Effect of estrogen and progesterone on lactating rat mammary glands: a light and electron microscope study

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Effect of estrogen and progesterone on lactating rat mammary glands: A light and electron microscope study

by

Richard Wayne Walker

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

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INTRODUCTION

The mammary gland represents an interesting tissue on which to conduct physiological research from several standpoints. During lactation it is engaged in active secretion of both the apocrine, merocrine and possibly the holocrine varieties; it is acted upon by every major hormone in the body, and it undergoes a tremendous growth, followed by involution at the end of the lactation period.

Endocrinologists have long been intrigued by the rapidity with which the mammary gland begins its milk secretion, almost simultaneously with parturition. Their investigations into the causes for this have led to numerous theories which attempt to explain the triggering mechanism that initiates lactation. These results have been clouded, however, by species differences among the animals investigated, the multiplicity of hormones which are known to influence both gland growth and lactation, and the various interpretations which always accompany the publishing of any scientific result.

It is generally accepted that in the rat, estrogen and progesterone are the two major hormones primarily concerned with mammary growth during pregnancy, while hormones of the anterior pituitary and other endocrine glands are more concerned with lactation. It is also known that exogenous gonadal hormones in physiological amounts inhibit lactation when administered to the dam during the lactating period.

The explanations suggested to explain this effect have ranged from an inhibition of prolactin release by the pituitary, involution of the mammary gland, failure of the milk ejection mechanism, reversion of the gland to a
growing stage during which milk production does not occur, an inhibition of milk secretion and numerous other explanations.

In an attempt to reduce the confusion and to determine which of the previous mechanisms might be eliminated in forming a hypothesis of mammary gland or milk inhibition under the influence of gonadal hormones, it seemed advisable to first examine the mammary glands of treated animals microscopically at the light and electron microscope levels.

Female rats undergoing their first lactation were obtained and given daily injections of estrogen and progesterone dissolved in corn oil. At various times during the first two weeks of lactation the dams were sacrificed and sample tissue taken from their mammary glands for microscope study.
REVIEW OF THE LITERATURE

Histology and Cytology of the Normal Mammary Gland

Bloom and Fawcett (1968, pp. 767-775) describe the mammary gland as a structure resembling a sweat gland which is laid down in the embryo of mammals along two lines, the mammary lines, extending from the axilla to the groin on either side of the midline on the ventral aspect of the thorax and abdomen. The mammary glands are paired, six pair being present in the rat, and are classed according to their structure as compound tubulo-glands consisting of lobes radiating from the mammary papilla or nipple. Each lobe is subdivided into lobules and further into alveolar ducts. The terminus of an alveolar duct is an evagination of a hollow ball of cells, the alveolus.

Prior to puberty, the mammary glands of the female and male are similar. At puberty the glands of the female undergo extensive development which is correlated with age and the state of the reproductive system. Throughout life there is little further change in the male. The mammary glands of various animals are microscopically similar (Waugh and van der Hoeven, 1962).

Helminen and Ericsson (1968a) describe four types of cells which are found in and among the alveoli of the lactating rat mammary gland; secretory epithelial cells, myoepithelial cells, pale cells and macrophages. The pale cells are not to be confused with the "washed-out" cells of Feldman (1961). Pale cells are thought by Helminen and Ericsson (1968a) to represent lymphocytes while the "washed-out" cells of Feldman (1961) possibly represent an epithelial cell undergoing disintegration.
Kurosumi et al. (1968) describe three cell types, two of which are constant, glandular cells and myoepithelial, and one which is temporary or wandering and which might have migrated in from the surrounding connective tissue or blood vessels. These latter cells are histiocytes, granular leucocytes or lymphocytes.

At the onset of pregnancy there is an abrupt change in the mammary gland epithelium. Feldman (1961) describes three phases in the growth of the mammary gland; an initial stage of cellular proliferation in which mitoses are frequently encountered, a colostrum forming phase, and the lactational phase. Traurig (1967) found that not only was there a proliferative response to pregnancy, first noted on day 3 in the mouse, but this response followed a bimodal distribution. This distribution of proliferative activity is possibly correlated with the rise in prolactin during early pregnancy, followed by the rise of a mammotrophic hormone from the day 12 mouse placenta (Traurig, 1967).

During lactation mitoses are absent from the lactating gland (Jeffer, 1935a; Greenbaum and Slater, 1957a; Weatherford, 1929). Using biochemical parameters of mammary gland growth, Greenbaum and Slater (1957a) found the increase in tissue nitrogen rose slowly during pregnancy and lactation, but rapidly over the period of parturition. Greenbaum and Slater (1957b), Kirkham and Turner (1953), Tucker and Reece (1963a), Griffith and Turner (1959b, 1961) and Anderson and Turner (1968) found an increase in nuclear deoxyribonucleic acid (DNA) during pregnancy and an increase in ribonucleic acid (RNA) during lactation. Griffith and Turner (1959a) found that deoxyribonuclease activity of the rat mammary gland increased during pregnancy. The preceding suggest that the total concentration of DNA
increased during pregnancy, reflecting an increase in cell numbers. Following parturition there is no increase in cell numbers, only an increase in cell size as shown by an increase in RNA/DNA (Greenbaum and Slater, 1957b) and total nitrogen during lactation (Kirkham and Turner, 1953; Tucker and Reece, 1963a).

The second phase, as described by Feldman (1961), is that of colostrum formation. Colostrum formation is defined as the formation of secretory products before the first emptying of the gland by suckling or milking (Mayer and Klein, 1961). Colostrum formation occurs prior to parturition as well as during involution. Grynfeltt (1937) distinguished between true milk secretion where suckling evacuates the gland versus the secretion of colostrum, evacuated spontaneously and for the most part resorbed in the mammary ducts.

The low levels of RNA during pregnancy in contrast to lactational levels suggest that little synthesis is accomplished during pregnancy (Greenbaum and Slater, 1957b). What materials are found within the alveoli are thought to have originated elsewhere and to have been transformed through the cytoplasm to the Golgi and from there to the acinar lumen (Feldman, 1961). During colostrum formation the alveolar epithelium acts primarily as a transport of serum proteins, largely gamma globulin, while during lactation its function is that of a protein producer (Dixon et al., 1961). During lactation the milk may be said to have a double origin. First those components which are synthesized by the epithelial cells, and secondly the contribution produced by diffusion from the circulatory system (Azimov, 1959).

It should not be assumed that secretion does not occur prior to
parturition or that secretion stops with involution. Girardie (1968) concludes that the protein granules which are found within the alveoli are neither typical of lactation nor of pregnancy. She reports that they have been identified in mammary gland cells of a few virgin mice, rats and guinea pigs and in rabbits 3 months after the end of lactation.

The secretion of lipid is also not confined to the period immediately following parturition. Jeffers (1935a, b) reports that lipid occurs both prior to parturition and during pseudo-pregnancy. Fatty secretion is also reported to occur in epithelial cells during pregnancy (Da Fano, 1922; Dempsey et al., 1947; Girardie, 1968). During late pregnancy and lactation there is an increase in lipase activity within the mammary gland and a decrease in the surrounding adipose tissue. This suggests a change in enzyme activity to divert dietary lipid from storage in adipose tissue to mammary tissue for milk formation (Hamosh and Scow, 1970).

The third phase of Feldman's (1961) scheme of the development of the mammary gland is the period of lactation. There have been numerous studies on the cytology of milk secretion at the light (Da Fano, 1922; Dempsey et al., 1947; Grynfeltt, 1937; Jeffers, 1935a, b; Weatherford, 1929) and electron microscope levels (Bargmann, 1959; Bargmann et al., 1961; Bargmann and Knoop, 1959; Feldman, 1961; Girardie, 1968; Helminen and Ericsson, 1968a; Hollmann, 1959; Hollmann, 1966; Keenan et al., 1972; Kurosumi et al., 1968; Murad, 1970; Stein and Stein, 1967; and Wellings et al., 1960). The secreting epithelial cell, based on the descriptions of the preceding authors, contains parallel-arrayed cisternae of well developed rough endoplasmic reticulum. There is also a prominent Golgi apparatus which contains small dark secretion granules of a
protein nature. It is generally assumed that milk protein particles are formed in Golgi vacuoles and are then transported to the luminar side of the cell (Hollmann, 1959; Wellings et al., 1960; Wellings and DeOme, 1961). Once they arrive, the membranes of the vacuoles appear to fuse with the apical membrane of the epithelial cell and the contents of the vacuole are thereby released into the acinar lumen (Helminen and Ericsson, 1968a). There is normally no loss of cytoplasm during protein granule discharge.

Based upon analysis of phospholipid content of various membranes of the lactating mammary epithelial cells, Keenan et al. (1972) believe that the Golgi function not only in product compartmentation, but also in cytomembrane differentiation. Golgi function in the transformation of membranes from ergastoplasmic-like to membrane-like. This can be easily seen when the Golgi vacuoles merge with the free cytoplasmic membrane surface thus creating a greater surface area on the luminal side of the cell.

One other feature of the apical surface is the presence of numerous microvilli (Bargmann et al., 1961; Azimov, 1959; Grynfeltt, 1937). The increase in cell surface brought about by the microvilli is thought to be related to the resorptive process (Bargmann et al., 1961). Azimov (1959) has shown that there is a considerable resorption of products from the alveolar lumen simultaneously with the absorption of milk precursors from the blood. His investigations involved radio-active isotopes of phosphorus, calcium and sulfur which were injected into one teat and could then be detected in milk drawn from a different teat. This process was intensified during milking.

The other major secretion product of the alveolar cells, the milk fat,
is thought not to originate within membrane bound vacuoles as does the milk protein. The exact origin of the fat droplets remains unknown but they first appear in the basal portion of the cell cytoplasm (Bargmann et al., 1961) in the vicinity of an accumulation of tubular or vesicular elements of smooth endoplasmic reticulum (Kurosumi et al., 1968). Stein and Stein (1967), using a radio-active precursor of mammary gland glycerides, could find no evidence that lipid droplets were in any way associated with the Golgi. After esterification of the precursor into glycerides in the rough endoplasmic reticulum an aggregation of lipid occurred which formed the characteristic lipid droplet (Stein and Stein, 1967).

Lipid droplets after formation are then transported to the apical portion of the cell where they protrude through the plasma membrane into the lumen. Each fat droplet is then pinched off carrying with it a small portion of the cell cytoplasm. The lipid droplet and accompanying cytoplasm, if any, is surrounded by a membrane whose origin is the apical cytoplasmic membrane of the epithelial cell (Bargmann et al., 1961).

The elaboration and secretion of the smaller molecular constituents of milk has not been visualized.

The second major cell which is found in the alveoli is the myoepithelial cell. These cells lie on the alveolar side of the basement membrane but usually beneath the epithelial cells. Myoepithelium covers the stromal surface of the epithelium of alveoli, ducts and cisternae and is generally more abundant than realized (Richardson, 1949). The cells are stellate on alveoli but are fusiform on ducts where they lie outside two layers of epithelium (Silver, 1954).
In the past it has been assumed that these cells were contractile due to the close structural resemblance they bore to smooth muscle cells (Bloom and Fawcett, 1968 pp. 263-270). Linzell (1955), by a direct microscopical examination of the mammary gland, observed the contraction of the alveoli and a widening of the ducts when oxytocin was applied. As further evidence of the relationship between myoepithelial cells and smooth muscle cells, Archer and Kao (1968) describe an immunohistochemical technique for the identification of actomyosin in myoepithelium. Puchtler et al. (1966, 1969) describe a staining technique which is specific for the group of proteins known as keratin-myosin-epidermis-fibrin (K-M-E-F) and which can be used for the identification of muscle fibers and myoepithelial cells. Myoepithelial cells also appear birefringent when viewed with polarized light (Goldstein, 1961; Puchtler et al., 1969).

Myoepithelial cells are found not only in mammary glands (Cross et al., 1958; Hebb and Linzell, 1970; Linzell, 1952, 1955; Murad and von Haam, 1967), but also in other exocrine glands such as salivary glands (Emmelin et al., 1968, 1969, 1970; Hubner et al., 1969a, b; Tamarin, 1966; and Travill and Hill, 1963), lacrimal glands (Leeson, 1960; Leeson and Leeson, 1971; Luciano, 1967; Kuhnel, 1968; Scott and Pease, 1959; and Yamauchi and Burnstock, 1967), in sweat glands (Goldstein, 1961; Ellis, 1965), in the prostate (Mao and Angrist, 1966) and in the kidney (Harper et al., 1969).

With the use of the electron microscope are seen Golgi membranes, rough and smooth endoplasmic reticulum, numerous mitochondria, glycogen deposits, microtubules, lipid pigment in and around the peri-nuclear zone and myofilaments similar to those found in smooth muscle. A close packing of the myofilaments in some areas produces dense zones
identical to those of smooth muscle (Bloom and Fawcett, 1968, pp. 767-
775). In addition to these features, surface caveoli appearing on the
stromal side of myoepithelial cells (Tamarin, 1966) have prompted some
authors (Tamarin, 1966; Leeson, 1960; Ellis, 1965; Murad and von Haam,
1967) to speculate not only on the role in contraction of the myoepithelial
cell, but also on its ability to act in pinocytosis, cell division, the
production of basement membrane and in impulse transmission.

Using a silver impregnation technique, Richardson (1949) and Linzell
(1952) have demonstrated the myoepithelial cell in stained sections.
Silver (1954), using a modified Gomori (1939) method was able to demonstrate
phosphatase activity in myoepithelial cells. Cross et al. (1958), Murad
and von Haam (1967), Travill and Hill (1963) and Grossman and Stratton
(1969) have shown ATPase activity within the cytoplasm and plasma mem-
brane. These authors also conclude that the myoepithelial cell functions
in contraction.

It has been widely accepted that myoepithelial cells were of ectodermal
origin (Richardson, 1949; Hubner et al., 1969a, b; Goldstein, 1961) and
that they were derived from either the alveolar cells or the duct cells of
the developing mammary gland. Scott and Pease (1959) dispute this, how-
ever, due to a lack of any transitional forms seen between epithelial
cells and myoepithelial cells. They conclude that there is no evidence
that the two are related, but they give no information concerning the
origin of the cells.

With regard to the remainder of the cell types found within the
mammary gland, Kurosumi et al. (1968) simplify the problem by dividing
all cells of the gland into two groups, permanent (epithelial and
myoepithelial) and temporary. Temporary cells have migrated in from the surrounding connective tissue and blood vessels and lack secretion granules, droplets and myofilaments. They are either histiocytes, granular leucocytes or lymphocytes (Kurosumi et al., 1968). Helminen and Ericsson (1968a) suggest two temporary cell types which are present, lymphocytes and macrophages. Jeffers (1935a) found leucocytes in all stages of lactation and noted a conspicuous increase during involution as did Helminen and Ericsson (1968b, c). Slater (1962) reported a rise in mammary DNA due to an increase in leucocytes in involuting tissue. Paape and Desjardins (1971) found an increase in leucocytes in mammary tissue following nursing. Okada (1957, 1958a) found a decrease in circulating blood eosinophil counts and an increase in lymphoid cells in involuting mammary tissue. Okada (1958b) found that ACTH induced a migration of and penetration of lymphoid cells into the mammary gland.

The evidence supports the idea that an invasion of leucocytes is a dynamic phenomena, and that they are drawn to the mammary gland during involution to combat a local inflammatory response caused by a buildup of milk pressure (Okada, 1957).

One other concept of leucocytic invasion during involution concerns the formation of the colostrum corpuscles or corpuscles of Donne which were first described in 1838 by Donne (as cited by C. W. Turner, 1952, Chapter 12). Colostral secretions are produced when there is a change in the hormonal balance as occurs in the mammary glands of the young at birth, the end of pregnancy or pseudo-pregnancy, during the ovarian cycle and at the end of lactation. During these times the hormonal lactogenic stimulus acts on the secreting cells and the gland is not emptied of its' contents. At this time a highly
typical cytological structure is formed, the colostrum corpuscle. This structure is unicellular, has a cytoplasm which contains a large number of fat globules and it may be found within the lumen of the alveoli or recovered from the alveolar contents at any time during involution (Mayer and Klein, 1961).

The origin of the colostrum corpuscle is still uncertain. Lee et al. (1968), who refer to colostrum corpuscles as foamy cells due to the presence of Oil Red O stainable fat, suggests that they are macrophages responsible for the removal of fat from the involuting gland. Their view is shared by Lascelles et al. (1969) even though phagocytosis of available fat could not be demonstrated in vitro by freshly harvested colostral cells. Okada (1956a, b) has shown that teat ligation caused an inflammatory process in the mammary gland resulting in an infiltration of lymphoid elements which were transformed into colostrum bodies. Again, however, the absorption of milk fat by these bodies was not demonstrated (Okada, 1956a).

Other investigators have supported the idea of an intra-alveolar origin for colostral cells. Maeder (1922) states that there is nothing to indicate that in the rat leucocytes have anything to do with the removal of secretion. Bratianu and Guerriero (1930) speculate on the possibility that epithelial cells of the alveoli themselves may become phagocytic and remove fat droplets during involution. Richards and Benson (1971c) suggest that macrophages might in reality be only epithelial cells. Hollmann and Verley (1967) and Verley and Hollmann (1967) feel that the colostrum bodies correspond to degenerating epithelial cells which become liberated in the alveolar lumen.
Histology and Cytology of the Involuting Mammary Gland

The normal trigger for the onset of involution in the mammary gland is the cessation of nursing. The immediate effect on the rat mammary gland following enforced weaning is an increase in the weight of the gland due to an accumulation of milk secretion (Ota, 1964). A significant weight difference was reached 6 hours after weaning which reached a maximum 12 hours after weaning, but 24 hours later the gland lost weight (Ota, 1964).

Maeder (1922) found that after weaning, stained sections of the gland showed practically the same appearance as those taken during lactation. After 3 days, the number of distended alveoli markedly decreased, but secretory activity was still present in some alveoli. At this stage a few pycnotic epithelial cell nuclei were also seen. At day 4 of involution the ducts were still well distended and now appeared more prominent. The alveoli had disappeared to such an extent that the stroma and parenchyma now appeared equal in amount. Secretory activity was still evident in some alveoli, but atrophy was almost universal. Pycnosis was more frequent.

By day 8 the glandular tissue was reduced to small chords with small or no lumina and tiny fat droplets were present in the epithelium as well as the lumina. Many nuclei were pycnotic and the nuclear substance greatly exceeded the cytoplasm in volume. By day 9 the gland approached the virgin state in appearance and the virgin condition was fully reached by days 13 to 28.

Da Fano (1922) noted that during involution the number of detached epithelial cells increased. This condition reached such proportions that a continuous passage of detached epithelial cells into the glandular lumina was described. Jeffers (1935a) noted that during lactation a variable
amount of cellular degeneration was found. She states that degenerating cells had no demonstrable cell membrane and that their cytoplasm extended into the lumen. She also commented on the presence of leucocytes which appeared in all stages of lactation and whose numbers increased during involution. Colostral cells are also found which was typical of involution (Mayer and Klein, 1961). Okada (1956a, b) also found colostrum bodies occurring at the later stages of pregnancy and lactation and at the whole stage of regression. Desquamating cells also found during lactation and regression were found not to be related to colostrum bodies (Okada, 1956a).

Okada (1957, 1958a, b) felt that involution produced an inflammatory condition within the mammary gland. This was followed by a penetration into the mammary gland by neutrophils and lymphoid cells and the appearance of colostrum cells. This suggested an influence by the adrenal cortex on involution. Adrenalectomized and normal nursing rats given exogenous adrenaocorticotrophin (ACTH) showed an increase in lymphoid cells in the region of the mammary alveoli suggesting a close relationship between the two types of cells.

Lee et al. (1968) and Lascelles et al. (1969) also felt that colostrum cells occurring during involution were a product of macrophage migration and fat phagocytosis.

Verley and Hollmann (1967) on the other hand found that during involution the gland tissue is reduced by degeneration and elimination of isolated cells without obvious rupture of the gland tree. Myoepithelial cells bridged the gaps left by necrotic cells and held the surviving cells together. Colostrum bodies corresponded to the degenerating epithelial cells and were eliminated in the acinar lumen.
At the level of the electron microscope, mammary gland epithelial cells, which are deprived of their hormonal stimulus in a situation such as might be encountered during weaning, would be expected to show ultrastructural alterations. In a study of such cells by Gagne and Pitelka (1970), the normal polarity of epithelial cells, in which the nucleus and the origins of fat globules were basically situated, was lost. Also changed were the apical locations of the Golgi and the protein vacuoles. Other alterations noted in this tissue culture study were a vesiculation of the endoplasmic reticulum, distortion of Golgi bodies and mitochondria, a disappearance of microtubules and a smoothing of cell surfaces (Gagne and Pitelka, 1970).

Richards and Benson (1971a) found that at an ultrastructural level, involution began with the removal of protein granules from the milk by structures known as stasis vacuoles located in the alveolar epithelial cells. Later stages of involution involved a progressive necrosis of the epithelial cells by auto-phagocytosis. Removal of fat droplets was accomplished by macrophage-like cells (Richards and Benson, 1971a). After removal of litters, mammary gland ultrastructure of the dams was progressively maintained by treatment with prolactin, prolactin plus growth hormone or prolactin plus ACTH. An increase in the number of fat droplets was noted following hormone treatment (Richards and Benson, 1971b). Reserpine treatment also increased the number of fat droplets seen in these cells. All treatments failed to alter the distribution of macrophage-like cells.

In a further investigation of the cells described as macrophages by Helminen and Ericsson (1968a), Richards and Benson (1971c) labelled macrophages found in involuting rat mammary glands with trypan blue or
horseradish peroxidase. This treatment showed the macrophages on the fourth day of lactation, the day the litters were removed from their mothers, to the fifth day of involution to be present in the connective tissue stroma. However, on the sixth day of involution they were congre­gated around the remaining islets of alveoli. Different cells of a non-epithelial origin were present on day 4 of lactation and increased numerically during the first two days of involution and then decreased. This second type of cell was similar in its staining reaction to the cells of the regional lymph node, and it is believed by the authors that this second cell type was a lymphocyte. Richards and Benson (1971c) further suggest that the macrophages reported in the literature were in reality epithelial cells which had become phagocytic.

An alternative to the hypothesis of migrating cells which phagocytize the luminal contents during mammary involution (Okada, 1958a, b; Lee et al., 1968; Lascelles et al., 1969) is also offered by investigators who believe that the end results of such phagocytosis, the colostrum body, is of epithelial origin. Hollmann (1966) found a fusion and rearrangement within the cytoplasm of the secreted protein formed the so-called "stasis vacuole". Hollmann and Verley (1967) believe that these secretory protein granules are resorbed and digested in the cytoplasm of the epithelial cells without apparent participation of lysosomes. The liquified homogenate so produced is then probably carried to the still well developed capillaries. Dying cells become vacuolated and are either eliminated as colostrum bodies or lysed and eliminated in the interstitium (Hollmann and Verley, 1967).

Helminen and Ericsson (1968b) found that two types of phagocytosis accompanied the involuting mammary gland. Autophagocytosis was indicated
by the presence of a structure known as a cytosegresome. Here a portion of the cell's own cytoplasm is digested resulting in the formation of myelin figures from the residual membranes. Heterophagocytosis is accomplished by an uptake of a portion of the epithelial cell cytoplasm by a macrophage. Cytosegrosomes produced by either process are transformed into cytosomes, structures containing dense and membrandeous material. Cytosegrosomes, which are extremely rare in lactating glands, increase as involution progresses. Autophagocytosis is more pronounced during early involution and heterophagocytosis during late involution (Helminen and Ericsson, 1968b).

Using electron microscopy, Brandes et al. (1969) were able to show the development of numerous lysosomes and areas of focal cytoplasmic degeneration in the glandular cells following enforced cessation of lactation in the rat. At 2 days following cessation of lactation very few areas of phosphatase activity could be detected, but activity was intense on day 4 post lactation. Necrotic cells still contained intact lysosomes including residual bodies with myelin figure formation.

Of the various biochemical parameters that have been used to describe the physiological state of the mammary gland, the measurement of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) have found wide acceptance. Since the DNA per nucleus was found to be consistent in virgin and pregnant mammary gland tissue (Griffith and Turner, 1957), and the DNA per mammary cell nucleus was not shown to differ significantly between pregnant and lactating rats (Tucker and Reece, 1962), the DNA content of the mammary gland has been used as an index of development. The ratio of RNA/DNA has also been used as a measurement of the protein synthesizing activity of the
cell (Tucker and Reece, 1963a). Using these measurements, it has been accepted that the DNA increase of the mammary gland throughout pregnancy is consistent with the findings that mitoses occur frequently in the developing gland during pregnancy (Jeffers, 1935a).

The increase in DNA in the mammary gland during lactation has been assumed to represent hyperplasia during early lactation (Tucker and Reece, 1963b). It is not entirely clear how this finding can be reconciled with the observations that mitoses do not occur during lactation (Weatherford, 1929; Greenbaum and Slater, 1957a). A possible explanation might be found in the investigations of Izuo et al. (1971). In studying the DNA content of alveolar cells of lactating mammary tissue they found that the gland contained as many as 13% tetraploid cells. This increase in cellular DNA above the normal diploid state may represent an attempt of the cell to meet physiological demands placed on it during lactation when mitoses are not possible.

Based on the DNA content of the mammary gland (Ota, 1964) three phases of involution have been proposed. In phase I, milk formation continues but then becomes weak and ceases. This phase lasts up to 24 hours. Phase II lasts for 4 to 6 days and is evidenced by the disappearance of active secretory tissue. Milk which is contained within the alveoli and ducts is rapidly resorbed. Phase III is represented by a collapse of the secretory tissue and a return to a rudimentary duct system. A decrease in RNA content during involution was thought related to a cessation of milk secretion (Zarzycki et al., 1969). The rise in DNA noted by some investigators at this time has been thought to be indicative of an increase in leucocyte invasion of mammary tissue during involution (Tucker and Reece, 1964).
Four lysosomal enzymes: acid phosphatase, aryl sulfatase, cathepsin D and acid deoxyribonuclease, were measured during involution in the rat mammary gland (Helminen and Ericsson, 1968d). The results showed an increase in all enzymes on day 2 with a maximum reached on days 3 to 5. Acid phosphatase and aryl sulfatase seemed to be present in the same lysosome. Stasis vacuoles containing protein secretion droplets seen during early involution did not show activity of acid phosphatase and aryl sulfatase and presumably are unrelated to lysosomes (Helminen and Ericsson, 1968c). Zarzycki et al. (1969) also found an increase in acid phosphatase related to involution. The presence of alkaline phosphatase found in the secretory sections of the gland suggest that the participation of this enzyme is involved with the resorption of the substances secreted with milk by the epithelial cells.

Hormonal Influence on Mammary Gland Development and Lactation

The literature is extensive on the subject of hormones which aid in the development of the mammary gland during pregnancy and the initiation and maintenance of lactation. It is beyond the scope of this review to evaluate all hormonal influence on the mammary gland, and only those hormones which are most important from the standpoint of subject of this paper will be considered in any detail. A more comprehensive review is provided by Denamur (1971).

Most investigators are in general agreement as to the requirements for the organ culture of mammary tissue. Elias (1959) found cortisol, prolactin and insulin produced a darkly stained secretion in intracellular vacuoles
in prelactating mouse mammary tissue. Anderson and Larson (1970) found that cortisol was necessary in addition to prolactin, aldosterone and insulin in order to induce the synthesis of β-lactoglobulin and β-casein. Turkington and Riddle (1970) noted that insulin and prolactin stimulated the formation of ribosomes and their organization into polyribosomes in mammary cells pretreated with insulin and hydrocortisone. Prolactin caused a marked stimulation in cultures with insulin which was, on another occasion, only slightly increased by the further addition of corticosterone (Mayne and Barry, 1970). Ceriana (1970a, b) found that the most effective hormone combination in stimulating casein-like synthesis in vitro consisted of insulin plus prolactin plus aldosterone plus progesterone. Ultrastructural studies of such cells revealed an organizational level similar to the mammary gland of a new born rat (Ceriana et al., 1970). El-Darwish and Rivera (1970) also found that the triad of insulin, prolactin and corticosterone to be the most effective in secretion development. The addition of prolactin was found to induce an increase in the number of polyribosomes bound to membranes (Gaye and Denamur, 1969).

It appears that the corticoids are responsible for the formation of the rough endoplasmic reticulum while prolactin and insulin are involved in the redistribution of organelles. Prolactin also promoted RNA synthesis and the completion of enzyme complements (Forsyth, 1971). Insulin alone is not capable of maintaining the initiation of mitotic activity or of inducing alveolar development. The addition of prolactin is necessary in tissue culture for the stimulation of alveolar development (Dilley, 1970).

In discussing the in vivo hormonal requirements of the mammary gland it must be remembered that species vary greatly in their responses to the
same hormone and in their hormone production. It would be foolhardy, therefore, to attempt an integrative theory of the hormonal mechanisms controlling lactogenesis and pregnancy (Denamur, 1971). As an example of the variations produced in mammary development, Cowie and Folley (1961) cite three categories of responses to an ovarian hormone. The first is exemplified by the mouse, rat, rabbit and cat. Endogenous estrogen produced mainly duct growth. In the second group are those animals in which endogenous estrogen caused both duct and lobule-alveolar growth. The guinea pig, cattle and goats are good examples of this category. In the last group are those species in which estrogen caused little or no mammary growth. Examples of this group are the dog and ferret.

Benson et al. (1957) found that in the guinea pig, better mammary gland development could be obtained if progesterone was administered simultaneously with estrogen. Moon et al. (1959) have shown that in the rat, mammary gland growth comparable to that of pregnant rats could be obtained with a daily dose of 1 µg estrogen and 2 mg of progesterone. Similar results were found in the mouse (Traurig and Morgan, 1964). Variability found among treated animals was explained as a result of the endogenous secretion of thyroxine, growth hormone, and glucocorticoids which synergized with the ovarian hormones (Moon et al., 1959). Lyons et al. (1958) found that prolactin and growth hormone were necessary along with estrogen and progesterone to produce full mammary development. Hamberger and Ahren (1964) have suggested that the ovarian hormones can act only when anterior pituitary hormones are present in the tissue. The pituitary hormones therefore exert a sensitizing or permissive action for the steroid hormones to complete lobulo-alveolar development. The thyroid has also been
implicated as exerting a limiting factor in mammary growth when estrogen and progesterone secretions were adequate (Moon and Turner, 1960).

Although many, and possibly all, hormones are in some way associated with milk production, if any one hormone had to be singled out as the hormone of lactation it would almost certainly be prolactin. Prolactin is produced in the pituitary and its secretion is chronically inhibited by a smaller molecular weight protein, prolactin inhibiting factor (PIF) (Meites, 1966). Normally the suckling stimulus during lactation induces the release of prolactin by reducing the amount of PIF in the hypothalamus. It is thought that ACTH as well as oxytocin is also released at this time (Johnson and Meites, 1958). The disruption of lactation following hypophysectomy confirms the earlier theory of Selye (1934) that prolactin released from the anterior pituitary as a result of the suckling stimulus is necessary for the maintenance of milk secretion (Cowie, 1957). Convey (1969) found an 88% reduction in the prolactin content of rat pituitaries following suckling. Amenomori and Nellor (1969) found a reduction in serum prolactin levels of dams following removal of litters. Johke (1971) also noted a rapid increase in serum prolactin following milking in the goat and sow.

Another potent hormone acting upon the prolactin content of the pituitary is estrogen. In vitro investigations have shown that when anterior pituitaries were cultured in the presence of estrogen, more prolactin was released into the medium than from control pituitaries incubated in the absence of estrogen (Meites et al., 1961; Nicoll and Meites, 1962; Gala and Reece, 1964) In vivo injections of exogenous estrogen confirm the in vitro increase in prolactin in response to estrogen
(Meites and Turner, 1942a; Ratner et al., 1963; Minaguchi et al., 1968; Chen and Meites, 1970). The best results for the release of prolactin were obtained with a dosage of from 0.1 to 5 µg of estrogen. Higher doses of from 10 to 500 µg produced smaller increases in serum prolactin (Chen and Meites, 1970).

Estrogen implants in the hypothalamic region also had a positive effect on pituitary prolactin content. Kanematsu and Sawyer (1962) found that estrogen implants in the posterior tuberal hypothalamus promoted synthesis and storage of prolactin while direct pituitary implants caused a release of prolactin. Ramirez and McCann (1964) have shown that implants into the median eminance region induced an increase in the release and synthesis of prolactin by the anterior pituitary.

The effects of other hormones, most notably progesterone, on pituitary prolactin content have also been studied (Reece and Bivins, 1942; Sar and Meites, 1968). The general effect seems to be one of no significant increase or reduction in pituitary prolactin. The amount of progesterone used in these studies has been large, however, in the range of 10-15 mg/day, and the possibility that progesterone may be converted to an estrogen in vivo before acting on the hypothalamus cannot be ruled out (Sar and Meites, 1968).

Although secretions are present in the alveoli of the mammary gland prior to parturition and after lactation ceases (Girardie, 1968), full lactation is normally not assumed to have started until after parturition. In fact, some authors regard parturition as the trigger for the initiation of lactation (Meites and Turner, 1948). Following parturition the placentae are lost along with any hormones which have been functioning
during pregnancy (Leonard, 1945). The ovaries also assume a different functional state after parturition, and the steroid hormones which they produce in large amounts during pregnancy are reduced drastically (Atkinson and Hooker, 1945). By removing the ovarian hormones by ovariectomy midway through pregnancy, Liu and Davis (1967) were able to induce lactation in the rat thus demonstrating the depressing action of the ovarian hormones on lactation.

Talwalker et al. (1961) were able to induce mammary secretion in the rat and rabbit by the administration of hydrocortisone acetate to pregnant rats suggesting that the level of adrenal corticoids may not be sufficiently high during pregnancy for lactation to start. Prolactin, oxytocin and adrenal corticotrophic hormone were ineffective. From this study it was felt that the ovarian hormones might depress synthesis of glucocorticoids and this prevented lactation during pregnancy.

The exact opposite was found by Meites and Turner (1948) who injected various hormones into the teats of pseudo-pregnant rabbits and found that only prolactin and whole pituitary extract could induce a localized lactation in the rabbit. ACTH was not effective in initiating lactation in this study. They concluded that lactation was not initiated during pregnancy due to insufficient prolactin secretion or synthesis.

Evidence for the initiation of lactation in suitably primed mammary gland tissue is provided through the investigations of Meites and co-workers. Injection of hypothalamic or cerebral tissue into estrogen primed rats induced lactation suggesting that the hypothalamus produced a release of prolactin in the primed rats sufficient to initiate lactation (Meites et al., 1960). Pituitaries, which are known to secrete prolactin
when removed from the inhibitory influence of the hypothalamus, were transplanted into rats whose mammary glands had been developed with estrogen injections. Of twenty-four rats receiving the transplanted pituitaries, twenty-three rats showed secretion in their mammary glands. These rats also possessed ovaries with large corpora lutea (Meites and Hopkins, 1960). Reserpine, which is assumed to induce secretion of prolactin, when injected into estrogen-primed rabbits produced mammary growth and lactation (Meites, 1957). Nonspecific stresses of intense heat, light and cold are also able to induce lactation in primed animals by promoting the release of prolactin and ACTH (Nicoll et al., 1960).

The absence or depression of pituitary prolactin during pregnancy (Meites and Turner, 1948; Buttle and Forsyth, 1971) provided a simple explanation of why lactation did not occur during pregnancy even though some studies have been made showing an increase in pituitary prolactin during the first two-thirds of pregnancy in the rat (Grosvenor and Turner, 1960). The problem of determining serum prolactin levels during pregnancy is further confused by reports of a compound originating in the placenta of the rat known as rat placental lactogen and which has mammotrophic activity in rats primed with estrogen (Shani et al., 1970).

Another hormone of major importance in lactation is the posterior pituitary hormone oxytocin. It was first shown in 1910 (Ott and Scott) that the active principle in the posterior pituitary greatly increased the secretion of milk when injected into the ear vein of a lactating goat. These results were interpreted by Turner and Cooper (1941) as indicating that the posterior pituitary contained a compound causing a contraction of the mammary gland enabling milk, which is otherwise not available, to be
removed. Of the compounds tested for a similar action, pitocin, pitressin, epinephrine, histamine, ergonomine and acetylcholine, only pitocin and acetylcholine caused complete evacuation of milk in a perfused lactating mammary gland (Petersen, 1942). The pituitary compound responsible for this milk let-down action has been identified as the octapeptide amid oxytocin and was synthesized by du Vigneaud et al. (1953).

Richardson (1949) proposed a network of myoepithelial cells surrounding the alveoli of the mammary gland which contracted in response to oxytocin released from the posterior pituitary during suckling. A neural pathway has been proposed and its existence proven which carries the suckling stimulus from the nipples to the pituitary (Eayrs and Baddeley, 1956; Richard et al., 1970).

The released oxytocin has a half-life of only several minutes (Ott and Scott, 1910), and it is inactivated by the liver, kidney and splanchnic vascular area (Ginsburg and Smith, 1959; Chaudhury and Walker, 1959; Aroskar et al., 1964).

Benson and Folley (1956, 1957; Benson et al., 1960) observed that mammary involution in the rat was retarded when exogenous oxytocin or oxytocin analogues were given. They interpreted their findings as suggesting that oxytocin could directly evoke the release of prolactin from the pituitary. This theory has been disputed by Meites et al. (1963) who found that oxytocin in no way increased the release of prolactin from cultured rat pituitaries. The mechanical action of the myoepithelium may have a more indirect action in retarding mammary involution by the expulsion of milk from the alveolar lumen. Levy (1964) has shown that rat milk contains an inhibitor to fatty acid synthesis. By forcibly expelling this
inhibitor from the lumen, further synthesis could be promoted and involution thus retarded.

It appears that estrogen and progesterone are necessary for gland development during pregnancy in the rat, but during lactation they have a depressing effect on the volume of milk produced by lactating dams. Since the same mechanism may be acting to suppress lactation both during pregnancy and lactation it is necessary to investigate this relationship in more detail as an aid in understanding the action of the gonadal steroids on the mammary gland during lactation.

Nelson (1935) found that the removal of ovarian grafts in the guinea pig induced lactation. Also lactation, in the presence of the pituitary, was initiated when estrogen injections were discontinued (Nelson, 1937). These results led him to establish a theory of estrogen inhibition of lactation during pregnancy. According to his theory, the high levels of estrogen released in late pregnancy suppressed the secretion or release of prolactin from the pituitary and also had a direct inhibitory effect on the mammary gland.

This theory might be challenged on two points. The first is that estrogen levels are not uniformly high in all species during pregnancy (Atkinson and Hooker, 1945; Yoshinaga et al., 1969). The second is that of the two threshold level of estrogen action (Folley and Malpress, 1948). Estrogen is thought to stimulate prolactin at low or microgram levels (Ramirez and McCann, 1964; Ratner et al., 1963; Bruce and Ramirez, 1970) and to be toxic or inhibitory at higher levels (Meites and Turner, 1942). Thus at the higher levels with which Nelson was dealing, it is understandable how he could establish an inhibitory effect from the dosage of
exogenous estrogen used.

By far the theory most quoted concerning inhibition of lactation during pregnancy is that of Meites and Turner (1942a, b, c, d, e, 1948; Meites et al., 1963; Meites, 1966) who report that prolactin levels are suppressed during pregnancy and are therefore insufficient to initiate lactation. Their theory in general states that high levels of estrogen and progesterone during pregnancy stimulate lobulo-alveolar development of the gland. Despite the fact that estrogen can increase prolactin content and release from the pituitary, progesterone is dominant over estrogen and serves to block the stimulating action of estrogen on pituitary prolactin. At parturition there is a sharp decline in progesterone (Hashimoto and Melampy, 1967; Csapo et al., 1971; Yoshinaga et al., 1969; Atkinson and Hooker, 1945; Kuhn, 1969) and the pituitary level of prolactin rises in response to the still present levels of circulating estrogen. It is the relative ratio of estrogen to progesterone that is thus important in initiating lactation. Reduced glucorticoids may also be a factor at this time (Barnawell, 1967) as is the fact that the tissue of the mammary gland during pregnancy may be refractory to the stimulatory action of prolactin (Meites and Sgouris, 1953; Meites, 1966).

The natural question raised in response to this theory is how animals may be simultaneously pregnant and lactating if the estrogen and progesterone block to prolactin theory is correct. Chatterton (1971) explains this seeming contradiction by noting that pregnancy is not necessarily incompatible with lactation. He further says that sufficient levels of estrogen may be missing during this time to inhibit prolactin, and progesterone in the absence of estrogen is not inhibitory (Meites and
Low levels of estrogen may also interfere with the metabolism of progesterone and thus the metabolites of progesterone necessary for inhibiting the synthesis of milk may not be produced in sufficient quantities. Bruce (1961) states that although an apparently normal lactation could be maintained during pregnancy in rats, toward the end of pregnancy lactation failed despite continued suckling.

Another question arises as to which of the various ovarian hormones is effective in inhibiting lactation. Kalra et al. (1970) found that a single injection of progesterone in estrogen-pretreated-ovariectomized females lowered serum prolactin levels while estrogen increased serum prolactin levels. Clemens et al. (1971) noted that progesterone raised the threshold for excitement of neurons involved in the release of prolactin while estrogen lowered it. Ben-David et al. (1964) have shown that estradiol stimulates while progesterone depresses prolactin release in vitro. Nelson (1937) found that lactation in response to exogenous pituitary extracts was prevented if estrogen was simultaneously administered. Herrenkohl (1971) has shown that progesterone injections during pregnancy effectively inhibited lactation in the rat while Bruce and Ramirez (1970) have shown that estrogen implanted into the mammary gland inhibited lactation.

An important step in the synthesis of lactose requires the synthesis of two protein components of the lactose synthetase enzyme. These proteins, galactosyltransferase known as the A protein and α-lactalbumin known as the B protein, are asynchronously present during pregnancy and lactation. The level of the A protein increased to a maximum toward the end of pregnancy while the B protein remained low during pregnancy but rose at the onset
of lactation (Turkington et al., 1968). The level of these two proteins is probably dependent on hormone levels during pregnancy and Turkington (1971a, b) has shown that progesterone is able to inhibit in vitro the induction of \( \alpha \)-lactalbumin.

The majority of the papers presented here suggest that the progesterone level is the main factor in determining the lactational state of the mammary gland. Estrogen also appears necessary, in physiological amounts, as Katzman et al. (1971) and Corvol et al. (1972) have shown that an estrogen pretreatment increased the uptake and retention of progesterone. Other studies (Reece and Bivins, 1942; Ben-David et al., 1965; Sar and Meites, 1968; Chen and Meites, 1970) in which progesterone has been reported to have a facilitatory effect upon milk secretion can be explained by the fact that the large doses of progesterone employed may be converted in vivo into estrogen and this stimulates rather than inhibits milk synthesis (Sar and Meites, 1968). The toxic effects of large doses of estrogen have already been mentioned.

Another ovarian hormone which may also be influencing lactation during pregnancy and also during exogenous estrogen and progesterone treatment is relaxin. Relaxin is a heat labile crystalline solid protein produced in the corpus luteum (Fevold et al., 1930; Herrick, 1928) uterus (Wislocki et al., 1957; Baker, 1948; Belt et al., 1971) and placenta (Hisaw and Zarrow, 1948). Its appearance during pregnancy and during the luteal phase of the estrous cycle (Hisaw, 1926; Hisaw and Zarrow, 1948) implies that estrogen and/or progesterone are associated with its rise in concentration during these periods. The main function of relaxin is assumed to be in the relaxation of the pubic ligaments prior to parturition.
This relaxation is thought to be as a result of a modification of the stromal connective tissue which the hormone renders, in synergism with estrogen, a short swollen ligamentous mass (Frieden and Velardo, 1952; Crelin, 1954; Jablonski and Velardo, 1957a, b, 1958). It has also been shown to inhibit uterine motility and stimulate uterine growth (Wada and Turner, 1963a, b).

The necessity of using intact rather than ovariectomized rats to demonstrate an estrogen-progesterone inhibition of lactation (Barsantini and Masson, 1947; Griffith and Turner, 1962) has led to the proposal that the estrogen-progesterone inducement of relaxin might be a factor in gland function (Wada and Turner, 1958). Estrogen, progesterone and relaxin have been shown to synergize in the development of the mammary gland (Wada and Turner, 1959a, b; Hamolsky and Sparrow, 1945). Trentin (1951), however, found little or no increase in the percent of positive mammary alveolar responses in ovariectomized mice treated with estrogen, progesterone and relaxin as compared to those treated with estrogen and progesterone only. Knox (1966) found that estrogen and progesterone acted synergistically to diminish the amount of milk available although the DNA content of glands from treated rats did not differ from those of controls. These DNA levels support an earlier paper by Griffith and Turner (1962) who concluded, in a similar study, that DNA levels were not different between intact lactating rats receiving exogenous estrogen and progesterone and controls. Since milk yield and thus pup weight gains were lower in dams receiving the hormones it appeared the effect might involve milk let-down and an interference with the normal contraction of the myoepithelial cells. Sawyer and Frieden (1952) demonstrated that relaxin-containing extract of sow ovaries
was able to cause a definite inhibition of the spontaneous motility of uteri from proestrous, estrous or pregnant rats. This inhibition did not, however, appear to reduce the sensitivity of the rat uterus to the oxytocic effects of pituitrin (Sawyer and Frieden, 1952).

To the extent that the action of smooth muscle of the rat uterus may be compared to the action of the mammary gland myoepithelial cells in response to oxytocin, Pose and Fielitz (1961) found that large doses of progesterone in vitro in the human, do not suppress uterine contractility in response to oxytocin as they do in the rabbit. On the other hand, Pinto et al. (1967) found that progesterone did diminish spontaneous activity of the uterus and the activity elicited by oxytocin. Based on the preceding reports it would appear that any such diminished activity of the uterus or of the myoepithelial cells of the mammary gland in response to progesterone is a direct local action on the contractile elements themselves.

In an attempt to demonstrate the in vivo effects of progesterone on oxytocin release, Nishimura and Manabe (1967), using a metreuynter, measured the oxytocin released in response to the Ferguson reflex (Ferguson, 1941). Estrogen and progesterone were administered by various routes prior to the investigation. These authors found that although the oxytocin sensitivity of the uterus was increased, no conclusions could be drawn concerning the effects of the gonadal hormones. In a similar investigation more conclusive results were obtained. Using goats, Roberts (1971) found that progesterone inhibited the vaginal distention release of oxytocin. This same mechanism may also be working as a progesterone inhibition of oxytocin release during pregnancy and may account for the
reports that under conditions of exogenous progesterone, the alveoli of the mammary glands of lactating rats appeared full of milk suggesting a failure in the milk let-down process (Griffith and Turner, 1962; Herrenkohl, 1971).

In conclusion this review has shown that exogenous estrogen and progesterone administered during lactation will cause a reduction in the milk output resulting in lower pup weight gains. The evidence presented here in general favors a reduced prolactin level as the cause, but other mechanisms such as an inhibition in milk synthesis caused by progesterone acting directly on the enzymes which control milk synthesis cannot be ruled out. An interference by progesterone on the milk let-down mechanism is also a possibility.
METHOD OF PROCEDURE

Pregnant Sprague-Dawley-Rolfsmeier rats undergoing their first pregnancy were obtained from the Dan Rolfsmeier Company, Madison, Wisconsin, and housed individually in an air conditioned room with a temperature of approximately 25°C and a lighting cycle of 14 hours of daylight. They were given Wayne Lab Blox and water ad libitum. The day their pups were born was counted as day zero of lactation. Subcutaneous daily injections of 1 μg estrogen and 3 mg progesterone dissolved in corn oil were begun on day 1 of lactation and continued throughout the period of study. On day 3 of lactation pup litters were randomly reduced to 6 pups. On days 4, 9 or 14 the dams were sacrificed using ether and tissue samples taken from their pectoral and abdominal-inguinal mammary glands and fixed as rapidly as possible. Dams given corn oil only served as controls.

Tissues were fixed in paraformaldehyde-glutaraldehyde fixative (Karnovsky, 1965) and washed overnight in cold 0.1M phosphate buffer followed by a post-fix in 1% osmium tetroxide (Palade, 1952) in 0.1M phosphate buffer (Millonig, 1961). Following the post-fix, tissues were rinsed in 0.1 phosphate buffer, dehydrated through a graded series of ethyl alcohol and propylene oxide and embedded in either Epon-Araldite (Anderson and Ellis, 1965) or pure Epon (Luft, 1961).

Sections 0.5-1 microns thick for light microscopy were cut on an LKB Ultrotome-II using glass knives and transferred to a drop of water on a cover slip and allowed to dry on a 55°C slide warmer. Sections were then stained on the slide warmer using methylene blue and Azure II (Leeson and Leeson, 1970), toluidine blue (Leeson and Leeson, 1970; Trump et al., 1961;
Lynn, 1965), Paragon-1301 (Paragon C. & C. Company, Bronx, New York) (Spurlock et al., 1966) or methylene blue and basic fuchsin (Aparicio and Marsden, 1969). Cover slips were placed on a drop of immersion oil on microscope slides and ringed with fingernail polish.

Silver sections for electron microscopy were cut on the same microtome and picked up on uncoated copper grids. Sections were stained with uranyl acetate (Watson, 1958) and lead citrate (Venable and Coggeshall, 1965) and examined on an RCA EMU-3F electron microscope.
RESULTS

Light Microscopy

Of the various stains used, methylene blue and Azure II in 1% borax (Leeson and Leeson, 1970) gave the most consistent results. The only disadvantage noted was that of a nearly monochrome reaction with mammary tissue. All structures appeared in shades of blue. Lipid, previously fixed in osmium, stained a blue-gray.

With respect to polychromasia, Paragon-1301 (Spurlock et al., 1966) proved superior to the methylene blue-Azure II combination. Paragon-1301 contains a red and blue component in an unknown solvent. The spectrograph of this stain resembled basic fuchsin and methylene blue. Unfortunately, the results with this stain, which when good resembled paraffin sections stained with hematoxylin and eosin, are capricious with mammary tissue when fixed as described in this paper. Occasionally, tissues stained with Paragon-1301 were entirely lacking in the blue component. At times, although both colors were present in the stained tissue section, the stain was very light; while at other times the staining in some sections was uneven.

Visually the most pleasing stain was a combination of equal portions of 1% methylene blue in 1% borax and Paragon-1301. These stains produced sections whose colors resembled those of the original Paragon-1301 without the disadvantages of uneven or spotty staining. The resulting combination produced sections whose cell cytoplasm was colored in shades of purple, the nuclei were outlined in a deeper purple, collagen fibers showed red to pink, fat globules were stained blue-gray and Epon surrounding the
section was clear.

In almost all cases a pre-treatment of the sections with a 1% solution of periodic acid (Richardson, Jarett and Finke, 1960) for 15 seconds on a 55°C hot plate resulted in a more uniform and intense stain. The use of the periodic acid is similar to the actions of H₂SO₄-H₂O₂ (Pool, 1969) and KMnO₄-oxalic acid (Shires, Johnson and Richter, 1969) and served to oxidize and solubilize the reduced osmium (Pool, 1969).

In all cases, staining was poor without the use of borax. It was necessary to dissolve each stain used, with the exception of the Paragon-1301 which was already in solution, in an aqueous 1% solution of sodium borate to achieve the high pH necessary for staining (Lee and Hopper, 1965; Bennett and Radimska, 1966) and thus avoid the affinity of a compound in the plastic for acid dyes. Staining, for example, with the acid dye eosin near neutral pH resulted in the entire tissue section uniformly taking up the dye without any differentiation even between clear Epon and tissue.

After sections were stained and mounted in conventional mounting media, it was thought desirable to weight the cover slips in order to reduce as much as possible the thickness of the mounting medium between the cover slip and the slide and then to dry the slides on a hotplate overnight. In many cases this treatment resulted in severe fading of the stained tissue, especially those slides stained with Paragon-1301.

In order to minimize fading, all sections were mounted in immersion oil and fingernail polish was used as a sealant around the cover slips. To insure that there was a minimum distance between the objective lens of the microscope and the tissue section and to avoid the necessity of having to weight the cover slips, sections were transferred from the glass knife
directly to cover slips and then heated to dryness on a hot plate at 55 °C. Staining was also carried out directly on the cover slip. No adhesive was used to mount the sections, and they generally adhered well to the cover slips. Epon-Araldite embedded sections adhered better than those embedded in straight Epon, but if moderate care was used in staining and washing, few if any sections were lost from either embedding procedure. Sections which adhered better seemed to stain better.

Tissues taken from lactating rats given corn oil injections through the 14 days of lactation appeared similar to tissues from animals given no injections during lactation. In general the picture seen is one of well distended and closely packed alveoli (Figures 1-3). Within the lumens of the alveoli may be seen the larger fat droplets, but individual milk protein droplets are not readily seen due to their small size. Occasionally, an alveolus may be found which showed necrotic cells (Figure 4). The cytoplasm of such cells stained poorly, the nuclei were pycnotic and a prominent nucleolus was usually evident.

Myoepithelial cells, which are abundant, were not easily distinguished from the overlying epithelial cells. Their presence may be inferred in cases where there is an obvious invagination of an alveolus suggesting that the myoepithelial cells was in a state of contraction at the time of fixation. In Figure 4, however, myoepithelial cells are more clearly seen when the nearby epithelial cells have become necrotic and thus stain poorly and the underlying myoepithelium is seen in a state of partial contraction producing an invagination of the alveolar wall. The ratio of necrotic to normal epithelial cells is normally small, but occasionally an alveolus may be found which contained several dying cells.
Tissue taken on the 4th day of lactation from animals given daily injections of estrogen and progesterone showed two major differences over tissues of normal lactating rats. The first was a collapse of the alveoli (Figure 5) as compared to the distended alveoli seen in Figures 1, 2 and 3. The second difference noted was the presence of occasional cytosegresomes as seen in Figure 6. The exact nature of these structures cannot be resolved at the light microscope level, and it is difficult to tell whether they are of hetero- or autophagocytic origin.

By the 9th day of lactation the number of alveoli which appeared collapsed had increased to approximately 25% of all alveoli examined. Within the contents of the lumina of such alveoli were seen many fat droplets (Figure 7), but other alveoli from the same animal may be found which appeared normal. At this stage a greater number of pale staining cells were seen among the epithelial cells of the alveoli (Figure 8). Such cells are seen only occasionally in tissues taken from normal lactating animals and probably represent macrophages.

By the 14th day of lactation the majority of the alveoli from animals receiving estrogen and progesterone had collapsed to the extent that there was little or no lumen present (Figures 9-12). Fat globules seen in tissue at this time were of two sizes. Larger globules had a diameter of from 4 to 17 microns and appeared to be associated with the remains of the luminal contents, although some droplets were still contained within the cell cytoplasm. Smaller droplets of approximately 2 microns in diameter were still within the cell cytoplasm in the basal portion of the cell.

Necrotic cells, which were occasionally seen even in control animals at all stages of lactation, increased in number as the period of injection
progressed. At the 14th day of lactation they are frequently encountered in animals receiving estrogen and progesterone and again were readily recognized by their pycnotic nuclei and the poor staining of their cytoplasm (Figure 13). Figure 14 shows an alveolus in which nearly all the cells have become pycnotic and are in the process of being sloughed into the lumen.

Mammary gland tissues taken 14 days after parturition from rats whose litters had failed on the first or second days of lactation were composed mainly of adipose tissue with scattered islands of disarranged epithelial cells among the adipocytes. Alveolar structures were not maintained and secretory activity was absent (Figure 15).

If, however, estrogen and progesterone injections were continued for 14 days in dams whose litters had failed on the first or second days of lactation, the integrity of the alveoli was maintained and fat globules, similar to the two sizes found in animals given estrogen and progesterone while lactating, were seen (Figure 16). The alveoli which were observed contained small or no lumina.

**Electron Microscopy**

Tissues embedded in Epon (Luft, 1961) proved superior in beam stability and in their ability to produce sections with fewer knife marks. Epon-Araldite (Anderson and Ellis, 1965) sections had a greater tendency to creep under beam illumination, especially at higher magnifications. These sections also seemed more prone to knife marks through tissue containing abundant lipid and otherwise in general appeared too soft. The majority of tissues were therefore embedded in Epon only.
Although sections were stained with either lead citrate and lead citrate plus uranyl acetate, the lead citrate alone did not produce enough contrast and all micrographs taken were of sections which were double stained.

The appearance of lactating rat mammary glands seen in this study is in good agreement with past reports which are presented in the literature review. The typical picture seen was that of a single row of epithelial cells, arranged in alveoli, secreting through their apical surfaces into the alveolar lumen (Figures 17, 18). Although myoepithelial cells were frequently observed, their small size in some sections made it understandable why they were not readily apparent at the level of the light microscope (Figures 17, 18). As in the case of light microscopy, their location may also be demonstrated by the infoldings of the epithelial alveolar wall producing a scalloped effect on the outside of the alveoli (Figure 20).

Epithelial cells in various stages of necrosis were also seen occasionally in normal lactating tissues (Figures 21, 22). The chromatin in such cells appeared dense at the outer rim of the nucleus mitochondria appeared swollen, the cytoplasm which stained lightly was seen, in some cases, to stream into the alveolar lumen and the endoplasmic reticulum was represented by short segments scattered throughout the cytoplasm. The endoplasmic reticulum seen in necrotic cells was in sharp contrast to the densely packed and well layered arrangement present in normal cells. The basal membrane still appeared intact after the loss of epithelial cells, and myoepithelial cells when present in the vicinity of a necrotic cell still adhered firmly to this outer limiting membrane.
(Figure 21) confirming Verley and Hollmann (1967) who suggested that the myoepithelial cells bridge the gap when necrotic cells are lost and hold the surviving alveolar cells together.

Occasionally cells which appeared to have suffered mechanical damage during fixation were encountered (Figure 23). They differed from the necrotic cells of Figures 21 and 22 in that the cisternae of the endoplasmic reticulum of the damaged cells appeared distended, the cytoplasm stained more nearly like that of normal epithelial cells and the entire cell appeared to have been separated from the basement membrane allowing milk to enter between the basement membrane and the basal surface of the cell. Another difference found between normal necrotic cells and those appearing to have suffered physical damage was the adherence of the myoepithelial cells to the basement membrane in the case of necrosis (Figure 21). The myoepithelial cells in Figure 23 appeared also to have been pulled from the basement membrane in a manner similar to the epithelial cells in such tissue.

In the third category of cells, which were infrequently seen, was a cell which appeared among the epithelial cells of the alveolus (Figure 24). The cytoplasm of such cells contained few organelles, stained lightly and probably corresponded to one of the wandering cells of Kurosumi et al. (1968). Fibroblasts and mast cells were also seen less frequently in the interstitium at all stages of lactation and during hormone treatment.

On the 4th day of lactation following daily estrogen and progesterone treatment, there was a noticeable increase in the number of wandering cells found among the epithelial cells (Figures 25, 26). Despite the suggestion of Figure 24 and the contention of some authors that wandering cells
migrate into the alveolar lumen, no wandering cells were seen in the alveolar lumina throughout any phase of this investigation. Nuclei of necrotic cells were, however, seen in the lumina both with the light and electron microscope.

Cytosegresomes, which are only rarely found in normal lactating glands are also seen at this time (Figure 28). They correspond to the descriptions of Helminen and Ericsson (1968b, c) and appear to contain remnants of rough endoplasmic reticulum and other nondescript membraneous structures. Lipid material was also seen, but infrequently.

Another structure which was first encountered during this stage was the stasis vacuole (Figure 27). The contents of these vacuoles were filled with milk protein droplets and appeared to have been formed by a fusion of several of the smaller vacuoles from the Golgi. Normally, milk protein droplets are thought to condense and form within the Golgi vacuoles and the vacuoles with the contained droplets migrate toward the apical surface where they are liberated into the alveolar lumen by merocrine secretion. Figure 27 shows that such a process is still possible, but in addition to this normal form of secretion, larger vacuoles are also formed. These structures have been described by Hollmann (1966) and are thought by him to indicate re-absorption of the secreted protein. Later stages may be seen which suggest that the contents of such vacuoles are condensed as if digestion were taking place (Figure 32). No evidence was found to indicate that these vacuoles could be formed by endocytosis.

The small lipid droplets seen in the base of the epithelial cells at the light microscope level may be found as early as the 4th day of lactation following estrogen and progesterone treatment (Figure 25), but
they are more commonly seen on the 9th and 14th days (Figure 30). They appeared to be formed in a similar manner to the larger lipid droplets and in agreement with other authors, no evidence was found suggesting that the Golgi function in their formation nor do the fat droplets appear membrane bound.

Stasis vacuoles and cytosegresomes seen on the 4th day of lactation in hormone treated animals persist through the 14th day (Figure 31). Necrotic cells similar to the ones found throughout normal lactation are also present in increasing numbers in hormone treated animals (Figures 26, 29).

It should be emphasized that the cytological differences observed between tissues taken on the 4th through the 9th days of lactation from rats given estrogen and progesterone appear to be one of a difference in the number of alveoli which exhibited the characteristics of wandering cells, cytosegresomes, stasis vacuoles and necrotic cells. Tissues taken from hormone treated animals on all days of this study showed normal epithelial cells and alveoli. Their numbers, however, decreased as the period of injection progressed.

On the 14th day of lactation hormone treated animals exhibited a cytological structure not seen on previous days. The presence of irregular shaped granules in membrane bound bodies was noted for the first time (Figure 32). These bodies have been previously described (Murad, 1970) in pregnant rat mammary glands and named "Gr" (Granular) particles. Gr bodies are similar in appearance to stasis vacuoles but contain densely packed granules, while stasis vacuoles usually were observed in the latter stages of their formation in which there was a clear space separating the digested protein granules from the membrane (Figure 32).
Figures 1-2. Normal lactating rat mammary glands taken on the 14th day of lactation showing well filled and distended alveoli. Paragon-1301, methylene blue. X440

Figure 3. Normal lactating rat mammary glands taken on the 14th day of lactation. In some cases lipid droplet formation occupied so much of the cellular volume that the droplets extended to the basal portion of the cell. Methylene blue, Azure II. X670

Figure 4. Normal lactating rat mammary glands taken on the 14th day of lactation. These alveoli are unusual in having such a high percentage of necrotic cells, but otherwise the remainder of this section appeared normal. The light staining of the necrotic cells enabled the myoepithelial cells (arrow) to be seen. Methylene blue, Azure II. X670
Figure 5. Mammary gland tissue from estrogen and progesterone treated animals taken on the 4th day of lactation. Although the majority of all alveoli examined appeared normal, isolated alveoli such as these (arrow) without the distended lumen typical of full lactation could be found. Methylene blue, Azure II. X440

Figure 6. Estrogen and progesterone treatment, 4th day of lactation. Alveoli with cytosegresomes (arrow) representing phagocytosis within an epithelial cell are occasionally found. Methylene blue, Azure II. X1750

Figure 7. Estrogen and progesterone treatment, 9th day of lactation. Collapsed alveoli are more frequently encountered. Lumina of such alveoli contain mostly lipid suggesting either reduced synthesis of some milk components or phagocytosis of lumen contents other than lipid. Paragon-1301, methylene blue. X480

Figure 8. Estrogen and progesterone treatment, 9th day of lactation. Pale cells (arrows) are frequently encountered among the epithelial cells at this stage. Methylene blue, Azure II. X670
Figures 9-12. Estrogen and progesterone, 14th day of lactation. The alveoli have collapsed to such an extent that only small lumina remain. Abundant fat droplets are present. The larger ones probably represent secretion prior to the effects of the hormone treatment while the smaller droplets (arrows) are apparently a result of such treatment. Figures 9 and 10: Paragon-1301, methylene blue. X440. Figures 11 and 12; Paragon-1301, methylene blue. X670
Figure 13. Estrogen and progesterone, 14th day of lactation. Necrosis of epithelial cells (arrow) continues to increase as hormone treatment progresses. Paragon-1301, methylene blue. X700

Figure 14. Estrogen and progesterone, 14th day of lactation. Epithelial cells have died and been sloughed off to such an extent that the alveolus is almost bare of functional cells. Paragon-1301, methylene blue. X1800

Figure 15. 14th day of involution, no hormone treatment given. Only a relatively few cells of the entire mammary gland remain; the rest having been sloughed into the alveolar lumina or been absorbed following disintegration. The nuclei seen here appear distorted, no alveolar structures remain and secretory activity is not evident. Paragon-1301, methylene blue. X670

Figure 16. 14th day of involution, estrogen and progesterone treatment given daily following pup removal on day 1 of lactation. The presence of abundant lipid droplets and alveolar structures similar to estrogen and progesterone animals on the 14th day of lactation suggests that the hormones are able to maintain the mammary gland in the absence of suckling. Methylene blue, Azure II. X700
Figure 17. Normal mammary gland, 14th day of lactation. Typical epithelial cells lying back to back in adjacent alveoli are shown. Protein secretion (arrow) is by fusion of the Golgi vacuoles with the cell membrane and liberation of the protein granules. M, mitochondria; ME, myoepithelial cell; L, lipid; G, Golgi; ER, endoplasmic reticulum; LU, alveolar lumen; N, nucleus. X14,000
Figure 18. Normal mammary gland, 14th day of lactation. Lipid droplets are about to be released into the lumen. Surface calveolae can be seen on the myoepithelial cells. L, lipid; C, capillary; LU, alveolar lumen; ME, myoepithelial cells. ×14,000
Figure 19. Normal mammary gland, 14th day of lactation. A lipid droplet has been released into the lumen containing a portion of the cellular cytoplasm from its cell of origin. L, lipid; G, Golgi; C, capillary. X14,000
Figure 20. Normal mammary gland, 9th day of lactation. Myoepithelial cells (arrows) in a state of contraction produce scalloped formations on alveolar walls. L, lipid; LU, alveolar lumen. X9,200
Figure 21. Normal mammary gland, 9th day of lactation. A necrotic cell is shown between two normal epithelial cells. The myoepithelial cell is firmly attached to the basement membrane (arrow). ME, myoepithelial cell. X14,000
Figure 22. Normal mammary gland, 14th day of lactation. A portion of a necrotic cell is seen adjacent to a normal epithelial cell. Necrotic cells at all stages of lactation appear similar. The remains of a tight junction (arrow) which normally bind epithelial cells together are still seen. X14,00
Figure 23. Damaged tissue from an otherwise normal mammary gland, 14th day of lactation. Both epithelial and myoepithelial cells appear to have been physically pulled from the basement membrane allowing milk to enter between the basement membrane and the epithelial cell. BM, basement membrane; R, red blood cell; ME, myoepithelial cell. X14,000
Figure 24. Normal mammary gland, 14th day of lactation. Wandering pale cells are occasionally seen among the alveolar cells. This one appears to have migrated in from the surrounding interstitium or the adjacent capillary (C). Note the lack of organelles and the pale staining of the cytoplasm. X17,000
Figure 25. Estrogen and progesterone, 4th day of lactation. Even at this early stage of treatment many of the features of more prolonged hormone administration may be found. Here an increase in the numbers of pale cells is noted along with the presence of small lipid droplets in the basal part of the cell. C, capillary; R, red blood cells; L, lipid droplet; SL, small lipid droplets; MA, pale cells; LU, alveolar lumen; CE, capillary endothelial cell nucleus; arrows, myoepithelial cells. X4,850
Figure 26. Estrogen and progesterone, 4th day of lactation. The number of necrotic cells was found to increase with continued hormone treatment. A wandering pale cell is also seen. NE, necrotic cells; CE, capillary endothelial cell nucleus; ME, myoepithelial cell; MA, pale cell. X7,550
Figure 27. Estrogen and progesterone, 4th day of lactation. Stasis vacuoles (S) are encountered at this time also. They resemble protein secretion vacuoles but are much larger suggesting a fusion of several small vesicles produced by the Golgi containing milk protein droplets. In this micrograph the normal release of protein granules (arrows) is still operative along with the continued increase in size of the stasis vacuole. X14,000
Figure 28. Estrogen and progesterone, 4th day of lactation. Cyto- segresomes, which were not seen during normal lactation, are encountered more frequently from the 4th day onward in treated animals. The cytoplasm surrounding the membranes in the process of digestion resembles that of the pale cells and thus this micrograph represents hetero- rather than auto-phagocytosis. X14,000
Figure 29. Estrogen and progesterone, 14th day of lactation. Necrotic cells such as these are commonly seen at this time in hormone treated animals. X7,550
Figure 30. Estrogen and progesterone, 14th day of lactation. Small lipid droplets (SL) are characteristically found in the basal part of the epithelial cells after prolonged hormone treatment. They are similar to the larger droplets of normal lactation but apparently are not released into the alveolar lumen. Their large numbers, in some cases, appear to disrupt the arrangement of well organized endoplasmic reticulum seen in normal lactation. X14,000
Figure 31. Estrogen and progesterone, 14th day of lactation. Cyto­segresomes seen on the 14th day of hormone treatment are similar to those found as early as the 4th day. The origin of the vacuole (V) is not certain. It resembles a stasis vacuole in which the contents have been eliminated by auto­digestion, but the presence of membrane-like residues in the interior may also suggest a cytosomese, or its derivative, a cytosome. X9,200
Figure 32. Estrogen and progesterone, 14th day of lactation. Two stasis vacuoles (S) are seen in which the milk protein droplets have apparently undergone a partial digestion and condensation. A new structure, identified as a granular particle (Gr) and previously described by Murad (1970) is seen for the first time. MA, pale cells; Gr, granular particle. X9,200
DISCUSSION

Maeder (1922) noted that one of the first histological indications of weaning was the presence of distended alveoli. The weight gain of the mammary gland and decrease in thickness of the alveolar wall were also noted by Ota (1964) and Okada (1956b) and taken as an early indication of weaning. These results suggest that the increased milk content within the alveoli accounted for the weight gain and also stretched the alveoli. The collapse of the alveoli as involution progressed was found as a universal feature by all investigators.

The appearance of distended alveoli seen at all times during normal lactation in the present study implies that more than adequate supplies of milk are being produced by lactating dams for pup litters which have been adjusted to 6 pups at the beginning of lactation.

One of the first results seen in this study following estrogen and progesterone administration to lactating dams was a collapse in isolated alveoli similar to that reported by other authors during the early stages of involution. This was first seen on day 4 of lactation, and though far from being universally found, progressed to the point that by day 14 the majority of alveoli seen in hormone treated animals had collapsed.

Several explanations for this observation may be suggested. The first is that the estrogen-progesterone combination simulates the hormonal conditions which prevail during pregnancy, and, according to the Meites-Turner (1942b) theory of lactation, milk production during pregnancy is prevented due to the low levels of serum prolactin. The release of pituitary prolactin in turn is prevented by the high levels of endogenous progesterone
during pregnancy or by the exogenous progesterone present in the current study.

The progesterone effect on reducing lactation may also be a local action of the mammary gland epithelial cells rather than mediated through prolactin release by the pituitary. Meites and Sgouris (1953) felt that the ovarian hormones were able to render the mammary gland refractory to the action of prolactin. Turkington (1971a, b) has shown that progesterone is able to inhibit the B protein of lactose synthetase.

The net result of any of the former mechanisms may be said to be an inhibition of lactation, or at least an inhibition of a specific portion of milk synthesis. As in the case of the condition of nonlactation during pregnancy in most species, the term inhibition implies a reversal when the inhibitory influence is removed. Preliminary studies (unpublished) underway in our laboratory show that when exogenous estrogen and progesterone treatment is stopped during lactation, the downward trend of pup weight gains is reversed.

Nothing in the current study reported on in this paper is in disagreement with the findings that exogenous ovarian steroids administered during early lactation can inhibit lactation. If anything, these findings confirm this theory by showing a reduced alveolar volume following hormone administration which, when the pups are still nursing, can be explained by a reduction in milk output. Whether the causes for this are local, involve the pituitary or involve some other endocrine organ are not clear at this point, nor can they be easily resolved by the methods used in the current study.

The reports (Herrenkohl, 1971; Bruce and Ramirez, 1970) that exogenous
estrogen or progesterone administration in rats during lactation interfered with milk let-down is not confirmed by the present study. The previous authors based their conclusions on histological appearances of mammary gland alveoli, pup weight gains and milk yields from pup stomach contents. Of the three, pup weight gains appear to be the best index of milk secretion. The appearance of distended alveoli cannot always be regarded as an index of normal lactation since, even before parturition and following weaning during the period of involution, the alveoli contain material for which the nebulous name of colostrum is applied. The name of "lactogenesis" which is equated with the initiation of milk secretion does not differentiate between special cases of lactation such as pre-partum milking, galactorrhea or witch's milk.

The discrepancies found by Herrenkohl (1971) and Bruce and Ramirez (1970) between the distended alveoli, indicating an adequate milk production, and low pup weight gains and low milk yields, indicating an insufficient method of milk let-down, were taken as an indication that synthesis was normal but that the pups failed to gain weight because of a failure of the milk ejection reflex. Their explanation, which implied an inhibition in oxytocin release, gains some support from the investigations of Roberts (1971) who found that exogenous progesterone did inhibit oxytocin release from the pituitary.

The results reported on in this current paper, up to the 4th day of hormone administration, again do not necessarily disprove the hypothesis of reduced oxytocin release, but other explanations seem more attractive, especially in view of the results obtained on later days of hormone treatment.
Tissues taken on the 4th day of lactation following hormone administration showed the well distended alveoli reported by others following hormone administration, but the current results did not differ from the appearance of control alveoli from animals given no, or only corn oil injections. Also, occasionally an alveolus could be found in which the myoepithelial cells were contracted suggesting that oxytocin, with a half-life of under 5 minutes, was still present in sufficient amounts to cause alveolar contractions. This was despite a delay of some 20 minutes during which time the pups were removed from their mothers, the dams sacrificed and the tissues fixed. Although it can be argued that it is the amount of the reduction in oxytocin that is important, the appearance of contracted myoepithelial cells suggests the presence of some circulating oxytocin.

Of more importance in explaining lower pup weight gains from dams receiving exogenous hormone treatments is the appearance of alveoli found at the 9th and 14th days of lactation. As early as the 4th day of treatment, an increase in the number of pale cells was found among the epithelial cells. These cells have been found during involution by Richards and Benson (1971b, c), Helminen and Ericsson (1968b), Jeffers (1935a), Okada (1957, 1958a) and others and given the various names of wandering cells, pale cells, macrophages, macrophage-like, lymphocytes, leucocytes and histiocytes. Regardless of the name used to describe these cells, they are seen in increasing numbers during mammary gland involution. As described by Helminen and Ericsson (1968c) and Richards and Benson (1971a) they probably represent macrophages. Okada (1956b) reported that involution or teat ligation in rats produced a stagnation of the alveolar milk and caused an inflammatory process in the mammary gland. In response to this condition
he believed that leucocytes were drawn to the region. He further has shown (1956a) that colostrum bodies were derived from these invading leucocytes.

The results presented in the current investigation do not demonstrate colostrum bodies in any stage of lactation either in control or hormone treated animals. The hormonal conditions of involution may be similar to the exogenous ovarian hormone administrations in that prolactin levels are low during involution due to the elimination of the suckling stimulus shown necessary to cause a release of pituitary prolactin (Convey, 1969). Exogenous progesterone is also thought to reduce serum prolactin levels. The conditions are different in that during hormone administration, milk is not allowed to remain in the alveoli and become stagnant, due to the continued suckling of the pups. If any colostrum bodies were formed in response to hormone treatment they might be eliminated when the milk was withdrawn by the pups.

The wandering cells are aptly described by Richards and Benson (1971a) as macrophage-like cells due to a degree of uncertainty as to their origin and function. Macrophages are derived from circulating monocytes (Weiss, 1972; pages 107-132). A salient feature is the presence of lysosomes. Although wandering cells were frequently seen, their origin and identity could not be readily determined. In blood smears, leucocytes are more easily identified from their color reactions with specified stains and by their morphology. At the level of the electron microscope, responses to these standard stains is not possible and the descriptive cellular morphology is often incomplete as the plane of section may miss an important identifying characteristic. The identity of leucocytes is further
complicated by the fact that their description, even at the ultrastructural level, is often based on the description of these cells in circulating blood or tissue culture. The morphology of such cells may differ radically when they invade tissues.

The cell most frequently encountered in the present study was one with few cytoplasmic organelles. Frequently lysosomes were not seen. This description most nearly fits that of a lymphocyte, but the cytoplasm-to-nucleus ratio favored the identification as a macrophage. The identification is further complicated by theories of blood cell formation which suggest that there is a continuum from small, medium and large lymphocytes, on to monocytes.

The function of these wandering cells is as obscure as is their identity. Okada (1956a), Lascelles et al. (1969) and Lee et al. (1968) favor a theory suggesting that the colostrum cells are derived from leucocytes. Jeffers (1935a) felt that replacement of lost epithelial cells occurred since the epithelial counts per alveolus remained high during lactation despite a normal loss of necrotic cells. The origins of such replacement cells might be found in the wandering cells since mitoses are not seen during lactation. Weiss (1972, pages 133-150) noted that lymphocytes, due to their lack of specialization, may be precursor cells. He presented evidence which showed that lymphocytes may undergo a conversion to plasma cells. Following leucocytic infiltration, plasma cells were found, in the current study, between mammary gland alveoli.

The net result of leucocytic infiltration cannot be fully assessed at this time. The only agreed upon fact by most observers is that during the onset of involution there is an increase in the numbers of alveolar
leucocytes. Such an increase may account for the observations (Griffith and Turner, 1962; Knox, 1966) that DNA levels of the mammary gland remain constant during estrogen-progesterone treatment, since the source of the DNA may be leucocytic rather than epithelial. In the present study, leucocytes were frequently found in mammary glands of estrogen and progesterone treated animals.

Another interesting cytological feature found at the 4th day of hormone treatment was the presence of what Helminen and Ericsson (1968b) describe as a cytosegresome. These structures represent phagocytosis either from within the cell or from other cells such as macrophages. Such phagocytosis, as reported by Helminen and Ericsson (1968b), is only rarely encountered during normal lactation but is frequently found during involution.

The presence of necrotic cells has frequently been mentioned by others (Jeffers, 1935a; Maeder, 1922; Feldman, 1961; Verley and Hollmann, 1967; Richards and Benson, 1971a) and appears to be a normal consequence of the cell cycle during lactation. This process may account for the observation that the nucleic acid content of the mammary gland is lower following milking (Kirkham and Turner, 1953); the assumption being that DNA present in leucocytes is lost at this time. The presence of intact but necrotic nuclei seen in the alveolus during the course of the current study, rather than leucocytes, appears to be the source of this DNA found in milk as leucocytes were not seen within alveolar lumina during normal lactation nor during periods of hormone administration.

Feldman (1961) suggested that these necrotic cells may in part be caused by tissue damage during fixation. Tissue damage as seen in this
current study appears to take the form as seen in Figure 23, and therefore the necrotic or pale cells of Feldman are considered to have occurred naturally during the course of lactation.

The increasing numbers of necrotic cells found in the current study is thus in agreement with others (Jeffers, 1935a; Maeder, 1922; Verley and Hollmann, 1967; Richards and Benson, 1971a) who note a progressive necrosis in the alveolar epithelium during involution.

Another structure which appeared during the period of hormone administration was the stasis vacuole (Figures 27 and 32). These structures too are characteristic of involuting mammary glands (Hollmann, 1966; Hollmann and Verley, 1967; Richards and Benson, 1971a). There are at least two possible explanations for their origins. The least likely, from the standpoint of the appearance of the vacuole in Figure 27, is that stasis vacuoles represent an endocytosis of the luminal contents. No evidence for this was seen in the current study. The second and more likely explanation is that they represent some failure of the release mechanism of protein secretion granules, followed by their fusion and rearrangement within the cytoplasm (Hollmann, 1966). The last phase in the stasis vacuole formation is the removal of the protein granules by digestion (Richards and Benson, 1971a). The results of this final stage can be clearly seen in Figure 32 where the protein granules have merged and been condensed in the center of the vacuoles.

Prolonged ovarian hormone administration to lactating dams produced two other changes not seen in mammary glands from normal lactating rats. The first of these was the presence of small lipid droplets which apparently were not secreted but are retained in the basal portion of the cell. The
appearance of these droplets is correlated with the length of the period of hormone administration. They were infrequently seen on the 4th day of lactation, but by the 14th day nearly all tissue samples from lactating rats receiving injections were composed of shrunken alveoli whose cells contained these small droplets.

The second difference, in addition to the ones already mentioned, was the appearance of a membrane bound structure composed of dense, tightly packed, irregularly shaped granules (Figure 32). These structures have been described previously (Murad, 1970) and arise from the endoplasmic reticulum during pregnancy in the mammary gland of the rat. They are considered to be proteinaceous and to be related to antibody formation (Murad, 1970).

The appearance of lipid within the gland is also associated with pregnancy. Jeffers (1935a) noted a large amount of fat in the pregnant mammary gland. Mayer and Klein (1961) state that fatty droplets appear in the cytoplasm of alveolar cells from the onset of pregnancy. Girardie (1968) found lipid synthesis in the pre-lactating gland. Luckey, Mende and Pleasants (1954) found more than double the amount of fat present in pre-partum colostrum as compared to normal milk.

These results show that lipid synthesis occurs readily in mammary glands of pregnant rats at which time the hormonal influence on the gland is similar to the levels of exogenous hormone used in the current study. Estrogen and progesterone administered during involution maintained the alveolar structures and induced synthesis of the small lipid droplets similar to the ones seen during lactation following 14 days of hormone administration (Figure 16).
CONCLUSIONS

Apparently three stages are operating in the lactating rat mammary gland during exogenous estrogen and progesterone administration. The first involves the release of prolactin and oxytocin from the pituitary following suckling. Prolactin is necessary for the maintenance of milk secretion and oxytocin is necessary for milk let-down. Since progesterone can effectively inhibit both the release of prolactin and the synthesis of lactose by inhibiting the induction of α-lactalbumin, the secretion of estrogen and progesterone may be said to inhibit lactation. Of secondary importance may be the reduction of oxytocin and thus an inhibition of milk let-down following suckling. The extent to which this may influence lactation is not known.

This present study neither proves nor disproves the inhibition of lactation by estrogen and progesterone during lactation. The near normal appearances of tissues taken at the end of the 4th day of hormone administration do not exhibit any differences at the ultrastructural level that could be interpreted as an inhibition of lactation as reported by others.

The second stage of hormone administration is evident from treatment from the 4th day onward and it is during this stage that structures are seen both at the light and ultrastructural level which suggest mammary gland involution. This conclusion is drawn from the appearance of collapsed alveoli, the increase seen in the number of necrotic cells and lymphocytes, the occurrence of cytosegresomes and stasis vacuoles, all of which are found during involution.

The ovarian hormones may function in this case to inhibit prolactin
secretion, or the effect may be one of a local action as the epithelial cells have been reported to be rendered refractory by estrogen and progesterone to the stimulatory action of prolactin. In any case, the net results are similar to the results obtained following weaning during which time prolactin levels would also be reduced.

A third stage is suggested by the persistence of fat synthesis and the appearance of granular structure similar to those seen during the later periods of pregnancy. It is suggested that this stage is promoted by exogenous estrogen and progesterone administration and possibly also by the induction of relaxin from the ovary by these hormones. Thus the older idea that estrogen-progesterone administration regresses the gland back to a growing stage finds some support from this investigation.

The newly developed radio immunoassay for prolactin would be helpful in resolving the problem of serum concentration of prolactin during ovarian hormone administration. Also helpful would be a better definition of what constitutes an inhibition of lactation and deciding at what point lactation ends and involution begins.
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