopsied on da 18 received no dexamethasone and were not milked. Mammary tissue from each cow was taken from four standardized zones (see Figure 1). Due to massive hemorrhage, tissue from zone four was not obtained from one cow on da 21. Biopsies were performed using a local anesthetic, procaine hydrochloride (2%) or using a general anesthetic, Kemithal or Surital; Surital was the most satisfactory. A block of tissue (1.5-2.0 cm³) was surgically removed at a depth of 3.0 to 5.0 cm; the wound was sutured closed. Tissue was trimmed of excess fat, fixed and stored in 10% neutral buffered formalin and sectioned at -35°C. Sections, 6 μ thick, were stained in Oil Red "O" (.5 gm Oil Red "O"/100 ml propylene glycol), counter stained in Harris Hematoxylin and blued in a saturated solution of lithium carbonate.

A modification of Chalkley's (1943) quantitative morphological analysis was used to determine the percent mammary tissue area composing eight classifications as follows: 1) no lipid droplet epithelia, 2) small lipid droplet epithelia, 3) mixed lipid droplet epithelia, 4) large lipid droplet epithelia, 5) alveolar lumen, 6) stroma, 7) adipose, and 8) duct. One hundred contact points were classified at a magnification of 400X; three replicate of 100 were made per slide. A total of 12 slides was classified per cow (3 slides/zone). Random selection of areas to be counted was accomplished by relocating unobserved coded slides. Five contact points in the ocular provided reference points used in the counting process - one central and four spaced equal distance from the center. A total of 31,500 contact points was characterized for this study.

Figure 1. Location of the 4 biopsy zones.



Munford's (1963a) morphological technique for determining the number of nuclei per alveolar cross section was performed at a magnification of 250. Alveolar cross sections were selected at random by using two fixed points, one in the upper right hand and the other in the lower left hand area of the microscopic field. The specimen was moved at random as described previously. Contacts with either fixed point were counted to determine the number of nuclei per alveolar cross section. When no alveolar cross sections were in contact with the fixed points, the process was repeated again. Twenty alveolar cross sections were counted on each of the three slides per zone totaling 240 alveolar cross sections per cow, 2100 cross sections for this study.

A split plot analysis of variance for disproportionate subclass numbers was performed to determine the effects of days, cows within days, zones, zone-day interaction, cow-zone interaction within days and slides within zone and cows on the occurrence of the dependent variables. Due to a completely missing subclass (zone 4 from one cow on day 21), the sum of squares for the cow-zone interaction within days was obtained as the difference between the cow-zone subclass sum of squares and the accumulated sums of squares for days, cows within days, zones and zone-day interaction. The cows within days mean square was used to determine the F value for day effect. The slides within zones and cows mean squares were used to determine the F value for cows within days, zone, zone-day interaction and cow-zone interaction within days. The error mean square was used to determine the F value for slides within zones and cows. Correlation coefficients among the dependent variables were computed from the cow-zone means.

RESULTS

I. Milk Production. Milk production was induced in all four cows treated. Degree of success was variable. Production records, based on 305 M.E. for 4 cows hormonally induced into lactation are presented in Table 1. Comparison of milk yields of 4 cows ranged from 33 to 74% of previous natural lactations.

II. Comparative Morphology. Percent area and standard deviation of tissue assigned as mammary epithelium, alveolar lumen, stroma, adipose and ducts from da 18, 21 and 23 by quantitative morphological analysis appears in Table 2. Mammary epithelium was further characterized as having no lipid droplets, small lipid droplets, mixed lipid droplets (small and large lipid droplets), large lipid droplets. Means and standard deviation for the number of nuclei/alveolar cross section from tissue on da 18, 21 and 23 also appear in Table 2. Micrographs of tissue from da 18, 21 and 23 appear in Fig. 2, 3 and 4. Tissue from da 18 was characterized by a large stroma (78.8%A), small alveolar lumen (2.5%A), and small lipid droplet epithelium (12.1%A) area, see Table 2, Fig. 2 and 5. Tissue from day 23 had the largest alveolar lumen (16.4%A), largest mixed lipid droplet epithelium (20.9%A) and the least stroma (53.0%A) area, see Table 2, Fig. 4 and 5. Tissue area assigned to alveolar lumen (7.7%A), small lipid droplet epithelium (6.2%), mixed lipid droplet epithelium (12.5%A) and stroma (69.7%A) for da 21 was intermediate, see Table 2, Fig. 3 and 5. Epithelia of individual alveoli were uniform in lipid droplet characteristics, see Fig. 4, 6, 7 and 8. Lipid droplet characteristics of epithelia within a lobule

Table 1. Comparison of 305 da M.E. milk production of 4 cows treated with progesterone, estradiol-17 β and dexamethasone to induce lactation with cows previous natural production.

Cow	305 da M.E. Production (kg)	% Previous Lactation
A	3989	74
B	2144	37
C	2999	65
D	2586	33
	$\bar{X} = 2930$	$\bar{X} = 52$

Table 2. Quantitative morphological summary of mammary tissue including the means and standard deviation for the percent of tissue area (%A) assigned to each of the 8 tissue classifications for da 18, 21 and 23, the means and standard deviation for the number of nuclei per alveolar cross section for da 18, 21 and 23 and the difference in %A for the 8 tissue classifications and number of nuclei per alveolar cross section from da 18 to 23.

Classifications	Day 18		Day 21		Day 23		Difference Day 18 to 23
	Means %A	SD	Means %A	SD	Means %A	SD	
Epithelia:							
No lipid droplets	.23	.7	.66	1.3	.25	.7	.02
Small lipid droplets	12.07	5.3	6.20	5.5	3.90	4.8	-8.17
Mixed lipid droplets	3.22	4.3	12.49	10.9	20.87	9.8	17.65
Large lipid droplets	.03	.2	.19	.6	1.62	4.6	1.59
Alveolar lumen	2.50	2.2	7.68	6.5	16.35	7.4	13.85
Stroma	78.82	6.9	69.74	13.0	52.95	10.6	-25.87
Adipose tissue	1.48	2.3	2.10	2.3	3.06	2.9	1.58
Duct area	1.68	3.0	.87	1.7	.94	2.0	-.74
	No. Nuclei	SD	No. Nuclei	SD	No. Nuclei	SD	
Nuclei/cross section	22.94	6.6	27.63	10.2	37.76	12.7	14.82

Figures 2, 3 and 4. Light micrographs of 6 μ thick frozen sections stained with Oil Red "O" and Harris Hematoxylin. 390X

Figure 2. Tissue from day 18 characterized by numerous small alveoli, small lumenal area and large stromal area.

Figure 3. Tissue from day 21 characterized by fewer large irregularly shaped alveoli and decreased amount of stroma.

Figure 4. Tissue from day 23 characterized by more uniformly developed alveoli, greatly decreased stromal area and increased lumenal area.

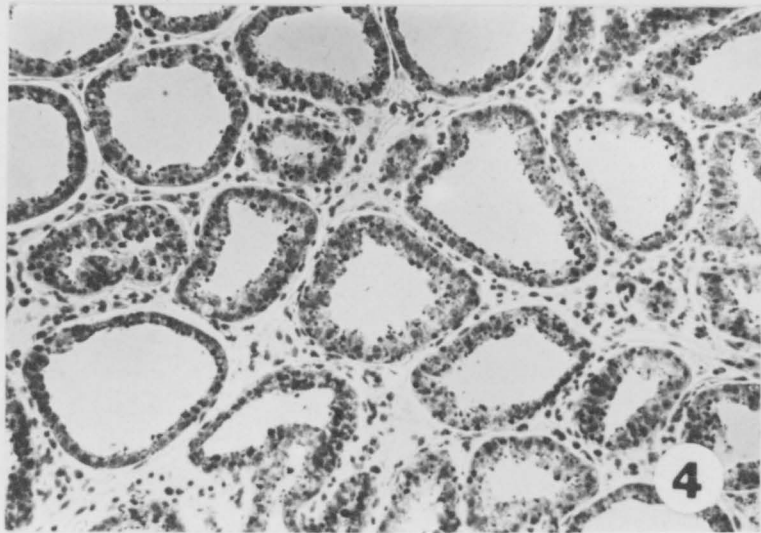
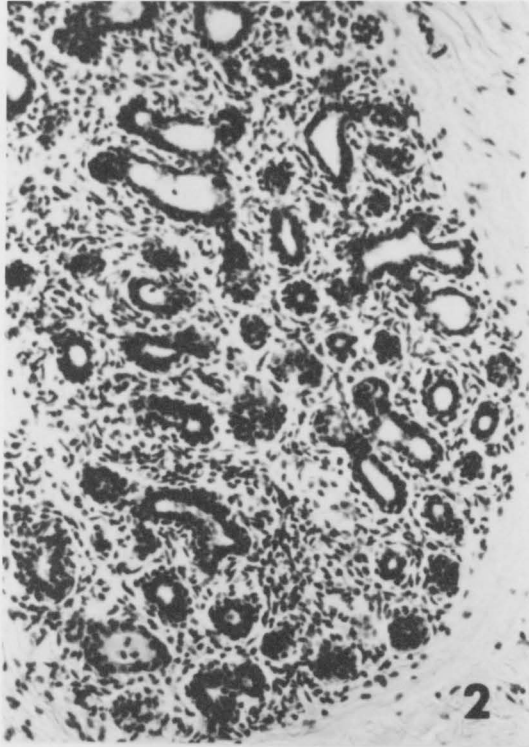
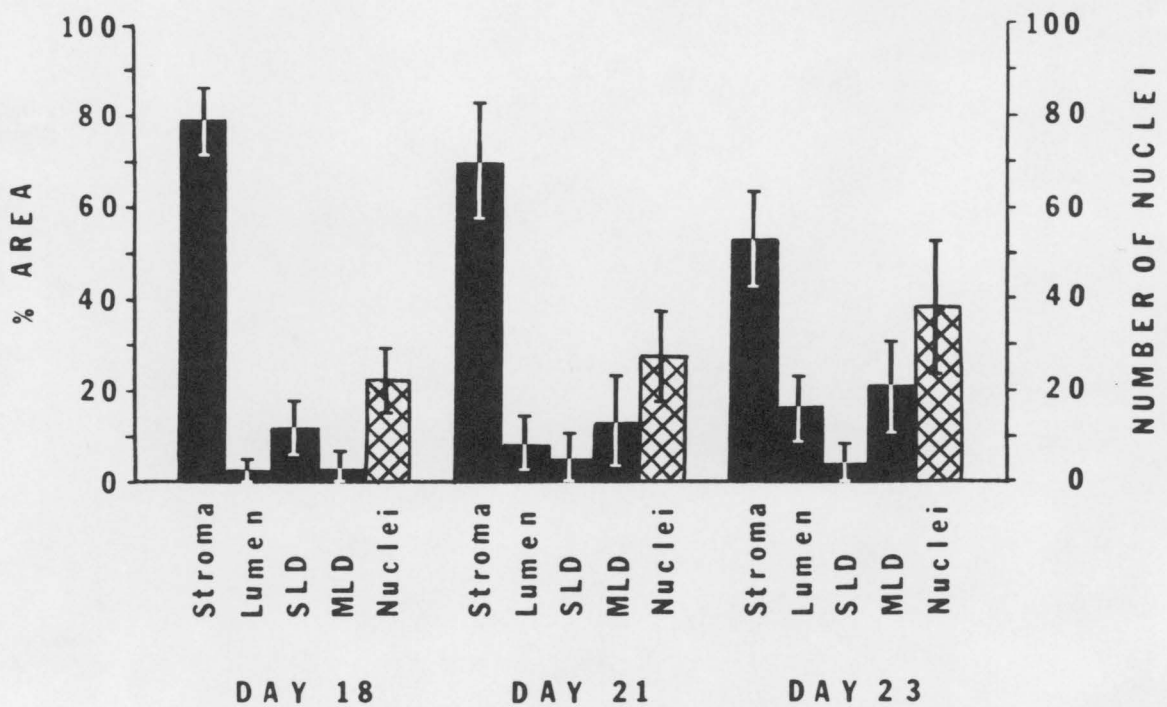


Figure 5. A histogram comparing number of nuclei per alveolar cross section and percent tissue area assigned as stroma, alveolar lumen, small lipid droplet epithelium (SLD) and mixed lipid droplet epithelium (MLD).



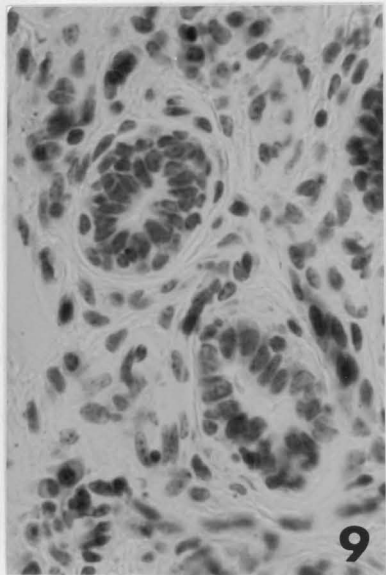
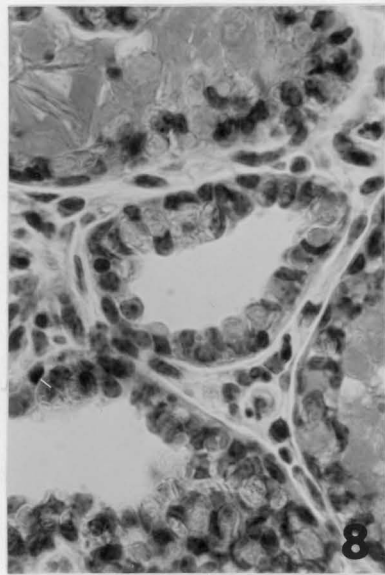
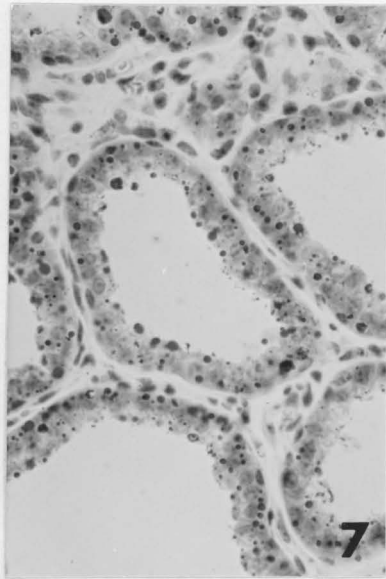
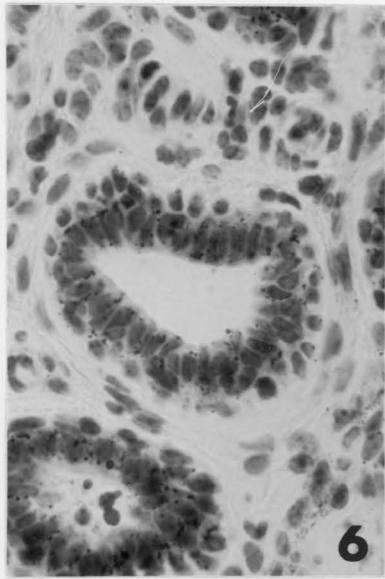
Figures 6, 7, 8 and 9. Light micrographs of 6 μ thick frozen sections of mammary tissue stained with Oil Red "O" and counter stained with Harris Hematoxylin. 1100X

Figure 6. Epithelium with small lipid droplets, characterized by a large stromal area, small luminal area and dark staining elongated nuclei.

Figure 7. Epithelium with mixed lipid droplets, characterized by small stromal area, large alveolar luminal area and light staining spherically shaped nuclei.

Figure 8. Epithelium with large lipid droplets characterized by a small stromal area, large luminal area and dark staining nuclei.

Figure 9. Epithelium with no lipid droplets characterized by a large stromal area, limited luminal area and dark staining elongated nuclei.



were not quantitated but, in general, were found to be uniform in appearance.

Epithelial nuclear morphology and estimated nuclear/cytoplasmic area ratio changed with sampling days. Epithelia with small lipid droplets and no lipid droplets had dark staining elongated nuclei with a large estimated nuclear/cytoplasmic ratio, Fig. 10. Epithelia with mixed lipid droplets had very light staining centrally located, spherical nuclei containing several nucleoli, Fig. 11. Epithelia with large lipid droplets had a dark staining centrally located spherically shaped nuclei with no prominent nucleoli, Fig. 12. Smaller estimated nuclear/cytoplasmic ratio was associated with spherically shaped nuclei seen in epithelia with mixed or large lipid droplets as compared to epithelia with no lipid droplets or small lipid droplets.

Areas classified as large lipid droplet epithelia (< 2%A), Fig. 8, no lipid droplet epithelia (< 1%A), Fig. 9, duct (< 2%A) and adipose (< 4%A) were small and relatively constant on each of the 3 da.

III. Large lipid droplets. Large lipid droplets stained orange and were irregular (wrinkled or lacy) in appearance in comparison to other lipid droplets, Fig. 8. This irregular appearance was characteristic of large lipid droplets in the cytoplasm and in alveolar lumina. During the staining process, most alveolar content was lost. When luminal content was preserved, it was found predisposed to large lipid droplet engorgement. Large lipid droplets in the cytoplasm or lumina were not found in association with more fully developed alveolar epithelia.

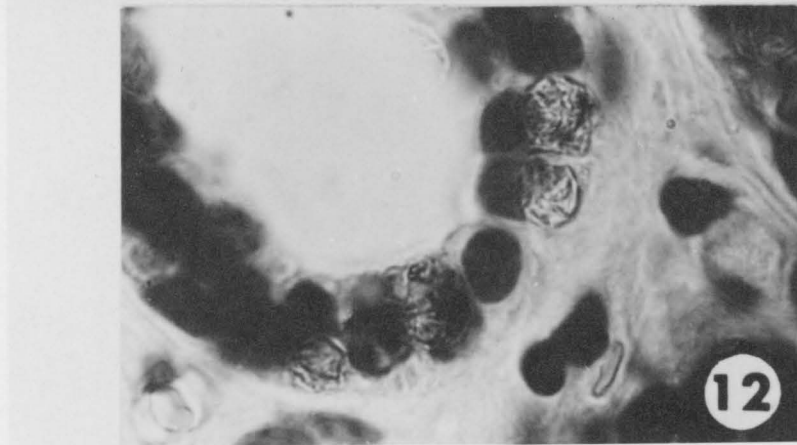
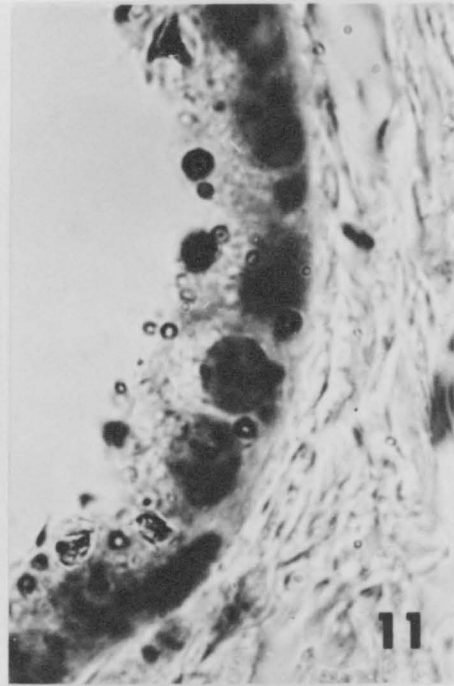
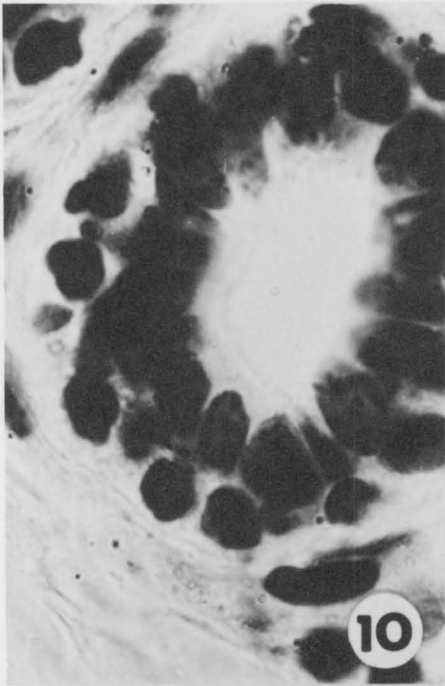
IV. Split Plot Analysis. Results from the split plot analysis are

Figures 10, 11 and 12. High magnification, light micrographs of 6 μ thick frozen sections of mammary tissue stained with Oil Red "O" and Harris Hematoxylin. 3250X

Figure 10. Dark staining elongated nuclei and a large nuclear/cytoplasmic ratio characteristic of no lipid droplet and small lipid droplet classes.

Figure 11. Spherically shaped nuclei, variable size lipid droplets and small nuclear/cytoplasmic ratio characteristic of mixed lipid droplet epithelia.

Figure 12. Spherically shaped nuclei and wrinkled lipid droplets within the cytoplasm characteristic of large lipid droplet epithelia.



summarized in Tables 3A and B. Correlation coefficients computed from the cow-zone means for all dependent variables are presented in Table 4. Days were found to be significant for small lipid droplet epithelia, luminal and stromal area and nuclei per alveolar cross section. Failure of the day effect to be significant for the large increase in mixed lipid droplet epithelia on da 21 and 23 is attributed to the large variation among cows within days.

Zones had a significant effect on all the dependent variables except the area assigned as alveolar lumen. Zones did not significantly ($P < .01$) affect alveolar lumen, indicating the area assigned as alveolar lumen was independent of the zone from which the tissue was taken. Thus, the single parameter measuring mammary development most accurately in this research is alveolar lumen.

A progressive change in mammary tissue was evident with time. Decrease in area assigned as small lipid droplet epithelia from da 18 to 23 was significant ($P < .05$). Mixed lipid droplet epithelia increased from da 18 to 23 and accounted for the greatest epithelial area on da 21 and 23; this increase was not significant at the .05 level. Correlation for the inverse change in small lipid droplet and mixed lipid droplet epithelia is $-.83$. On da 21 and 23, there is a significant ($P < .05$) increase in the area assigned as alveolar lumen. A correlation of $.76$ between alveolar lumina and mixed lipid droplet epithelia and a correlation of $-.75$ between alveolar lumina and small lipid droplet epithelia was found.

Stromal area decreased significantly ($P < .05$) from da 18 to 23,

Table 3 A. Mean square (MS) from the analysis of variance for the dependent variables epithelia characterized as no lipid droplets (NLD), small lipid droplets (SLD), mixed lipid droplets (MLD) and large lipid droplets (LLD).

Source	d.f.	<u>NLD</u> M.S.	<u>SLD</u> M.S.	<u>MLD</u> M.S.	<u>LLD</u> M.S.
Days	2	2.16	1,884.96 ^a	8,415.31	83.62
Cows (Days)	6	1.94 ^a	335.03 ^b	1,779.97 ^a	52.41 ^a
Zone	3	24.72 ^b	358.73 ^b	992.89 ^a	98.23 ^b
Days X Zone	6	1.89 ^a	51.22	346.13 ^a	76.07 ^a
Zones X Cows (Days)	17	1.58 ^a	134.59 ^a	254.27 ^a	48.43 ^a
Slide (Cow Zone)	70	.69 ^a	26.43 ^a	26.66 ^a	3.18 ^a
Error	280	.34	10.81	13.39	1.40

^a P < .05

^b P < .01

Table 3 B. Mean square (MS) from the analysis of variance for the dependent variables luminal area (LA), stroma area, adipose area, duct area and nuclei per alveolar cross section (NUC).

Source	d.f.	LA M.S.	Stroma M.S.	NUC M.S.	Adipose M.S.	Duct M.S.
Days	2	5,357.23 ^a	18,687.12 ^a	41,756.79 ^a	67.37	19.31
Cows (Days)	6	775.52 ^b	2,554.75 ^b	4,441.01 ^b	26.82 ^b	9.01
Zone	3	55.05	394.45 ^b	1,315.40 ^b	42.46 ^b	54.24 ^a
Days X Zone	6	58.35	428.65 ^b	650.11 ^a	16.14 ^a	11.63
Zone X Cows (Days)	17	114.84 ^b	214.24 ^a	670.44 ^a	19.44 ^a	20.27 ^a
Slide (Cow Zone)	70	25.07 ^b	74.29 ^a	247.67 ^a	5.81 ^b	9.49 ^a
Error	280	12.75	36.26	87.75	4.41	3.87

^a P < .05

^b P < .01

Table 4. Correlation coefficients computed from the cow zone means for the dependent variables epithelia areas classified as no lipid droplets (NLD), small lipid droplets (SLD), mixed lipid droplets (MLD), and large lipid droplets (LLD) and lumenal area (LA), stroma area, nuclei per alveolar cross section (NUC), adipose area and duct area.

Dependent Variables	dependent variables							
	NLD	SLD	MLD	LLD	LA	Stroma	NUC	Adipose
SLD	.35							
MLD	-.35	-.83						
LLD	.10	-.14	-.03					
LA	-.17	-.75	.76	.26				
Stroma	.23	.73	-.90	-.23	-.91			
NUC	-.11	-.60	.65	.14	.74	-.71		
Adipose	-.28	-.59	.55	-.07	-.26	-.53	.27	
Duct	-.27	.13	-.08	-.14	.74	.09	-.12	< .01

but still accounted for the largest percent area of all tissue classes on da 23. Concurrent and nearly equal to this decrease in stromal area was the combined increase in alveolar luminal area and mixed lipid droplet epithelia. Negative correlations between stroma and alveolar lumina ($r = -.91$) and stroma and mixed lipid droplet epithelia ($r = -.90$) were obtained. Correlation between the areas assigned as stroma and small lipid droplet epithelia was .73.

Figure 5 shows significant ($P < .05$) increase in the number of nuclei per alveolar cross section as sampling days increased. Also, an increase in the number of nuclei per alveolar cross section is positively correlated to luminal area ($r = .74$) and mixed lipid droplet epithelia ($r = .65$) and negatively correlated to stromal area ($r = -.71$) and small lipid droplet epithelia ($r = -.60$).

Biological variation was also found among cows within days and between zones within cows. Variation within a single zone among cows within days is evident by the significant ($P < .05$) day/zone interaction for all the dependent variables except small lipid droplet epithelia and duct. The heterogeneous histological structure among zones within mammary glands is evident by a significant ($P < .05$) zone/cow within days interaction for all dependent variables.

DISCUSSION

Lactation was induced in 4 cows treated with progesterone, estradiol-17 β and dexamethasone; production from these induced lactations averaged 52% of the previous lactation. Stage of mammary development was histologically described for da 18, 21 and 23 following the initiation of the hormone treatment. Immature tissue on da 18 had vast stromal area, limited luminal area, irregular shaped nuclei, greater estimated nuclear/cytoplasmic ratio and no lipid droplet or small lipid droplet epithelia. In comparison, the more mature tissue on da 23 was characterized by diminished stromal area, enlarged luminal area, large spherically shaped nuclei with several nucleoli, lesser estimated nuclear/cytoplasmic ratio and more mixed lipid droplet epithelia. A significant difference was found in the stage of development from da 18 to 23. Variations among cows were found on da 23 in the amount of area assigned as stroma, mixed lipid droplet epithelia and luminal area and the number of nuclei/alveoli.

Previously, lactation has been successfully induced in the bovine by administering diethylstilbestrol and progesterone (Reineke et al., 1952; Hancock et al., 1954), estradiol benzoate and progesterone (Turner et al., 1956) and more recently estradiol-17 β and progesterone (Smith et al., 1971; Smith and Schanbacher, 1973; Smith et al., 1973; Erb et al., 1973). Milk production yields from induced lactation were variable, ranging from 0 to 105% of the animal's previous lactation (Smith and Schanbacher, 1973). Administration of progesterone and estradiol-17 β initiated lactation in 70% (Smith and Schanbacher, 1973) and 79% (Smith et al., 1973)

of treated cows. Using progesterone, estradiol-17 β and dexamethasone in our study all cows initiated lactation yielding 33 to 74% of their previous lactation.

Morphological studies in goats have shown a high correlation between milk production and secretory epithelial surface area developed during pregnancy (Cowie et al., 1952) and by hormonal induction (Benson et al., 1955). In our study, the sum total of secretory epithelia classes increased from 15.6% to 19.5% to 26.6% on da 18, 21 and 23, respectively. The progressive increase in secretory epithelia per unit area on da 21 and 23 demonstrates that tissue from our study follows a similar sequence of development as shown in the goat (Cowie et al., 1952; Benson et al., 1955).

Total glandular tissue and number of alveolar nuclei per histological section were significantly correlated with DNA increases during pregnancy in the rat and mouse (Munford, 1963b). DNA measures made during pregnancy show secretory tissue proliferates at a greater rate than stroma in the rat (Harkness and Harkness, 1956; Munford, 1963b) and mouse (Munford, 1963b; Nicoll and Tucker, 1965). Decreases in stromal area and the increases in epithelial and luminal area with time, in our study, correlates with these indices of maturation in the rat and mouse mammary tissue.

Munford (1963a) found the number of nuclei per alveolar cross section increased approximately two fold between da 20 of pregnancy and da 1 of lactation in the mouse and rat. An increase of 15 (1.6X) nuclei per alveolar cross section occurred between da 18 and 23 in the pre-

sent study paralleling the increase in nuclei/alveoli that occurs about the time of parturition (Munford, 1963a). A similar increase in nuclei per alveoli occurs about the time of parturition in the goat (Naito *et al.*, 1955), guinea-pig (Naito, 1958) and cow (Altman, 1945).

At parturition in the rat and mouse, Munford (1963a) observed a decrease in the number of alveoli per unit area and an increase in alveolar diameter. A significant ($P < .05$) increase in the percent area assigned as alveolar lumen and increased number of nuclei per alveolar cross section on da 21 and 23, found in the current study, parallels Munford's (1963a) findings.

Epithelia with lipid droplets of variable size predominate throughout lactation, Saacke and Heald (1969). In our study, mixed lipid droplet epithelia increased while small lipid droplet epithelia decreased with time. We interpret this reciprocal change in epithelial types as small lipid droplet epithelia being replaced with mixed lipid droplet epithelia. Concurrent with the change in lipid droplet morphology, the nuclear morphology of these cells changed from elongated dark staining nuclei on da 18 to spherically shaped nuclei with prominent nucleoli on da 23.

Large negative correlations between stromal and luminal area and stroma and mixed lipid droplet epithelia area, in our study, indicates that most of the decrease (25.9%) in stroma area and small lipid droplet epithelium (8.2%) can be accounted for by a nearly equal concurrent increase (33%) in the sum total of other epithelial classes and luminal area. Mixed lipid droplet epithelia and luminal area accounted

for 53 and 42% of this increase, respectively. Although it was not quantitated, it appeared that most if not all of the decrease in stroma per unit area resulted from a displacement of stroma in the perivascular spaces rather than in interlobular connective tissue. This was evident by a change in perivascular stroma width and an unchanging interlobular connective tissue width. In subsequent studies, of this nature, division of the stroma class into perivascular space and interlobular connective tissue would be useful in quantifying the displacement of stroma.

Epithelia with large irregular lipid droplets were infrequent but increased with time. The increase was not significant at the .05 level. Large irregular lipid droplets may represent a form of lipid catabolism, colostrum lipid secretion, transient early stage of secretory development, or a minor form of lipid secretion.

Limited area classified as duct in our study is attributed to the following: 1) the biopsy zones represent the peripheral regions of the gland where a limited number of larger ducts would be expected 2) small ducts cut in cross section are hard to distinguish from alveoli, particularly terminal ducts which have a secretory epithelia and 3) tissues with large ducts are very difficult to section on a freezing microtome and maintain through the staining process.

Day 23 tissues were the most developed mammary tissues, as measured by the parameters in this study, and were similar in composition to two weeks prepartum mammary tissue from one cow reported by Kinsella and Heald (1972). Two week prepartum tissue, in their study, consisted of

57.8% stroma, 10.8% alveolar lumen and 29.1% total epithelium. Induced tissue from our study on da 23 had less area assigned as stroma (52.9%) and more area assigned as alveolar lumen (16.3%). In comparison, induced mammary tissue development on da 23 is equal to or slightly more advanced than the level of development of two week prepartum tissue.

Milking on da 21 probably had an indirect stimulatory effect on the morphological appearance of tissue on da 23 in our work. The milking process in the normal parturient cow is known to stimulate the release of oxytocin (Momongan and Schmidt 1970) and prolactin (Koprowski and Tucker 1971). Under experimental conditions, prolactin promotes organelle differentiation and polarization of organelles (ultrastructural development) in vitro in the mouse (Mills and Topper, 1970). Likewise, exogenous prolactin in pseudopregnant rabbits (Fiddler et al., 1971; Tobon et al., 1972) promotes complete ultrastructural development and milk secretion. During milk removal, the alveoli are emptied. Physical removal of the secretory product from alveoli may stimulate cellular development or remove an inhibitory factor. The gland was found to greatly enlarge in most cows several days to weeks after initiation of milking. Therefore, a variable factor in this work could have been the cows individual ability to provide prolactin to the gland or the glands varying response to prolactin stimulation.

Milk yield and morphological development were variable in our study. From this we suggest that improved success of induced lactation will require consistent development of more epithelial cells at

a higher level of epithelial differentiation before first milking. Since gross gland development, cell development and lactation yields are variable under steroid therapy, additional exogenous lactogenic hormones, particularly prolactin, should be considered as having a possible role in reducing variability of induced lactations.

SUMMARY AND CONCLUSIONS

Thirteen cows were hormonally treated to induce lactation. Milk production from 4 of the induced cows averaged 52% of their previous lactations. Mammary biopsies were taken from the remaining 9 cows for histological study during the latter part of the induction period. Area assigned as lumen and mixed lipid droplet epithelia increased with time where as stroma and small lipid droplet epithelia decreased with time. Increased luminal area was the best indicator of mammary development. Small lipid droplet epithelia appeared to be replaced by mixed lipid droplet epithelia in the more mature tissue. Histological variation between cows was evident on all days studied. This histological variation indicates that the individual cows are not responding uniformly to the treatment. It was concluded that improved success of induced lactation will require consistent development of more epithelial cell at a higher level of differentiation. Since cell development and lactation yields are variable under steroid therapy, prolactin or some other lactogenic hormone should be considered in further research of induced lactation.

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APPENDIX

Table 1. Historical information on experimental animals including date of first hormone treatment, age in month when first hormone treatment was given, body weight, day of treatment when biopsy was performed, previous production level (ME) and length of dry period prior to first hormone treatment.

Cow	First Treatment	Age Mo	Body Weight (kg)	Biopsy Day	Previous Production (kg)	Dry Period (day)
3	7/30/73	81	675	N/A	7950	>30
23A	8/1/73	49	560	N/A	5400	>30
23B	2/14/72	47	560	N/A	4585	>30
55	7/26/72	64	670	N/A	7743	>30
470	10/5/72	65	700	21	8020	>30
472	10/6/72	66	700	18	7920	>30
606	1/23/73		600	18		18
673	11/27/72	35	550	23	6363	30
690	3/9/73	41	480	21		60
707*	12/20/72	30	590	23	N/A	N/A
708**	2/2/73	31	450	18		34
745*	2/11/73	33	320	21	N/A	N/A
762*	2/11/73		340	23	N/A	N/A

* Heifer: no previous lactation

** Heifer: milked for four months and turned dry

N/A Not Applicable

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ABSTRACT

Thirteen cycling, nonlactating dairy cows were hormonally treated to induce lactation. Starting 7 da after estrus and continuing for 7 da progesterone and estradiol-17 β dissolved in absolute ethanol were administered twice daily. Daily dose level for progesterone and estradiol-17 β was .25 mg and .01 mg/kg body weight, respectively. Intramuscular injections of dexamethasone (.028 mg/kg body weight) were given on da 18, 19 and 20 after initial treatment. Mammary tissue biopsies were taken from 9 cows on da 18, 21 and 23 after initiation of treatment (3 cows each da). Histological analysis was performed (> 30,000 contacts) to determine the average percent tissue area composed of epithelium, stroma, lumen, adipose and duct. Difference in percent area between da 18 thru 23 was as follows: (1) epithelium +11.1%, (2) stroma -25.9%, (3) lumen +13.9%, (4) adipose +1.58 and (5) duct -.74%. Also, an increase of 15 nuclei/alveoli occurred between da 18 thru 23. On da 18 small lipid droplet epithelia predominated but was replaced with mixed lipid droplet epithelia by da 23. Da 18 epithelia had elongated dark staining nuclei. Epithelia on da 23 had spherically shaped nuclei containing several nucleoli and a decreased nuclear/cytoplasmic ratio when compared to da 18 epithelia. Four of the hormonally treated cows were allowed to lactate. Production from these four cows ranged from 33 to 74% of the previous lactation. Production and histological development were variable in cows treated with estrogen, progesterone and dexamethasone.