RATES OF CELL DIVISION IN NORMAL AND MALIGNANT MAMMARY GLAND TISSUE IN THE RAT

A Thesis

Presented to

Faculty of Graduate Studies and Research

The University of Manitoba

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

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by

Ruth Elizabeth Grahame, M.D.

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The rates of epithelial cell division in the mammary glands of female Sprague-Dawley rats were determined, in the virgin state, during pregnancy, lactation and involution, using the colchicine method. In addition, the rates of cell division were ascertained in mammary tumors induced in female Sprague-Dawley rats by oral administration of 7, 12 dimethylbenz(a)anthracene.

In the first series of virgin rats, the mitotic rate for a twenty-four hour period was 1.05 per cent in stage I of estrus; the average six-hour mitotic rate (10:00 a.m. to 4:00 p.m.) was 0.26 per cent.

In the second series, the daily mitotic rate in the mammary glands of rats pregnant for twelve days was ascertained to be 13.20 per cent, implying a twelve-fold increase over the mitotic rate of the virgin animal. The average six-hour mitotic rate (10:00 a.m. to 4:00 p.m.) was 4.35 per cent.

In the third series, the mitotic rates in the mammary glands of rats pregnant four, seven, fourteen and eighteen days were determined during six-hour periods from 10:00 a.m. to 4:00 p.m.. These rates were compared with the six-hour mitotic rates, (10:00 a.m. to 4:00 p.m.), of rats pregnant for twelve days. On day four, the mean mitotic rate was low (0.43 per cent). The rate attained the highest level at the mid-point of pregnancy, that is, on day twelve (4.35 per cent). It declined by the fourteenth day to 2.01 per cent, and further to 1.02 per cent by the eighteenth day. This signified that cellular proliferation in the rat mammary gland was most rapid during the first half of pregnancy,

after the fourth day, and continued during the second half of pregnancy at a slower rate.

In another series of experiments, the six-hour mitotic rates of mammary glands of rats on the second day of lactation were observed to average 0.51 per cent. This indicated that the activity in the lactating gland was directed toward secretion rather than cell proliferation.

The mitotic rates of involuting mammary glands of rats, seven days after weaning, averaged 0.73 per cent for the six-hour period, which was somewhat higher than in the virgin animal.

The mitotic rates of mammary gland neoplasms, induced by dimethylbenz(a)anthracene administration ranged from 0.39 per cent to 8.43 per cent for six-hour periods, in a total of nineteen tumors from sixteen animals. The six-hour mitotic rates of most of the tumors, (Grades I - III) however, ranged between 0.53 per cent and 3.05 per cent, compared with the average mitotic rate of 4.35 per cent for the six-hour period on day twelve of pregnancy. This signified that neoplasms of the mammary gland in the rat did not necessarily proliferate more rapidly than the normal mammary gland epithelium in particular phases of physiological activity.

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CHAPTER I

INTRODUCTION

It is the purpose of this thesis to report the rates of epithelial cell division in the mammary glands of female Sprague-Dawley rats, determined by the colchicine method. In this investigation the mammary gland has been studied in four stages of physiological activity, i.e. in the virgin animal, during pregnancy, lactation and involution. Further, the rates of cell division in mammary tumors induced in the same strain of rats by the administration of 7, 12 dimethylbenz(a)anthracene (DMBA) has also been determined. These rates have been compared to the rates of epithelial cell division in the normal mammary tissue.

The mitotic rates of many cell populations in the rat have previously been determined (Bertalanffy, 1964), including those of the female genital tract epithelia (Bertalanffy and Lau, 1963). However, a search of the literature has failed to reveal a complete investigation of the mitotic rates of the epithelium of the mammary gland. Laguchev (1962) investigated the mitotic rates of the mammary glands of mice during different phases of the estrous cycle, and Echave Llanos and Piezzi (1963) demonstrated diurnal variation of mitotic rate in the mammary glands of pregnant mice. Reece and Warbritton (1953), using colchicine, observed that the rate of cell division was greatest at the mid-point of pregnancy in the rat mammary gland. This, however, was reported merely in a brief abstract. It therefore seemed desirable to make a more complete study of the rates of cell division in the normal mammary gland of the rat during different physiological states.

Previous attempts have been made to ascertain whether a relationship existed between the incidence of malignancy in the various organs and the degree of mitotic activity of the tissues of origin (Leblond, Storey and Bertalanffy, 1951). They observed that a number of organs showing a high mitotic rate, for example, stomach, skin and blood-forming organs, also showed a high incidence of malignancy. A notable exception is the small intestine (Leblond and Stevens, 1948; Bertalanffy, 1960; Bertalanffy and Nagy, 1961). On the other hand, they observed that organs with a low mitotic rate showed a low incidence of primary cancer in general, for example, endocrine glands and the liver. Although the mitotic rates reported in the above experiments were determined mainly in animal experimentation, in instances where the rates were ascertained in the same cell populations both in man and in rodents an almost identical rate was observed in both species (Bertalanffy, 1964). This apparent relationship between mitotic rate of the host tissue and the incidence of malignancy was an additional reason for investigating the rates of cell division in the mammary gland.

Furthermore, because carcinoma of the breast frequently occurs in women and is a leading cause of death (Manitoba Cancer Treatment and Research Foundation Report, 1963-64), much research work has been done using rat mammary tumors induced by 7, 12 dimethylbenz(a)anthracene (DMBA). These induced tumors are easily produced, and like some mammary cancers in humans are hormone sensitive (Dao, 1964). It was therefore one of the aims of the present study to investigate the

rates of cell division in the induced tumors of the mammary gland to determine whether they are greater or lesser than the mitotic rates which occur in the normal mammary gland during its various physiological states.

CHAPTER II

REVIEW OF THE LITERATURE

I. METHODS OF ASSESSING MAMMARY GLAND GROWTH

i. Whole Mount Method

Cowie and Folley (1947), Cowie (1949) and Silver (1953a) used whole mounts of the mammary gland to estimate size and growth. Mammary glands of the rat were dissected out under a low-power binocular microscope. A line was traced around the periphery of each gland on to cellophane, and then the tracing was placed over millimetre-squared paper. Flux (1954) used the same method to study the mammary glands of mice. This method does not provide information on the contribution of cellular proliferation, secretion or cellular hypertrophy to the observed growth.

ii. DNA Method

This is a chemical method used for determining growth in tissues. It is based on the fact that the DNA content of cell nuclei is constant in the somatic cells of the same species. (Boivin, Vendrely and Vendrely, 1948; Mirsky and Ris, 1949). DNA estimation therefore came to be used as a reference standard directly related to the number of cells present (Davidson and Leslie, 1950; Davidson, 1960). It was a chemical unit which could be used as a measure of cell multiplication. Numerous experiments using the DNA method have been performed to estimate the growth of the mammary gland in various physiological states. The disadvantage of this method is that it measures indiscriminately the contributions of all the cellular components of the mammary

gland. The DNA derived from connective tissue and vascular elements could contribute significantly to the total DNA estimation in the early phases of mammary growth (Traurig and Morgan, 1964a). The infiltration of leukocytes in lactation likewise could affect results obtained by this method (Greenbaum and Slater, 1957).

iii. Tritiated Thymidine ($T\text{-}H^3$) and Autoradiography

Thymidine has been shown to be a specific precursor for DNA and is incorporated into the DNA molecule during synthesis prior to mitosis. Tritiated hydrogen bound to thymidine ($T\text{-}H^3$) is incorporated into DNA, thus labeling the nuclei about to divide. The label is retained, and becomes diluted only through subsequent cell division (Hughes et al, 1958). Cells labeled by $T\text{-}H^3$ are visualized by radioautography (Robertson, Bond and Cronkite, 1959). Hence the percentage of cell nuclei in a population of cells demonstrating an incorporation of $T\text{-}H^3$ will provide an index of proliferation, or radioactive index (Messier and Leblond, 1960). This technique has been used to study many cell populations (Messier and Leblond, 1960), including the epithelium of the mammary gland of the mouse (Traurig and Morgan, 1964a), and also spontaneous mammary tumors in the mouse (Mendelsohn, 1964). However, it has not, so far as can be determined, been used to study the mammary gland epithelium, or mammary tumors of the rat.

iv. Colchicine

Colchicum, extracted from the seeds, flowers and corms of the

saffron plant, Colchicum autumnale, has been used since ancient times to treat gout. The crystalline substance, colchicine, an alkaloid, extracted from colchicum, has been observed in relatively recent times to arrest mitosis in metaphase both in plants and animals, apparently by interfering with spindle formation. Therefore, one of the most striking features of colchicine, when injected into animals, was the accumulation of mitoses arrested at metaphase. This accumulation of mitotic cells was soon understood to be most useful in the analysis of growth by cellular multiplication, especially in those tissues in which mitoses are rare. Colchicine, by arresting cells in metaphase, allowed these "colchicine metaphases" to progressively accumulate in a given tissue for a specified period of time thus facilitating the estimation of cellular growth (Eigsti and Dustin, 1955).

The technique of colchicine treatment has been described many times (Leblond and Stevens, 1948; Leblond and Walker, 1956; Stevens-Hooper, 1961; Bertalanffy, 1964). The optimal colchicine dosage for rats has been found to be 0.1 mgm. per one hundred grams of body weight (Bertalanffy and Leblond, 1953), and the most suitable time interval between injection and killing of the animals was determined by the same authors to be four to six hours, depending upon the magnitude of mitotic activity of the tissue under study. The method of determining the daily mitotic rate will be outlined in Chapter III.

The possibility that colchicine could exert a depressive or stimulating action has been investigated by counting prophases in colchicine-treated and untreated animals, using particularly the small

intestine which has a high mitotic activity. It has been found that a similar incidence of prophase nuclei occurs in both the colchicine-treated and the control rats (Stevens-Hooper, 1961), indicating that colchicine in proper dosage, did not alter the incidence of cells entering mitosis. It was also determined that colchicine arrested dividing cells in metaphase after a lag period. The duration of the latter varied from tissue to tissue, but has been found not to alter greatly the estimated daily mitotic rates (Bertalanffy and Lau, 1962a), and therefore is usually disregarded.

A comparison between the colchicine and the $T\text{-}H^3$ techniques in the study of cell population cytodynamics, was made by Bertalanffy (1964). According to this author, the $T\text{-}H^3$ technique requires fewer animals, data are ascertained more quickly, and newly formed cells are permanently labeled for further study. The advantage of the colchicine technique is that it can be used in any laboratory, and with greater safety for the staff. It furnishes data on actual percentages of cells dividing during certain periods, and a single precise figure of the turnover time of cell population. Both techniques yield data of equal validity.

II. NORMAL RAT MAMMARY GLAND

i. Embryology and Early Postnatal Development

In a series of articles, J.A. Myers (1916, 1917, 1919a and b) described the fetal and early postnatal development of the mammary gland in female albino rats. Myers (1919a) found the first indication of mammary gland development in a rat embryo of eleven days' gestation. This was a faint light streak on either side of the ventral midline extending from the anterior limb bud near the site of the future axilla to the inguinal region near the posterior limb bud. The streak was composed of a single layer of cells which were larger than those of the adjacent epidermis. Subsequently, several layers of cells were formed along the streak, which was then called the milk line. A further proliferation of cells, called hillocks, occurred at sites along the mammary line, corresponding to the number of glands for the species, and the intervening line disappeared. Hillocks were ellipsoid and sank into the mesenchyme until they were attached to the overlying epidermis only by a thin cord of cells (15 day, 9 hour fetus). This cord later became the primary duct (Myers, 1919a). A single bud of epithelial cells formed at the deep surface of the hillock, and by dichotomous branching formed the ducts. (This is in contrast to the mammary gland of the human in which sixteen to twenty-four buds develop; Hamilton, Boyd and Mossman, 1962).

By nineteen or twenty days' gestation a pit was formed by cornification and desquamation of epithelial cells on the surface overlying

the hillock, and a papilla-like eminence, the nipple primordium, appeared at the bottom of the pit (Myers, 1917). At birth, the nipple was usually level with the surrounding epidermis. It became elevated four or five days after birth, and continued gradual growth until the ninth week. The sulcus around the nipple in new born rats is the apparent remnant of the embryonic mammary pit which persists in adult rats as the epithelial ingrowth or hood. (Figure 1.)

At eighteen or nineteen days of fetal life, lumina began to form in the terminal portions of the ducts. At birth a lumen was present in all ducts distal to the intradermal portion of the primary duct, which was not canalized until the end of the second postnatal week (Myers, 1919b).

The connective tissue stroma arose from the cells of the surrounding mesenchyme, and masses of subcutaneous fat developed soon after birth. Slight amounts of secretion were present in the lumina after birth.

Growth and branching of ducts continued after birth with an unusually rapid rate during the ninth week, which, according to Myers (1916), corresponded to puberty. As will be shown, this fact was apparently of significance in the successful production of mammary carcinomas in Sprague-Dawley rats by the oral administration of 7, 12 dimethylbenz(a)anthracene (DMBA).

More recently it has been demonstrated by Cowie (1949) and Silver (1953a), that increased growth of the mammary gland occurred around the third week after birth, and that it was dependent on the

presence of ovarian hormones.

ii. Adult Virgin

The mammary glands of the adult white rat are represented on the surface by six pairs of nipples, three thoracic, one abdominal and two inguinal pairs. Astwood, Geschickter and Rausch (1937) observed the first thoracic glands to be anterior to the forelimbs. The second and third thoracic glands were spread out between the muscular planes over the sides of the thorax and into the axillae. The abdominal and first and second inguinal glands occupied a thick fat pad situated in the inguinal region and extending from above the iliac crest to the ischial tuberosity. Myers (1916) observed in examining one hundred female rats, that twelve mammary glands (six pairs) was the normal number for the white rat, but it ranged between ten and thirteen. The second thoracic glands were absent most frequently.

Maeder (1922) described the microscopic appearance of the mammary gland of the adult virgin rat to be composed of ramifying ducts and alveolar end buds or end pieces, embedded in adipose connective tissue. Surrounding the end buds and small ducts was a thin layer of dense connective tissue which increased in amount as the ducts grew in size. Blood vessels and macrophages were present in the stroma, as were mast cells. (Figures 2 and 3)

Sutter (1921) described cyclic changes in the mammary gland of the rat, brought about by the estrous cycle. Using whole mounts, he observed at the end of the proestrus that the mammary tree exhibited

long slender branches with a few twigs showing bud-like projections. During estrous there was a pronounced growth in the mammary tree. Twigs sprouted and new buds appeared. Following estrus, regression occurred. Cole (1933), studying whole mounts of mouse mammary gland, described similar cyclic changes. Laguchev (1962) observed that in female mice of the C_{57} strain the stage of the estrous cycle could be determined in nearly every case by study of histological sections of mammary glands: "In proestrus and estrus nodules of proliferating epithelium appeared at the ends and along the course of the small ducts of the gland. These were always absent in the stage of diestrus. Desquamation of epithelial cells into the lumen of the alveoli and ducts and a decrease in the size of the epithelial cells composing the luminal segments was observed." However, this pattern, he noted, was obscured in rats of the C_3H strain.

Weatherford (1929) described the low cuboidal cells of the epithelium lining of the mammary gland ducts. The apices of these cells were narrower than the bases. The cytoplasm was pale and stained slightly basophilic with haematoxylin and eosin. The nuclei, containing one or more nucleoli, were ovoid and lay toward the basal end of the cell. He noted desquamated cells, and an acidophilic substance in the lumina of some ducts; mitotic figures occurred in both ducts and alveolar buds.

Myoepithelial cells, not readily demonstrated by routine techniques, were described by Myers (1919b) and by Silver (1954). The latter applied the technique of Gomori (1939) to demonstrate myoepi-

thelial cells of the parotid, as well as the mammary glands. Richardson (1949) devised a silver impregnation method for the demonstration of the extensive proliferation of myoepithelial cells around all the alveoli and ducts of the mammary epithelium of lactating goats. He believed these cells to be a discrete tissue, different in form and location from smooth muscle, and to develop in pregnancy with the development of the rest of the gland. Linzell (1952), using the silver technique of Richardson (1949), studied the mammary glands of a variety of animal species, including the rat. He noted a stellate variety of myoepithelial cells forming a complicated irregular network around the alveoli at the base of the secretory cells. Surrounding the ducts they became spindle-shaped, and were arranged longitudinally between the epithelial cells and the basement membrane. Dempsey, Bunting and Wislocki (1947) stated that myoepithelial cells were less evident in the rat during pregnancy and lactation, and mitoses of these cells during growth phases were absent. This appeared to differ from the observations by Richardson (see above) and Rees and Eversole (1964), who stated that myoepithelial cells "of course" increased in number together with the epithelial cells during pregnancy and lactation. It is interesting to note at this time that Huggins, Briziarelli and Sutton (1959) observed great numbers of myoepithelial cells in carcinomas of the mammary gland of the rat, induced by the administration of a carcinogen (3-methyl cholanthrene). These cells were demonstrated by means of the alkaline phosphatase reaction.

iii. Pregnancy

The normal period of gestation in rats has been estimated to be between twenty-two and twenty-three days (Farris and Griffith, 1962). The mammary gland of pregnant rats has been studied by many investigators. Weatherford (1929) described the microscopic appearance of the gland. He noted the increase in size and number of the alveoli after conception. The epithelial cells of the alveoli became columnar, and by the tenth day of pregnancy vacuoles appeared in the cells. As pregnancy continued, the cells became swollen and distorted by the accumulation of secretory material. The cells lining the smaller ducts also contained vacuoles, indicating a limited secretory activity. Weatherford observed that by twenty days gestation, the apical ends of many cells bulged into the lumina, which were of greater diameter and contained secretion. During pregnancy the connective tissue surrounding the alveoli was observed to be scanty, while a relatively thick sheath of connective tissue enveloped the ducts. An increase in vascularity was observed as well.

Weatherford (1929) and Roberts (1921) stated that cellular proliferation was extremely rare in the mammary gland epithelium during the second half of pregnancy. However, Jeffers (1935) observed mitoses frequently between days twelve and nineteen of pregnancy. (Figures 4 to 14.) More recently, Griffiths and Turner (1961) and Tucker and Reece (1963a), using the DNA method to estimate cell growth, observed that mammary hyperplasia occurred throughout pregnancy. The greatest

increase in mitotic activity in the mammary gland of rats was stated to be at the mid-point of pregnancy by Reece and Warbritton (1953), using the colchicine method, and by Kirkham and Turner (1953), and Tucker and Reece (1963a), using the DNA method.

iv. Lactation

According to Maeder (1922), the glands in the lactating rat appeared as an almost continuous sheet in loose fatty connective tissue just beneath the panniculus carnosus. It extended on either side of the midline from neck to anus, but was interrupted for a short distance just below the lower border of the ribs.

In early lactation, the alveoli increased considerably in size mainly because the lumina were greatly distended by milk secretion. (Figure 15.) The epithelial cells of different alveoli showed different stages of activity. Many were flattened due to the distention of the lumina with secretion, but others were columnar with apical ends protruding into the lumen. (Figure 16.) Many leukocytes were present beneath and within the epithelial layer of the alveoli. A variable degree of cell degeneration was observed in glands of lactating animals (Jeffers, 1935).

According to Weatherford (1929), secretion was most active between the eighth and tenth days after parturition, when more cells were in the process of elaborating secretion than at any time during the previous week. After the tenth day of lactation the secretory activity of the cells gradually lessened, and by the fourteenth day the cells

showed less bulging, and fewer of them were columnar. On the twentieth day of lactation, he observed very few secretory vacuoles, and the appearance of the cells was similar to that during the first few days of gestation, apart from degenerated cells shed into the lumina.

Emmel, Weatherford and Streicker (1926) described a leukopenia in the circulating blood of lactating albino rats. This was attributed to an increased passage of lymphoid cells and other leukocytic elements into the mammary gland at that time. Also, Gardner and Dougherty (1944) demonstrated a marked leukopenia in lactating mice during nursing.

Reece and Warbritton (1953) observed little mitotic activity in the rat mammary gland during lactation, as did Maeder (1922). However, Jeffers (1935) observed an occasional mitotic figure, and demonstrated an increase in the number of cells per alveolus in lactation. More recently, Kirkham and Turner (1953), Brookreson and Turner (1959), Moon (1962), and Tucker and Reece (1963b), using the DNA method, demonstrated such an increase in the number of cells to occur in the mammary gland of the rat during lactation.

Greenbaum and Slater (1957) reported a doubling of the DNA content of the rat mammary gland occurring between the end of pregnancy and the third day of lactation. Although some of this increase in DNA was undoubtedly due to the leukocytic infiltration, these authors did not believe it to account for all of the increase. They suggested that a wave of mitoses might occur about the time of parturition, which subsided within a few hours. They quoted unpublished

results of Richardson, Slater and Greenbaum who, using colchicine, demonstrated the occurrence of a wave of cell division of relatively short duration some thirty hours after parturition. Further, they cited a personal communication from Lewin and Lewin who observed a rise in the number of nuclei /mg. wet tissue of mouse mammary gland from 136,000 to 260,000, during the period from parturition to the second day of lactation.

v. Involution

Involution of the mammary gland of the albino rat following weaning, was studied by Myers and Myers (1921). After the litters had been nursed for three weeks, the gross changes in the involuting mammary gland were studied at intervals following weaning. Myers observed at six hours following weaning, that the glandular tissue became enlarged due to the accumulation of milk secretion; this situation persisted for the following forty-eight hours. At the end of two and three weeks, the glands very closely resembled those of the adult virgin rat. The observations by Maeder (1922), Weatherford (1929), and Jeffers (1935) were roughly similar. They did not note any evidence of secretory activity after the first week of involution.

Maeder (1922) observed that the proportion of stroma and parenchyma was about equal on the fourth day. The nuclei were more irregular in outline and stained deeply. Many were pyknotic and fragmented. He also observed that in parous rats the ducts were longer and more ramified, and the blood vessels more numerous, than in the

virgin animal. (Figures 17 and 18.)

Williams (1942) noted active participation of polymorphonuclear leukocytes in phagocytosis early in involuting mouse mammary glands, followed subsequently by immigrating macrophages. Mayberry (1964), observed also in involuting mouse mammary glands, that macrophages congregated around and within atrophic alveoli and small ducts. These were probably active in removing retained secretion and necrotic epithelial debris. Also, plasma cells, lymphocytes, and neutrophils were increased in number.

Using DNA determination as an indication of cellular hyperplasia in the rat mammary gland, Greenbaum and Slater (1957), and Kirkham and Turner (1953) noted a decrease in DNA content in involution; a similar observation was made on mice by Anderson and Turner (1963). The latter noted the DNA content at twenty days of involution was similar to that of the virgin mouse. Slater (1962) observed an increase in DNA content of the rat mammary gland during the first two days of involution, followed by a decrease. The rise was probably brought about by enhanced leukocytic infiltration.

Tucker and Reece (1963c) reported that the total DNA content in six abdominal-inguinal mammary glands of rats, one day after weaning, did not differ significantly from that of glands on the twenty-first day of lactation; a significant decrease occurred three days after weaning, however. On the twenty-first day of involution the DNA content was similar to that of the sexually mature rat.

vi. Endocrine Control

The mammary glands, common to all mammals, belong to the category of sex organs participating in reproduction of the species. The mammary glands of female rats grow slowly from birth, with a slight spurt in growth occurring in the fourth week. At eight or nine weeks of age they grow more rapidly, and during the fertile age show a waxing and waning, corresponding to the cyclic changes of the ovaries, uterus and vagina. During pregnancy the glands increase further in size and become functionally active, producing milk secretion for the nourishment of the infant after birth. Following weaning the mammary glands involute, regressing to a state similar to that of the virgin gland.

It has been realized since 1895, that the control of the mammary gland in its various physiological states, and the interrelationship with other organs of reproduction, is endocrine (Turner, 1939). According to Jacobsohn (1961), the hormones believed to be involved in the mammary gland development are the gonodotropic hormones of the anterior pituitary and placenta, and the steroid hormones produced in the ovaries, adrenal cortex and placenta. In addition it appears that also thyroxine and insulin affect mammary growth indirectly by altering the metabolic environment (Jacobsohn, 1961; Cowie and Folley, 1961).

Using female hooded Norway rats, Cowie (1949) observed an increase in allometric growth of the mammary gland at some point between twenty-two to thirty days of age, the specific growth rate of the glands becoming three times that of the body surface. Ovarian hormones were

required for this phenomenon to occur. Silver (1953a) produced allometric growth at this age in ovariectomized hooded Norway rats by administering exogenous estrogens. Prior to three weeks of age, anterior pituitary extract was required in addition to estrogens to produce an increase in growth (Silver, 1953b). Myers (1916) demonstrated that a rapid growth occurred in the mammary glands of female albino rats at about nine weeks of age. This time corresponded to puberty. This observation was confirmed also by Astwood, Geschickter and Rausch (1937). Neither Myers nor Astwood noted the earlier growth described by Cowie and Silver.

After nine weeks, the endocrine fluctuations of the estrous cycle in the fertile rat coincided with fluctuations in growth of the mammary gland. These fluctuations were noted in the rat mammary gland by Sutter (1921) and Astwood et al. (1937). In mice, the fluctuations were noted by Cole (1933) and more recently by Laguchev (1962).

It has been discovered and widely accepted that estrogens are, in general, responsible for the growth of mammary gland ducts, whereas progesterone is necessary for complete lobular-alveolar growth to occur (Turner, 1939). More recently, Traurig and Morgan (1964a) have confirmed once again this concept on mice with the aid of radioautography. Laguchev (1959), showed that lack of ovarian hormones caused a cessation of mitotic division of the mammary gland epithelial cells in mice. Moreover, Bresciani (1964, 1965), also has shown that administration of estradiol-17β and progesterone for three or four days did not only initiate cell division in the mammary gland of mice, but also

accelerated a step of this process--the duplication of DNA. The steroid hormones of the ovary were much less effective in stimulating growth of the mammary glands in the absence of the pituitary (Petersen, 1944; Lloyd, 1962).

In 1943, Lyons demonstrated that in hypophysectomized-oophorectomized Long-Evans rats, estrogen, progesterone and prolactin were required to produce lobular-alveolar development, but the degree of development was less pronounced than that in the ovariectomized rat with intact pituitary, receiving estrogen and progesterone. The addition of somatotropic hormone to estrogen, progesterone and prolactin in hypophysectomized-ovariectomized rats increased the lobular-alveolar development to a condition corresponding to that of the latter half of pregnancy (Lyons et al., 1952). If this tetrad was injected for seven to ten days into hypophysectomized-ovariectomized rats, followed by the injection of prolactin, somatotropin, thyroxine and cortisone for one week, further development ensued, and finally milk secretion. Furthermore, the combination of hormones used in the first week was effective in the absence of adrenals, ovaries and pituitary, if NaCl was added to the drinking water (Lyons et al., 1953). In 1957, Lyons studied the local action of pituitary and ovarian hormones on the mammary glands of hypophysectomized-ovariectomized rats, confirming his previous findings.

Bates et al. (1964) observed in intact rats of N.I.H. Fischer strain that prolactin caused marked growth of the mammary gland without apparent milk production, while prolactin, somatotropic hormone and ACTH together produced large milk-filled glands. Cowie and Lyons (1959),

using hypophysectomized-ovariectomized-adrenalectomized hooded Norway rats, noted that the regression of mammary glands which followed the triple operation, was halted and duct growth resumed, after the addition of estrogen, progesterone, somatotropic hormones and corticoids. On withdrawing these hormones, local injection of prolactin caused secretion in the mammary glands if corticoids were administered systemically.

In addition to ovarian, anterior pituitary and adrenocortical hormones, Ray et al. (1955) discovered that the rat placenta contained a substance or substances with luteotropic, mammotropic, lactogenic and crop-stimulating properties, and also some evidence of a somatotropic hormone-like principle, which effect mammary gland growth.

The effect on the mammary gland of hormones of the testis varied in different species, and was similar to those exerted by certain hormones of the adrenal cortex (Jacobsohn, 1961). Using gonadectomized animals, Hamberger and Ahrén (1964) found that hormones of the adrenal cortex were necessary for the response of the rat mammary gland to testosterone. More recently, Jacobsohn and Norgren (1965) observed that mammary gland response to androgens was dependent on concomitant actions of estrogens and adrenal cortex steroids.

The necessary presence of the anterior pituitary for the initiation and maintenance of milk secretion has been known for many years (Cowie and Folley, 1961), and it has now been established that prolactin and probably somatotropic hormone are the most essential principles in this reaction (Lloyd, 1962). Although lactation persisted after

adrenalectomy, removal of these glands had a marked inhibitory effect (Turner, 1939). Less inhibition occurred in the absence of the thyroid, while ovariectomy did not appear to have any deleterious effect on lactation (Cowie and Folley, 1961). Parathyroid hormone, through its role in calcium metabolism, and insulin, also, affected milk secretion (Cowie and Folley, 1961).

If prolactin and somatotropic hormone are the necessary principles, then some inhibitory factor must be present during pregnancy to prevent the production of enough prolactin to cause synthesis of milk in large amounts. Many hypotheses have been proposed, suggesting possible co-operative and inhibiting actions of various hormones on milk secretion, but none of these has definitely been proved (Cowie and Folley, 1961). In addition, biochemical changes occur in mammary tissue near the time of parturition (Rees and Eversole, 1964), and the effect of suckling must be considered as well.

The ejection of milk is thought to be mediated through a neuro-humeral pathway. The suckling or milking stimulus is carried from the nipple to higher centres by sensory nerves. This is thought to cause an out-pouring of neurohypophyseal hormones. It is believed that oxytocin is the specific humoral agent (Cowie and Folley, 1961), and that it causes contraction of the myoepithelial cells surrounding the alveoli and ducts (Richardson, 1949).

III. CARCINOGENESIS

General

A carcinogen is an agent or process which significantly increases the yield of malignant neoplasms in a population. There are three main groups of carcinogens, (1) Ionizing radiation, (2) Viruses, and (3) Chemicals.

1) Radiation

It has been established that most forms of radiation, including sunlight, ultraviolet light, hard and soft X-rays and various forms of fundamental particles such as α and β rays, (helium nuclei and electrons), may induce tumors (Clayson, 1962).

2) Viruses

The role of viruses in producing tumors in animals was reviewed by Dmochowski (1957) and Martin (1964). The latter reviewed work on the interactions of hydrocarbon carcinogens with viruses and nucleic acids in vivo and in vitro. The subject has recently been reviewed again in a symposium by Rowe, Bryan, Lennette and MacMahon (1965). So far there is an almost total lack of direct evidence linking human cancer with oncogenic viral agents.

3) Chemicals

In 1932, Cook, Hieger, Kennaway and Mayneord were the first to show that a pure chemical was capable of inducing cancer in experimental animals. Since then, over five hundred chemicals have been demonstrated

to induce tumors in one or more species of experimental animals (Clayson, 1962). Many of the chemical compounds fall into a few groups of substances such as:

a) The polycyclic aromatic hydrocarbons, for instance, 3,4-benzpyrene, 3-methylcholanthrene (MCA) or 7, 12 dimethylbenz(a)anthracene (DMBA).

b) The aromatic amines and azo compounds, present in certain dyes.

c) The biological alkylating agents, such as the nitrogen mustards.

d) Other chemically diverse substances which produce tumors at a specific site or possess specific physiological activities, for instance, certain hormones.

Carcinogenesis in the Rat Mammary Gland

Carcinogenesis in the rat mammary gland is of particular interest in research because:

1) Mammary tumors can be induced readily in young animals.

2) Induced tumors in the rat mammary gland are sensitive to hormonal influences.

3) Viral factors have so far not been unequivocally demonstrated (Dao, 1964).

In the rat the incidence of spontaneous adenocarcinoma of the mammary gland is low in most strains, and develops late in life (Ratcliffe, 1940; Shay et al., 1949; Davis et al., 1956; Huggins, Morii

and Grand, 1961; Sydnor, Butenandt, Brillantes and Huggins, 1962). Huggins, Grand and Brillantes (1961) reported only two spontaneous mammary cancers among about 20,000 untreated female Sprague-Dawley rats younger than eight months. In contrast to the "spontaneous" appearance of rat mammary carcinoma, mammary gland neoplasms can be induced readily with polycyclic hydrocarbons at an early age (Huggins, Grand and Brillantes, 1961). Many investigators, for example, Huggins, Briziarelli and Sutton (1959), have observed that these mammary tumors produced by chemical carcinogens are hormone-sensitive. So far, viral factors have not been demonstrated in rat mammary cancer (Dao, 1964).

Rat mammary cancer has been produced by:

i. Radiation.

ii. A variety of chemicals, including hormones applied in various ways.

i. Radiation

Female albino rats are known to be prone to develop mammary gland malignancies after a single exposure to ionizing radiation (Shellabarger et al., 1957; Cronkite et al., 1960; Huggins and Fukunishi, 1963b). The last authors observed a significant number of sarcomas following such treatment. They compared the morphology of mammary carcinomas induced by radiation and by polycyclic hydrocarbons, and noted comparable histological appearance and a similar regression after ovariectomy. Application of x-irradiation was not as effective in producing mammary cancers as were certain chemical carcinogens, however.

ii. Chemicals

Although a variety of chemicals has been used to produce mammary cancer in the rat (Noble and Cutts, 1959), the two most commonly used were the polycyclic hydrocarbons, 3-methylcholanthrene (MCA) and 7, 12 dimethylbenz(a)anthracene (DMBA).

The effect of race-strain factors in the production of mammary cancers in rats was investigated by Sydnor, Butenandt, Brillantes and Huggins (1962) and Boyland and Sydnor (1962). They observed that DMBA was more effective in inducing mammary carcinoma in female Sprague-Dawley rats than in Long-Evans, Wistar, August or Chester Beatty strains. The Marshall strain of rats was resistant to treatment.

Howell (1960) showed that normal male rats did not develop breast tumors following administration of DMBA; Huggins, Grand and Brillantes (1961) made a similar observation with MCA application.

Methods of Administering Chemical Carcinogens

i. Application to the Skin

Orr (1955) painted the skin of three different strains of rats with DMBA, and produced mammary neoplasms in seventy-three per cent of the animals, almost all of which were adenocarcinoma. He failed to produce mammary tumors in rats by MCA application. Howell (1960) also produced mammary tumors by painting the skin of rats with DMBA.

ii. Subcutaneous Injection

Dunning et al. (1940) produced tumors, mainly sarcomas, by

injecting polycyclic aromatic hydrocarbons subcutaneously into breast tissue. Other workers (Noble and Cutts, 1959), applying a variety of hydrocarbons, produced similar tumors. Dao (1964) injected MCA into mammary tissue, resulting mainly in sarcomas.

iii. Intravenous Injection

Geyer et al. (1953) induced adenocarcinomas and benign tumors in the mammary glands of Wistar and Sprague-Dawley rats by multiple intravenous injections of DMBA. This procedure was subsequently repeated in Sprague-Dawley rats by Huggins, Morii and Grand (1961). A single intravenous injection of DMBA produced mammary tumors in one hundred per cent of the rats; in sixty-eight per cent of the animals multiple tumors appeared (Huggins and Fukunishi, 1963b).

iv. Intraperitoneal Administration

Huggins and Fukunishi (1963a) observed in fifty day old female Sprague-Dawley rats, that intraperitoneal administration of DMBA was just as effective as intravenous injection in producing mammary tumors.

v. Oral Administration

Shay et al. (1949, 1961) discovered that oral administration of MCA to female Wistar rats produced mammary carcinoma. Dao and Sunderland (1959) also produced mammary carcinomas with intragastric administration of MCA, as did Huggins, Briziarelli and Sutton (1959), and Daniel and Prichard (1961, 1963a and b, 1964a). In 1961, an important advance was made by Huggins, Grand and Brillantes, who

demonstrated that a single dose (twenty mgm.) of DMBA, dissolved in sesame oil, and administered by gastric tube to fifty day old female Sprague-Dawley rats, produced mammary cancer within sixty days, in one hundred per cent of the rats.

Production of Mammary Carcinoma by Hormones

Cutts and Noble (1964) and Cutts (1964) produced carcinoma of the mammary gland in a hooded strain of rats by the subcutaneous implantation of estrone pellets. The latent period was long, and the tumors regressed after removal of the implanted pellets.

Histology of the Tumors

In 1959, Huggins, Briziarelli and Sutton examined 680 mammary gland tumors induced by oral MCA. Of these, 678 were carcinomas. The remaining two tumors were fibrosarcomas, that developed after a much longer time than the carcinomas. The carcinomas exhibited a similar cytologic pattern. The acini were lined with many layers of epithelial cells arranged to form gland-like structures, with papillary formations projecting into the lumina. The lumina of these gland-like structures contained eosinophilic material of protein and carbohydrate nature. The alkaline phosphatase reaction revealed large numbers of myoepithelial cells irregularly arranged around and within the neoplastic glands. Dao and Sunderland (1959) noted that oral administration of MCA to Sprague-Dawley rats produced only mammary gland carcinomas. Their histologic appearance was similar to that described by Huggins et al. (1959).

Howell (1960) stated that practically all mammary gland tumors, produced by the administration of DMBA to the skin, were adenocarcinoma. He noted that the histological structure varied between tumors, and even between different parts of the same tumor. The structure ranged from poorly differentiated solid sheets of cells, to alveolar-type growth resembling the lactating breast. Most tumor tissue was composed of duct-like structures, sometimes with cysts and intracystic papillary growth, however.

Young, Cowan and Sutherland (1963) described a similar histological picture in mammary tumors following oral administration of DMBA. Almost all the mammary gland tumors were adenocarcinoma, but their cytodifferentiation varied from rat to rat, tumor to tumor and within the same tumor, from area to area. Some of the tumors regressed spontaneously.

The histology of mammary tumors induced by DMBA was studied also by Daniel and Prichard (1964b). In addition to the usual type of tumor, they observed milk-secreting adenomata, six to twelve months after the administration of carcinogen.

Stevens, Stevens and Currie (1965) attempted to correlate the growth characteristics, histology, and the rate of nucleic acid synthesis in mammary tumors produced by DMBA. They divided the DMBA induced mammary gland tumors into three types with a close correlation of their growth characteristics.

Grade A - Poorly differentiated tumor tissue with some acinar formation, and numerous mitotic figures.

Grade B - Reasonably well formed acinar or duct-like structures, some papillary processes, and not as many mitotic figures as Grade A.

Grade C - Well differentiated, often cystic, acinar or duct-like structures, with a single layer of flattened epithelium. Infrequent mitotic figures. These had the appearance of a "benign adenoma".

There was no correlation between these histological grades, the proportion of tumor occupied by stroma, and the nature and number of inflammatory and phagocytic cells infiltrating the stroma. There were varying proportions of mast cells, neutrophils and eosinophil polymorphs, lymphocytes, plasma cells and macrophages. The lumina of some ducts and acini were distended with homogeneous eosinophilic material, containing desquamated epithelial cells, macrophages, neutrophils, and cellular debris. Most of the growing tumors belonged to Grade A, the rest to Grade B. Grade C were mainly "regressing" tumors (Young and Cowan (1963)). As might be expected DNA synthesis was most rapid in the least differentiated, and most rapidly growing tumors.

Effect of Hormones on Induced Rat Mammary Tumors

The rôle of hormones in the development and growth of induced tumors of the rat mammary gland has been studied by many investigators. It was observed that most of the tumors induced by MCA or DMBA were hormone sensitive. In general, such induced tumors regressed permanently or temporarily following ovariectomy, hypophysectomy, and

administration of androgens, estradiol-17β with progesterone, or equine gonadotropin. Accelerated growth of mammary cancers occurred in pregnancy, pseudopregnancy, and with the administration of progesterone.

The effect of ovariectomy on the growth of tumors was studied by Howell (1960), Huggins, Briziarelli and Sutton (1959), Huggins, Grand and Brillantes (1959), Dao and Sunderland (1959), Shay, Gruenstein and Kessler (1961), Dao (1962), Daniel and Prichard (1963b) and Young, Cowan and Sutherland (1963). It is interesting to note that tumors induced by irradiation also regressed after ovariectomy (Huggins and Fukunishi, 1963b). Administration of ovarian hormones to ovariectomized rats, after tumor regression, reactivated the tumor (Huggins, Briziarelli and Sutton, 1959). The effect of hypophysectomy was observed by Huggins, Grand and Brillantes (1959), Huggins and Briziarelli (1959), Huggins, Briziarelli and Sutton (1959) and Daniel and Prichard (1963a). Hypophysectomy was more effective than ovariectomy in retarding growth of tumors (Huggins, Briziarelli and Sutton (1959); Daniel and Prichard (1963a)).

The effect of the administration of testerone in causing tumor regression was investigated by Huggins, Briziarelli and Sutton (1959), Shay, Gruenstein and Kessler (1961) and Kovacs (1965). Further, it has been observed that male rats did not develop mammary cancer following MCA or DMBA (Dao and Sunderland, 1959; Howell, 1960).

A combination of progesterone and estradiol-17β in proper dosage was shown to decrease the incidence of mammary tumors, or to cause existing tumors to regress (Huggins, Grand and Brillantes 1959, 1961;

Huggins and Yang, 1962; Huggins, Moon and Morii, 1962).

Also, equine gonadotropin was effective in preventing mammary cancer from developing (Huggins and Briziarelli, 1959; Huggins, Grand and Brillantes, 1959).

The acceleration of tumor growth during pregnancy was studied by Dao and Sunderland (1959), Dao, Bock and Greiner (1960), Huggins, Grand and Brillantes (1961), Huggins and Yang (1962), Huggins, Moon and Morii (1962). The effect of pseudopregnancy was similar to that of pregnancy (Dao and Sunderland, 1959).

It was observed that the administration of progesterone also accelerated the onset and growth of mammary tumors (Huggins and Briziarelli, 1959; Huggins, Briziarelli and Sutton, 1959; Huggins, Grand and Brillantes, 1959; and Huggins, Moon and Morii, 1962).

The effect of estradiol-17β alone appeared to depend on dosage. Large doses apparently blocked carcinogenesis, while small amounts had a permissive effect (Huggins, Briziarelli and Sutton, 1959; Huggins and Yang, 1962; Huggins, Moon and Morii, 1962).

The administration of ovarian hormones reactivated rat mammary carcinomas which had been induced by DMBA and caused to regress by ovariectomy (Huggins, Briziarelli and Sutton, 1959). In contrast, induced mammary tumors that had spontaneously regressed were not re-activated by the administration of ovarian hormones (Young, Cowan and Sutherland, 1963). The addition of growth hormone and cortisone to progesterone and estradiol-17β also failed to reactivate spontaneously regressing tumors, (Young and Cowan, 1963).

CHAPTER III

MATERIALS AND METHODS

Test Animals

Adult female Sprague-Dawley rats (Holtzman Rat Company, Madison 5, Wisconsin), ranging in body weight from 220 - 250 grams, were used in the experiments, apart from Experiment VI. In Experiment VI, as required by the technique applied, the animals were younger. The animals were fed Victor Fox food pellets and given unlimited tap water to drink. They were exposed to a daily cycle of twelve hours of light and twelve hours of darkness.

The Colchicine Technique

The colchicine technique was applied for ascertaining the mitotic rates in the normal mammary gland and in the mammary gland tumors. The details of this technique are discussed on page 7. The colchicine was obtained from Inland Alkaloid Inc., Tipton, Indiana. It was dissolved in distilled water, and injected, subcutaneously, in a dosage of 0.10 mgm./100 gm. body weight, in the interscapular region.

Carcinogen

The carcinogen for the induction of mammary tumors in Experiment VI was 7, 12 dimethylbenz(a)anthracene--$C_{18}H_{10}(CH_3)_2$, (DMBA). It was obtained from Eastman Organic Chemicals, Rochester 3, New York. In accordance with the technique of Huggins, Grand and Brillantes (1961), and Huggins and Yang (1962), twenty mgm. of DMBA were dissolved in two c.c. of sesame oil, and administered by gastric tube to each of fifty female rats, aged fifty days.

Sacrifice of Animals and Histological Technique

The animals were killed by chloroform six hours after colchicine injection. In the series of normal mammary glands (Experiments I - V), the abdominal and first inguinal mammary glands were fixed in Davidson's fixative, as were the mammary gland neoplasms of the tumor series (Experiment VI). All tissues were routinely prepared. Paraffin sections were cut at 5μ or 7μ, and stained with haematoxylin and eosin.

Counting

All cell counts were made by the investigator, in order to minimize error. Using a binocular microscope and a hand tally counter, more than 2,000 nuclei were counted under oil immersion for the tissue of each animal, and the numbers of colchicine metaphases that occured in the counted microscopic fields were recorded separately. Prophases were included with the interphase cells. Telophases and anaphases did not occur following colchicine administration. From these data, the percentage of cells that entered into division during each six-hour period was calculated for each animal.

Diurnal Variation

In order to detect possible diurnal variations in mitotic activity, it was necessary to determine the mitotic rate of the epithelial cells both during day and night. This was done by spacing evenly over a twenty-four hour period, four six-hour groups. For such

twenty-four hour series, (Experiments I, II* and VI), sixteen animals were used. They were divided into four groups of four rats each. The rats of each group were injected with colchicine, the first group at 10:00 a.m., the second group at 4:00 p.m., the third group at 10:00 p.m., and the fourth group at 4:00 a.m.. The animals were killed exactly six hours after injection. By this method, not only diurnal variations in mitotic rate can be determined, but the daily mitotic rate can be ascertained as well.

Daily Mitotic Rate

This was determined by averaging the percentages of colchicine metaphases of the four rats comprising each six-hour group. The summation of the four six-hour average percentages yielded the percentage of cells entering mitosis during one twenty-four hour period; that is, the daily mitotic rate of the cell population. This procedure was applied in Experiments I, II and VI. In the remaining experiments, the mitotic rate was determined only for the six-hour period between 10:00 a.m. and 4:00 p.m..

Estrous Cycle

As fluctuations of mitotic rate have been observed during different phases of the estrous cycle in the majority of epithelia lining the genital tract of the rat (Bertalanffy and Lau, 1963), the virgin rats in

*In Experiment II, 17 rats were used.

Experiments I and VI were followed through several estrous cycles by the vaginal smear technique, and then injected and killed during the first eighteen hours of estrous--stage I of estrus (Bertalanffy and Lau, 1963).

Experiment I. Determination of the rate of epithelial cell division in the mammary gland of virgin rats.

Sixteen rats in stage I of the estrous cycle were divided into four groups of four animals each. Each group represented a six-hour period in the twenty-four hour day. The animals were injected with colchicine and killed according to the procedure outlined above.

Experiment II. Determination of the rate of epithelial cell division in the mammary gland of twelve-day pregnant rats.

A twenty-four hour series composed of four groups of four rats each*, similar to that described in Experiment I was performed with rats on the twelfth day of pregnancy.

Experiment III. Determination of the rates of epithelial cell division in the mammary glands of rats at different stages of pregnancy.

The mitotic rates of mammary glands of rats pregnant four days, seven days, fourteen days and eighteen days were determined during six-hour periods, that is, from 10:00 a.m. to 4:00 p.m.. Pregnancy was confirmed after killing.

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*One group contained five rats.

Experiment IV. Determination of rates of epithelial cell division in the mammary glands of lactating rats.

Eight rats, on the second day of lactation following parturition, were injected with colchicine at 10:00 a.m., and killed at 4:00 p.m.. The mitotic rates were determined for this six-hour period.

Experiment V. Determination of rates of epithelial cell division in the involuting mammary gland of rats.

Twelve rats were allowed to nurse their litters for twenty-one days. The litters were then removed. After another seven days, the rats were injected with colchicine at 10:00 a.m. and killed at 4:00 p.m.. The mitotic rates were determined for this six-hour period.

Experiment VI. Determination of rates of cell division in mammary tumors induced by the administration of DMBA.

Each of a group of fifty female rats, aged exactly fifty days, was administered twenty mgm. of 7, 12 dimethylbenz(a)anthracene (DMBA) in two c.c. sesame oil by gastric tube, (8F rubber catheter), in accordance with the method of Huggins and Yang (1962). The animals were examined by palpation for mammary tumors, twice weekly, starting twenty days after the carcinogen was administered. Of the animals which developed mammary tumors, sixteen were used in a twenty-four hour series similar to Experiments I and II. All the animals were in Stage I Estrus at time of injection and killing. The tumors were examined and graded by a pathologist.

The grading of the tumors, by the consulting pathologist, was based primarily on the estimation of the degree of cell anaplasia. In addition, the tumors were classified histologically, using the terminology of human breast cancer, e.g., infiltrating lobular carcinoma, non-infiltrating medullary carcinoma, etc..

Statistical Methods

For each experimental series, the arithmetic means were calculated for each sample, as well as sample variances and standard deviations, the latter as measures of the amount of variation present in the data. Means were compared among treatments, (days), and were judged significant or not significant depending on the magnitude of the difference compared to the amount of variation.

CHAPTER IV

RESULTS

EXPERIMENT I

In this series the mitotic rates of mammary gland epithelium were determined in four six-hour groups of four rats each. The groups were spread over a twenty-four hour period. The animals were in Stage I Estrus. The mitotic rates for the four six-hour periods are shown in Table I. The average mitotic rate for the six-hour period from 10:00 a.m. to 4:00 p.m. was 0.26 per cent. The daily mitotic rate was determined by the summation of the average six-hour percentage of the four subgroups in this experiment, and proved to be 1.05 per cent. Analysis of variance disclosed no evidence of diurnal fluctuation in the mitotic rate. (Figures 2 and 3.)

EXPERIMENT II

In this experiment, the rates of epithelial cell division were determined in the mammary glands of rats pregnant twelve days. This was a twenty-four hour series similar to Experiment I. The results of the experiment are tabulated in Table II. The average mitotic rate for the six-hour period from 10:00 a.m. to 4:00 p.m. was 4.35 per cent. The summation of the four mean six-hour percentages yielded the daily mitotic rate of 13.20 per cent. Analysis of variance disclosed statistical evidence of diurnal variation in the twenty-four hour period. (Figures 8, 9 and 10.)

TABLE I

MITOTIC RATES OF MAMMARY GLAND
EPITHELIUM OF VIRGIN RATS (STAGE I ESTRUS)
DURING A TWENTY-FOUR HOUR PERIOD

Time of Day	Number of Nuclei	Number of Metaphases	Percentage of Metaphases	Mean
10 a.m.	2071	3	0.14	
to	2018	4	0.20	0.26
4 p.m.	2004	3	0.15	
	2028	11	0.54	
4 p.m.	2045	4	0.20	
to	2203	6	0.27	
10 p.m.	2091	1	0.05	0.32
	2038	15	0.74	
10 p.m.	2053	2	0.10	
to	2029	3	0.15	0.17
4 a.m.	2210	6	0.27	
	2054	3	0.15	
4 a.m.	2091	6	0.29	
to	2023	10	0.49	0.30
10 a.m.	2032	2	0.10	
	2006	6	0.30	

Daily Mitotic Rate -- 1.05% ± 0.20

TABLE II

MITOTIC RATES OF MAMMARY GLAND EPITHELIUM OF 12-DAY PREGNANT RATS DURING A 24-HOUR PERIOD

Time of Day	Number of Nuclei	Number of Metaphases	Percentage of Metaphases	Mean
10 a.m.	2088	85	4.07	
to	2178	88	4.04	
4 p.m.	2142	98	4.58	4.35
	2123	77	3.63	
	2177	118	5.42	
4 p.m.	2082	66	3.17	
to	2127	79	3.17	3.33
10 p.m.	2086	84	4.03	
	2194	65	2.96	
10 p.m.	2070	43	2.08	
to	2103	66	3.14	2.68
4 a.m.	2086	59	2.83	
	2169	58	2.67	
4 a.m.	2073	65	3.14	
to	2107	47	2.23	2.84
10 a.m.	2083	61	2.93	
	2085	64	3.07	

Daily Mitotic Rate - 13.20% ± 0.50

EXPERIMENT III

In this experiment the rates of epithelial cell division in the mammary glands of rats pregnant four, seven, fourteen and eighteen days were determined for the six-hour period from 10:00 a.m. to 4:00 p.m.. Table III presents the results of this experiment. The 10:00 a.m. to 4:00 p.m. group of twelve-day pregnant rats from Experiment II was also included in Table III. The average mitotic rate for the six-hour period (10:00 a.m. to 4:00 p.m.) in the mammary gland of rats pregnant four days was 0.43 per cent, and for rats pregnant seven days, 3.99 per cent. At day twelve of pregnancy the six-hour rate was 4.35 per cent. The rate fell to 1.02 per cent on day eighteen of pregnancy. The variation among the five six-hour groups was statistically significant. (Figures 4 to 14.)

EXPERIMENT IV

In this experiment the rates of epithelial cell division in the mammary glands of lactating rats were determined on the second day after parturition. The mitotic rates for the six-hour period from 10:00 a.m. to 4:00 p.m. are tabulated in Table IV. The average mitotic rate for the six-hour period was 0.51 per cent. (Figures 15 and 16)

TABLE III

MITOTIC RATES OF MAMMARY GLAND EPITHELIUM OF RATS ON VARIOUS DAYS OF PREGNANCY DURING A SIX-HOUR PERIOD

Time of Day	Number of Nuclei	Number of Metaphases	Percentage of Metaphases	Mean	
Day 4	2033	6	0.29		
10 a.m.	2111	16	0.76		
to	2074	16	0.77		
4 p.m.	2042	22	1.08		
	2086	2	0.01	0.43	
	2010	3	0.15		
	2037	5	0.25		
	2012	4	0.20		
Day 7	2204	118	5.35		
10 a.m.	2201	81	3.68		
to	2072	68	3.28	3.99	
4 p.m.	2164	85	3.93		
	2251	84	3.73		
Day 12*	2088	85	4.07		
10 a.m.	2178	88	4.04		
to	2142	98	4.58	4.35	
4 p.m.	2123	77	3.63		
	2177	118	5.42		
Day 14	2087	51	2.44		
10 a.m.	2105	48	2.28		
to	2130	37	1.74	2.01	
	2308	40	1.73		
	2097	39	1.86		
Day 18	2155	24	1.11		
10 a.m.	2143	29	1.35	1.02	
	2054	18	0.88		
	2037	15	0.74		± 0.50

*Day 12 figures taken from Table II.

TABLE IV

MITOTIC RATES OF MAMMARY GLAND EPITHELIUM OF RATS ON DAY 2 OF LACTATION DURING A SIX-HOUR PERIOD

Time of Day	Number of Nuclei	Number of Metaphases	Percentage of Metaphases	Mean
10 a.m. to 4 p.m.	2107	43	2.04	
	2130	4	0.19	
	2060	6	0.29	
	2043	18	0.88	0.51 ± 0.21
	2314	4	0.17	
	2229	1	0.04	
	2398	9	0.37	
	2022	2	0.09	

EXPERIMENT V

The mitotic rates of mammary gland epithelium in involution were ascertained on the seventh day after weaning. The results are shown in Table V. The average mitotic rate for the six-hour period from 10:00 a.m. to 4:00 p.m. was 0.73 per cent. (Figures 17 and 18.)

EXPERIMENT VI

The rates of cell division in mammary tumors induced by the administration of DMBA were ascertained in this experiment.

Of the fifty rats administered DMBA by gastric tube, twenty-five animals died of respiratory infections, most within thirty days following the administration of the carcinogen. The infections may have been the result of inhalation of the DMBA-oil suspension. In the remaining twenty-five rats, single or multiple tumors developed within one hundred days of DMBA administration in all but three animals. The sites of the mammary gland tumors varied from the neck to the inguinal region along the milk line. The size ranged from three mm. to forty mm. in diameter.

Sixteen of the tumor-bearing animals were used in Experiment VI. One or more tumors were excised from each animal, a total of nineteen tumors. To exclude possible variations of mitotic rate during the estrous cycle, the animals were injected and killed while in Stage I of estrus. They were divided into four groups, each one covering a different period of the day, in an attempt to determine whether daily

TABLE V

MITOTIC RATES OF MAMMARY GLAND EPITHELIUM OF RATS IN INVOLUTION DURING A SIX-HOUR PERIOD

Time of Day	Number of Nuclei	Number of Metaphases	Percentage of Metaphases	Mean
10 a.m. to 4 p.m.	2023	1	0.05	
	2108	0	0	
	2026	0	0	
	2097	0	0	
	2094	4	0.19	
	2061	15	0.73	
	2072	2	0.10	
	2081	4	0.19	0.73 ± 1.05
	2025	10	0.49	
	2076	35	1.68	
	2078	56	2.06	
	2072	67	3.23	

TABLE VI

MITOTIC RATES OF NINETEEN DMBA-INDUCED RAT MAMMARY TUMORS DURING A TWENTY-FOUR HOUR PERIOD

Time of Day	Animal Number	Tumor Size (Diam.)	Number of Nuclei	Number of Metaphases	Percentage of Metaphases
10 a.m.	18	10 mm.	2065	25	1.21
to	19	8 mm.	2060	46	2.18
4 p.m.	20	12 mm.	2193	56	2.55
	8a	14 mm.	2224	68	3.05
	8b	8 mm.	2041	16	0.78
4 p.m.	2	6 mm.	2045	34	1.66
to	5	15 mm.	2199	16	0.75
10 p.m.	4	18 mm.	2074	46	2.21
	9	8 mm.	2075	45	2.16
10 p.m.	11a	40 mm.	2331	54	2.31
to	11b	10 mm.	2063	14	0.67
4 a.m.	10	8 mm.	2024	22	1.08
	6*	8 mm.	2016	8	0.39
	3	5 mm.	2059	53	2.57
4 a.m.	16	5 mm.	2138	39	1.82
to	21	15 mm.	2186	114	5.21
10 a.m.	23	8 mm.	2288	192	8.43
	14a	20 mm.	2067	11	0.53
	14b	3 mm.	2066	33	1.59

* All tumors were adenocarcinomas except for #6, which was proliferative disease.

mitotic fluctuations occurred in the tumors. The results are tabulated in Table VI. It is obvious that because of the wide range in mitotic activity in the various tumors, no evidence of diurnal variation in cell division was apparent. Neither was it possible to calculate a daily mitotic rate of the tumors as has been done, for example, for Walker carcinosarcoma, and fibrosarcoma 1F16F-5LFM in rats (Bertalanffy and Lau, 1962b), and for spontaneous adenocarcinoma of the mammary gland in C_3H/HeJ mice (Bertalanffy, 1963). Therefore, the tumors were arranged according to their grade and mitotic activity as shown in Table VII.

The tumors were examined and graded by a pathologist. All were adenocarcinomas except for one. The grades corresponded very closely to the mitotic rates of the tumors. A correlation between tumor size and rate of cell division was not apparent. (Figures 19 to 24.)

TABLE VII

SIX-HOUR MITOTIC RATES OF NINETEEN DMBA-INDUCED RAT MAMMARY TUMORS

Animal Number	Tumor Size (Diam.)	Percentage of Metaphases	Grade
23	8 mm.	8.43	III-IV
21	15 mm.	5.21	III-IV
8a	14 mm.	3.05	III
3	5 mm.	2.57	II-III
20	12 mm.	2.55	III
11a	40 mm.	2.31	III
4	18 mm.	2.21	III
9	8 mm.	2.16	II-III
19	8 mm.	2.18	II-III
16	5 mm.	1.82	II
2	6 mm.	1.66	II
14b	3 mm.	1.59	II
18	10 mm.	1.21	II
10	8 mm.	1.08	II
8b	8 mm.	0.78	I-II
5	15 mm.	0.75	I-II
11b	10 mm.	0.67	II
14a	20 mm.	0.53	Mitoses uncommon
6*	8 mm.	0.39	Proliferative disease
		Mean -- 2.17%	± 1.90

*All tumors were adenocarcinomas except for one which was proliferative disease.

CHAPTER V

DISCUSSION

Experiment I. In this experiment the mitotic rates of the mammary gland epithelium were determined in virgin Sprague-Dawley rats in Stage I of estrus during a twenty-four hour period. A twenty-four hour pilot experiment by the investigator, disregarding the estrous cycle, indicated considerable variation in mitotic rate, ranging from 0.19 per cent to 2.05 per cent for a six-hour period. In addition, Bertalanffy and Lau (1963) had noticed fluctuations in mitotic rate during the estrous cycle in the majority of epithelia lining the female genital tract of the rat. Because of these observations, the animals used in Experiment I were followed through several successive estrous cycles, using the vaginal smear technique, to make sure that the sixteen animals had regular cycles and were in the first eighteen hours of the estrous cycle, (stage I estrus), for the duration of the experiment.

It can be noted from Table I that the mitotic rate of mammary gland epithelium of the rat in stage I of estrus is low (average for the six-hour period from 10:00 a.m. to 4:00 p.m. was 0.26 per cent). This result was not expected. Laguchev (1962), investigating the mitotic rates in the mouse mammary gland, had noted a higher rate of cell division in the proestrus and estrus phases of the cycle than in the diestrus phase in C_{57} mice. These observations corresponded to the observations of Sutter (1921) and Cole (1933) who, using the whole mount method, noted a pronounced growth in the mammary tree of rats and mice during the estrus phase. This growth regressed following estrus. Laguchev noted, however, that the pattern observed in C_{57} mice was obscured in C_3H mice. He thought this was due to an endocrine

imbalance. In fact he found in some animals of the C_3H strain that the mitotic rate was higher in diestrus than in estrus. It is the intention of the present investigator to pursue this study further. To date, the mitotic rate in diestrus has been investigated in five rats. The six-hour mitotic rate for the period from 10:00 a.m. to 4:00 p.m. ranged from 0.24 per cent to 2.48 per cent, compared to an average of 0.26 per cent in stage I of estrus. This would indicate that the mitotic rate in diestrus may be higher than in estrus in Sprague-Dawley rats. It will be necessary to divide diestrus into two twenty-four hour periods in order to make a more accurate assessment of the mitotic rate in this phase, which lasts approximately forty-eight hours in the rat (Long and Evans, 1922; Bertalanffy and Lau, 1963). In addition, the mitotic rates in proestrus and metestrus will be investigated as well.

Diurnal fluctuations of mitotic activity have been observed in some tissues of the rat (Bertalanffy, 1960). However, in other tissues this phenomenon has not been apparent (Bertalanffy and Lau, 1962a). In the present experiment there is no statistical evidence of diurnal fluctuation in the mammary gland of the rat in stage I of estrus. This apparent absence of diurnal variation may be due to the low mitotic rate, rendering any fluctuations undetectable.

Experiment II. In this experiment, mitotic rates were determined in the mammary gland epithelium of rats pregnant twelve days, during a twenty-four hour period. The daily mitotic rate of 13.20 per cent is

about a twelve-fold increase over the daily mitotic rate of the mammary gland epithelium of the virgin rats in Experiment I, which was 1.05 per cent. (Table II.) An increase in rate in pregnant rats was, of course, expected. Other investigators (see Chapter II, pp. 15 and 16) had noted this increase in mitotic activity especially around the mid-point of pregnancy. It is interesting to note that the time to time variation in the twenty-four hour period is statistically significant, and therefore a diurnal fluctuation in mitotic activity is apparent. The peak of activity occurred during the six-hour period from 10:00 a.m. to 4:00 p.m., while the lowest mitotic activity occurred during the period 10:00 p.m. to 4:00 a.m..

Experiment III. The mitotic rates for the six-hour period (10:00 a.m. to 4:00 p.m.) in rats pregnant four, seven, twelve, fourteen and eighteen days is shown in Table III. Animals pregnant four days exhibited considerable individual variations of mitotic rate, ranging from 0.10 per cent to 1.08 per cent. The fact that the average mitotic rate was low, (0.43 per cent), in the four-day pregnant rats, confirms the work of Tucker and Reece (1963a) who, using the DNA method, found only a 1.10 per cent increase in mammary gland growth over the virgin in hooded Norway rats by day four of pregnancy, while by the eighth day there was a twenty per cent increase. The observation of a mitotic rate of 1.08 per cent in one animal in the present experiment suggests that day four may mark the beginning of the hormonal stimulation of the mammary gland in pregnancy, the level prior to this date being not

sufficiently high to cause proliferation of the epithelial cells exceeding significantly that occurring in the virgin animal of stage I of estrus.

By day seven of pregnancy, the average mitotic rate for the six-hour period from 10:00 a.m. to 4:00 p.m. had increased to 3.99 per cent, and further, by the twelfth day of pregnancy to 4.35 per cent. Two days later, on day fourteen of pregnancy, the rate decreased to 2.01 per cent, while by the eighteenth day of pregnancy the mitotic rate had declined to 1.02 per cent for the six-hour period. These findings are in accord with the observations of Jeffers (1935), Reece and Warbritton (1953), Kirkham and Turner (1953), Griffiths and Turner (1961) and Tucker and Reece (1963a), detailed in Chapter II, page 15.

The present experiments reveal that during the first half of pregnancy, in the course of the rapid development in the mammary gland, the mitotic activity in the mammary gland becomes greatly augmented. Its peak is attained around the mid-point of pregnancy, on or about day twelve, and thereafter declines. However, parenchymal development in the mammary gland continues during the second half of pregnancy, although at a slower rate. This is indicated by the slower but still comparatively pronounced cell proliferation (Table III).

Experiment IV. In this experiment eight rats were administered colchicine on the second day following parturition. The mitotic rate of the mammary gland parenchyma was determined during the six-hour period from 10:00 a.m. to 4:00 p.m. (Table IV). The finding of a low

average mitotic rate of 0.51 per cent for the six-hour period, was not unexpected. It implied that during lactation the activity of the gland is directed mainly toward secretion, and formation of new cells subsides to a low level. The low rate of cell proliferation is not a general finding, however. Thus some previous investigators have found evidence of an increase in cell division in the mammary gland of rats and mice during the early stages of lactation. Greenbaum and Slater (1957), using the DNA method, have reported a doubling of the DNA content of the rat mammary gland between the end of pregnancy and the third day of lactation, and cited a personal communication from Lewin and Lewin, who observed a rise in the number of nuclei/mgm. wet tissue of mouse mammary gland from 136,000 to 260,000, during the period between parturition and the second day of lactation. Greenbaum and Slater (1957) suggested that a wave of mitoses, of short duration, might occur at the time of parturition and subside within a few hours. In the present investigation a higher mitotic rate of 2.04 per cent was observed only in one rat on the second day of lactation. Further investigation of the mitotic rates in the mammary gland around the time of parturition, and in early lactation, is clearly necessary to elucidate this phenomenon. In addition, mitotic rates should be determined at various intervals throughout lactation.

Experiment V. In this experiment, the mitotic rates of the mammary gland epithelium was determined on the seventh day after weaning. The average mitotic rate for the six-hour period (10:00 a.m. to 4:00 p.m.)

in the involuting rat mammary gland on this day was 0.73 per cent. Nine of the thirteen rats in the experiment showed a mitotic rate ranging from zero to 0.73 per cent, while three of the rats exhibited significantly higher rates, ranging from 1.68 per cent to 3.23 per cent for the six-hour period (Table V).

Slater (1962) observed an increase in DNA content of the rat mammary gland during the first two days of involution, thought to be due to an increase in leukocyte concentration. This increase was followed by a decrease in DNA content. A similar observation was made by Tucker and Reece (1963c), who also stated that by the twenty-first day of involution the DNA content had decreased to an amount similar to that of the sexually mature virgin rat. A search of the literature, however, has failed to reveal evidence of an increase in DNA content after the first few days of involution. The high rate in the three rats on the seventh day of involution, in the present series, may indicate a hormonal imbalance in these animals, maintaining the mitotic activety at a high level since the early part of pregnancy.

Experiment VI. In this experiment the mitotic rates of rat mammary tumors induced by oral DMBA administration were determined in a total of nineteen tumors from sixteen rats. It is apparent from Tables VI and VII that the mitotic rates of most of the mammary tumors studied in the present investigation were similar to those of the mammary gland epithelium of the pregnant rat (Tables II and III). An exception was one infiltrating carcinoma with a high mitotic rate of 8.43 per cent

for the six-hour period.

The relationship between hormones and DMBA in the induction of mammary tumors, and the role of hormones in the promotion and maintenance, or regression of mammary tumor growth in the rat, has been of great interest in research. Out of the vast amount of literature on the subject are several observations of particular interest in the present experiment.

One observation of interest is the optimal time of administration of the single dose of carcinogen to female rats. This has been determined to be between fifty to sixty-five days of age (Huggins, Grand and Brillantes, 1961). It was reported in Chapter II, page 21, that eight or nine weeks of age in the rat corresponded to puberty. Therefore, for maximum induction of tumors by DMBA, the carcinogen must be given before or during the onset of sexual maturity in virgin rats. If the carcinogen is administered after sixty-five days of age, the incidence of mammary tumors is dramatically reduced (Huggins, Grand and Brillantes, 1961).

Dao (1964) reported extensive studies of the concentration and clearance of polycyclic hydrocarbons in the fat, mammary gland and other tissues of the rat. The adipose tissue of the mammary glands apparently acts as a storage depot for the hydrocarbons and there is a slow rate of clearance from this tissue.

Furthermore, the observation that mammary cancer can be induced in certain strains of rats by the administration of estrogens has not been studied extensively. The "intriguing question remains to be answered:

How do estrogens induce neoplasia?" (Dao, 1964).

Furthermore, Huggins and Yang (1962) pointed out the "remarkable similarity in geometric pattern (but not in molecular thickness) between carcinogenic polynuclear aromatic hydrocarbons and the base pairs of nucleic acids.....A molecular model was made of guanine-cytosine, and a frame was constructed to surround it. In this frame similar atomic models of progesterone, testosterone, and estradiol-17β fit neatly". Similarly, they noted, mammary carcinogens, for instance, DMBA, could be inserted within the frame.

Dao (1964) stated: "The carcinogenic effects of steroid hormones are not yet understood. The steric resemblance between polycyclic hydrocarbons and steroid hormones suggests that the two types of compounds may act at the same sites in biological systems." It is the opinion of this author that carcinomas induced by the hydrocarbons may be the result of interference with normal steroid functions.

CHAPTER VI

SUMMARY

Experiment I. The mitotic rate of the mammary gland in virgin Sprague-Dawley rats in stage I of estrus was low.

Experiments II and III. After conception, the mitotic rate in the mammary gland of the rat increased, reaching its peak about the mid-point of pregnancy. Thereafter, the rate declined, but considerable mitotic activity was nevertheless maintained in the second half of pregnancy.

Experiment IV. On the second day of lactation following parturition, the mitotic rate of the mammary gland was low, indicating that the activity of the cells is directed toward secretion rather than proliferation. In one animal a higher mitotic rate was apparent. Other workers have found some evidence of a short-lived "wave" of mitoses following parturition. This problem remains to be elucidated.

Experiment V. In the involuting rat mammary gland on the seventh day after weaning, the mitotic rate was low. Exceptions to this were three animals that exhibited a much higher rate. This may have been due to a hormonal imbalance.

Experiment VI. The mitotic rates in most of the rat mammary tumors induced by oral DMBA administration were similar to the mitotic rates in the mammary gland epithelium of pregnant rats. In one tumor the mitotic rate was higher than in the mammary gland of pregnancy. This would indicate that the majority of mammary tumors induced in the rat by DMBA

administration, do not proliferate faster than the mammary gland epithelium of pregnant rats.

BIBLIOGRAPHY

Anderson, R.R., and Turner, C.W.: Involution of the mammary gland duct and lobule alveolar systems in the mouse. Proc. Soc. exp. Biol. Med., 113:333-334, 1963.

Astwood, E.B., Geschickter, C.F., and Ralisch, E.O.: Development of the mammary gland of the rat. Amer. J. Anat., 61:373-395, 1937.

Bates, R.W., Milkovic, S., and Garrison, M.M., Effects of prolactin, growth hormone and ACTH, alone and in combination, upon organ weights and adrenal function in normal rats. Endocrinology, 74: 714-723, 1964.

Bertalanffy, F.D.: Mitotic rates and renewal times of the digestive tract epithelia in the rat. Acta anat. (Basel), 40:130-148, 1960.

.....: Mitotic rate of spontaneous mammary gland adenocarcinoma in C_3H/HeJ mice. Nature, 198:496-497, 1963.

.....: Tritiated thymidine vs. colchicine technique in the study of cell population cytodynamics. Lab. Invest., 13: 871-886, 1964.

Bertalanffy, F.D., and Lau, Chosen: Mitotic rate and renewal time of the corneal epithelium in the rat. Arch. Ophthal., 68:546-550, 1962a.

.....: Rates of cell division of transplantable malignant rat tumors. Cancer Res., 22:627-31, 1962b.

.....: Mitotic rates, renewal times, and cytodynamics of the female genital tract epithelia in the rat. Acta anat., 54:39-81, 1963.

Bertalanffy, F.D., and Leblond, C.P.: The continuous renewal of the two types of alveolar cells in the lung of the rat. Anat. Rec., 115: 515-542, 1953.

Bertalanffy, F.D., and Nagy, K.P.: Mitotic activity and renewal rate of the epithelial cells of human duodenum. Acta anat., 45:362-370, 1961.

Boivin, A., Vendrely, R., and Vendrely, C: Compt. rend., 226:1061, 1948. Cited by Vendrely (1955).

Boyland, E., and Sydnor, K.L.: The induction of mammary cancer in rats. Brit. J. Cancer, 16:731-739, 1962.

Bresciani, Francesco: DNA synthesis in alveolar cells of mammary gland: Acceleration by ovarian hormones. Science, 146:653-655, 1964.

Bresciani, Francesco: Effect of ovarian hormones on duration of DNA synthesis in cells of the C_3H mouse mammary gland. Exp. Cell Res., 38:13-32, 1965.

Clayson, David B.: Chemical Carcinogenesis. London, J. and A. Churchill Ltd., 1962.

Cole, H.A.: The mammary gland of the mouse during the oestrus cycle, pregnancy and lactation. Proc. roy. Soc. B., 114:136-161, 1933.

Cook, J.W., Hieger, I., Kennaway, E.L., and Mayneord, W.V.: The production of cancer by pure hydrocarbons. Part I. Proc. roy. Soc. B., 111:455, 1932. Cited by Clayson, 1962.

Cowie, A.T.: The relative growth of the mammary gland in normal, gonadectomized and adrenalectomized rats. J. Endocrin., 6:145-157, 1949.

Cowie, A.T., and Folley, A.S.: The role of the adrenal cortex in mammary development and its relation to the mammagenic action of the anterior pituitary. Endocrinology, 40:274-285, 1947.

.....: The Mammary Gland and Lactation. In: Young, Wm. C.: Sex and Internal Secretions, ed. 3, Vol. I, Baltimore, Williams and Wilkins (1961), pp. 590-642.

Cowie, A.T., and Lyons, W.R.: Mammogenesis and lactogenesis in hypophysectomized ovariectomized, adrenalectomized rats. J. Endocr., 19: 29-32, 1959.

Cronkite, E.P., et al.: Studies on radiation-induced mammary gland neoplasia in the rat. Radiat. Res., 12:81-93, 1960.

Cutts, J. Harry: Estrone-induced mammary tumors in the rat. II. Effect of alterations in the hormonal environment on tumor induction, behaviour and growth. Cancer Res., 24:1124-1130, 1964.

Cutts, J. Harry, and Noble, R.L.: Estrone-induced mammary tumors in the rat. II. Induction and behaviour of tumors. Cancer Res., 24: 1116-1123, 1964.

Daniel, P.M., and Prichard, M.M.L.: The production of mammary tumors in rats by feeding with 3MCA. Brit. J. Cancer, 15:828-832, 1961.

.....: The response of experimentally induced mammary tumors in rats to hypophysectomy and to pituitary stalk section. Brit. J. Cancer, 17:446-453, 1963a.

.....: The response of experimentally induced mammary tumors in rats to ovariectomy. Brit. J. Cancer, 17:687-690, 1963b.

Daniel, P.M., and Prichard, M.M.L.: Production of mammary tumors with 3MCA. Nature, 201:578-580, 1964a.

.....: Three types of mammary tumor induced in rats by feeding with DMBA. Brit. J. Cancer, 18:514-520, 1964b.

Dao, Thomas L.: The role of ovarian hormones in initiating the induction of mammary cancer in rats by polynuclear hydrocarbons. Cancer Res., 22:973-981, 1962.

.....: Carcinogenesis of Mammary Gland in the Rat. In: Homburger, F.: Progr. exp. Tumor Res., 5: Basel/New York, Karger (1964), pp. 157-216.

Dao, T.L., Bock, F.G., and Greiner, M.J.: Mammary carcinogenesis by 3MCA. II. Inhibitory effect of pregnancy and lactation on tumor induction. J. nat. Cancer Inst., 25:991-1003, 1960.

Dao, T.L., and Sunderland, H.: Mammary carcinogenesis by 3MCA. I. Hormonal aspects in tumor induction and growth. J. nat. Cancer Inst., 23:567-585, 1959.

Dempsey, E.W., Bunting, H., and Wislocki, G.B.: Observations on the chemical cytology of the mammary gland. Amer. J. Anat., 81: 309-341, 1947.

Davidson, J.N.: Chemical Methods for Nucleic Acid Estimation. In: Davidson, J.N.: Biochemistry of the Nucleic Acids, ed. 4, London, Methuen and Co. Ltd. (1960), pp. 93-103.

Davidson, J.M., and Leslie, I.: A new approach in the biochemistry growth and development. Nature, 165:49-53, 1950.

Davis, R.K., Stevenson, G.T., and Busch, K.A.: Tumor incidence in Sprague-Dawley female rats. Cancer Res., 16:194-197, 1956.

Dmochowski, Leon: The Part Played by Viruses in the Origin of Tumors. In: Raven, R.W.: Cancer, Vol. I, Toronto, Butterworth and Company (1957), pp. 214-305.

Dunning, W.F., Curtis, M.R., and Eisen, M.J.: The carcinogenic activity of methylcholanthrene in rats. Amer. J. Cancer, 40:85-127, 1940.

Echave Llanos, J.M., and Piezzi, R.S.: Twenty-four hour rhythm in the mitotic activity of normal mammary epithelium on normal and inverted lighting regimes. J. Physiol., 165:437-442, 1963.

Eigsti, O.J., and Dustin, Pierre Jr.: In Colchicine--In Agriculture Medicine, Biology and Chemistry. Iowa State College Press, Ames, Iowa, 1955.

Emmel, V.E., Weatherford, H.L., and Streicher, M.H.: Leucocytes and lactation. Amer. J. Anat., 38:1-40, 1926.

Farris, E.J., and Griffith, J.Q. Jr.: In: The Rat in Laboratory Investigation, ed. 2, New York, Hafner Publishing Co. (1962).

Flux, D.S.: The effect of adrenal steroids on the growth of the mammary glands, uteri, thymus and adrenal glands of intact, ovariectomized and oestrone-treated ovariectomized mice. J. Endocr., 11:238-254, 1954.

Gardner, W.U., and Dougherty, J.H.: The circulating leucocytes of suckling and non-suckling lactating mice. Anat. Rec., 88:432-433, 1944.

Geyer, R.P., et al.: Effect of dose and hormones on tumor production in rats given emulsified 9, 10, dimethyl-1, 2-benzathracene intravenously. Cancer Res., 13:503-506, 1953.

Gomori, G.: Microtechnical demonstrations of phosphatase in tissue sections. Proc. Soc. exp. Biol. Med., 42:23-26, 1939.

Greenbaum, A.L., and Slater, T.F.: 1. Studies on the particulate components of rat mammary gland. 2. Changes in the levels of nucleic acids of the mammary glands of rats during pregnancy, lactation and mammary involution. Biochem. J., 66:155-161, 1957.

Griffiths, D.R., and Turner, C.W.: Normal growth of rat mammary glands during pregnancy and early lactation. Proc. Soc. exp. Biol. Med., 106:448-450, 1961.

Hamberger, L., and Ahren K.: Influence of adrenal cortex on growth process in the rat mammary gland. J. Endocr., 30:171-179, 1964.

Hamilton, W.J., Boyd, J.D., and Mossman, H.W.: In: Human Embryology, ed. 3, Cambridge, England, W. Heffer and Sons, Ltd. (1962), pp. 429.

Howell, J.S.: The chemical induction of breast tumors in the rat: Hormonal factors in tumour production. Brit. J. Cancer, 14: 657-667, 1960.

Hughes, W.L., et al.: Cellular proliferation in the mouse as revealed by autoradiography with tritiated thymidine. Proc. nat. Acad. Sci., 44:476-483, 1958.

Huggins, C., and Briziarelli, G.: Prevention of mammary cancer by endocrinologic methods. Science, 129:1285, 1959.

Huggins, C., Briziarelli, G., and Sutton, H.: Rapid induction of mammary carcinoma in the rat and the influence of hormones on the tumors. J. exp. Med., 109:25-42, 1959.

Huggins, C., and Fukunishi, Ryo: Mammary and peritoneal tumors induced by intraperitoneal administration of 7, 12 dimethylbenz(a)anthracene in newborn and adult rats. Cancer Res., 23:785-789, 1963a.

.....: Cancer in the rat after single exposures to irradiation or hydrocarbons. Age and strain factors: Hormone dependence of mammary cancers. Radiat. Res., 20:493-503, 1963b.

Huggins, C., Grand, L.C., and Brillantes, F.P.: Critical significance of breast structure in the induction of mammary cancer in the rat. Proc. nat. Acad. Sci., 45:1294-1300, 1959.

.....: Mammary cancer induced by a single feeding of polynuclear hydrocarbons, and its suppression. Nature, 189: 204-207, 1961.

Huggins, C., Moon, R.C., and Morii, S.: Extinction of experimental mammary cancer. I. Estradiol-17 and progesterone. Proc. nat. Acad. Sci., 48:379-386, 1962.

Huggins, C., Morii, S., and Grand, L.C.: Mammary cancer induced by a single dose of polynuclear hydrocarbons: Routes of administration, Ann. Surg., 154:315-318, 1961.

Huggins, C., and Yang, N.C.: Induction and extinction of mammary cancer. Science, 137:257-262, 1962.

Jacobsohn, Dora: Hormonal Regulation of Mammary Gland Growth. In: Kon, S.K., and Cowie, A.T.: Milk: The Mammary Gland and Its Secretion, New York, Academic Press (1961), pp. 126-160.

Jacobsohn, D., and Norgren, A.: Estrogens and corticoids in relation to mammary gland growth in male rats. Proc. Soc. exp. Biol. Med., 118:1106-1109, 1965.

Jeffers, K.R.: Cytology of the mammary gland of the albino rat. I. Pregnancy lactation and involution. Amer. J. Anat. 56:257-273, 1935.

Kirkham, Wm. R., and Turner, C.W.: Nucleic acids of the mammary glands of rats. Proc. Soc. exp. Biol. Med., 83:123-126, 1953.

Kovacs, K.: Effect of androgenisation on the development of mammary tumors in rats induced by the oral administration of 9, 10, dimethyl-1,2-benzanthracene. Brit. J. Cancer, 19:531-537, 1965.

Laguchev, S.S.: The cell division pattern of mammary gland epithelium at various stages of the estrous cycle in mice of high and low cancer lines. Biull. Eksp. Biol. Med., 53:318-321, 1962.

.....: Biull. Eksp. Biol. Med., 12:103, 1959. Cited by Laguchev, S.S., and Kargina-Terent'eva, (1963).

Laguchev, S.S., and Kargina-Terent'eva, R.A.: Ovarian hormones as a factor essential to mitosis of epithelial cells of reproductive organs. Biull. Eksp. Biol. Med., 56:1131-1133, 1963.

Leblond, C.P., and Stevens, C.E.: The constant renewal of the intestinal epithelium in the albino rat. Anat. Rec., 100:357-371, 1948.

Leblond, C.P., Storey, W.F., and Bertalanffy, F.D.: Rate of cell renewal of organs. A comparison with cancer statistics. Acta Un. int. Cancer., 7:692-694, 1951.

Leblond, C.P., and Walker, Bruce E.: Renewal of cell populations. Physiol. Rev., 36:255-276, 1956.

Linzell, J.L.: The silver staining of myoepithelial cells, particularly in the mammary gland, and their relation to the ejection of milk. J. Anat., 86:49-57, 1952.

Lloyd, C.W.: The Control of Mammary Growth. In: Williams Textbook of Endocrinology, ed. 3, Philadelphia, Saunders (1962), pp. 462-464.

Long, Jos. A., and Evans, Hubert M.: The oestrus cycle in the rat and its associated phenomena. Memoirs of the University of California, University of California Press, Berkeley, California, 1922.

Lyons, W.R.: In: Essays in Biology in Honor of Herbert M. Evans. University of California Press, Berkeley, California, 1943. Cited by Cowie and Folley, 1961.

Lyons, W.R., et al.: The enhancing effect of somatotropin on the mammary growth induced in rats with estrin, progestin, and mammotropin. J. clin. Endocr., 12:937, 1952.

Lyons, W.R., et al.: Some of the hormones required by the mammary gland in its development and function. J. clin. Endocr., 13:836-837, 1953.

Lyons, W.R., et al.: Local action of pituitary and ovarian hormones on the mammary glands of hypophysectomized and oophorectomized rats. Anat. Rec., 127:432-433, 1957.

Maeder, Leroy M.A.: Changes in mammary gland of albino rat during lactation and involution. Amer. J. Anat., 31:1-26, 1922.

Manitoba Cancer Treatment and Research Foundation: Report, April 1, 1963 to March 31, 1964.

Martin, Christopher M.: Interactions of Hydrocarbon Carcinogens With Viruses and Nucleic Acids in Vivo and In Vitro. In: Homburger, F.: Progr. exp. Tumor Res., 5: Basel/New York, Karger (1964), pp. 134-156.

Mayberry, H.E.: Macrophages in post-secretory mammary involution in mice. Anat. Rec., 149:99-112, 1964.

Mendelsohn, M.L.: Autoradiographic measurement of tumor growth. Acta Un. int. Cancer, 20:1400-1401, 1964.

Messier, B., and Leblond, C.P.: Cell proliferation and migration as revealed by radioautography after injection of T-H^3 into mole rats and mice. Amer. J. Anat., 106:247-285, 1960.

Mirsky, A.E., and Ris, Hans: Variable and constant components of chromosomes. Nature, 163:666-667, 1949.

Moon, Richard C.: Mammary gland cell content during various phases of lactation. Amer. J. Physiol., 203:939-941, 1962.

Myers, J.A.: Studies on the mammary gland. I. Growth and distribution of the milk ducts and the development of the nipple in the albino rat from birth to ten weeks of age. Amer. J. Anat., 19:353-389, 1916.

.....: II. The fetal development of the mammary gland in the female albino rat. Amer. J. Anat., 22:195-223, 1917.

.....: Studies on the mammary gland VI. The development of mammary gland from its earliest appearance until the period of pregnancy. Amer. J. Dis. Child., 18:4-13, 1919a.

.....: The histology of the mammary gland in male and female albino rats from birth to ten weeks of age. Amer. J. Anat., 25:395-436, 1919b.

Myers, F.J., and Myers, J.A.: Studies on the mammary gland VIII. Gross changes in the mammary gland in female albino rat during involution. Anat. Rec., 21:74, 1921.

Noble, R.L., and Cutts, J.H.: Mammary tumors of the rat: A review. Cancer Res., 19:1125-1139, 1959.

Orr, J.W.: British Empire Cancer Campaign. 33rd Annual Report, 235-236, 1955.

Petersen, W.E.: Lactation. Physiol. Rev., 24:340-371, 1944.

Ratcliffe, H.L.: Spontaneous tumors in two colonies of rats of the Wistar Institute of Anatomy and Biology. Amer. J. Path., 16:237-254, 1940.

Ray, E.W., et al.: Rat placental hormonal activities corresponding to those of pituitary mammotropin. Endocrinology, 56:359-373, 1955.

Reece, R.P., and Warbritton, V.: The rate of cell division in mammary glands of rats. Journal of Dairy Science, 36:589, 1953.

Rees, E. Douglas, and Eversole, Amy: Rat mammary gland metabolism relative to epithelial and connective tissue content. Amer. J. Physiol., 207:595-600, 1964.

Richardson, K.C.: Contractile tissues in the mammary gland, with special reference to myoepithelium in the goat. Proc. roy. Soc. Biol., 136:30-45, 1949.

Roberts, F.L.: Changes in the mammary gland of the albino rat during second half of pregnancy. Papers from the Mayo Foundation and Medical School, Vol. I, W.B. Saunders, 1921. Cited by Weatherford (1929).

Robertson, J.S., Bond, V.P., and Cronkite, E.P.: Resolution and image spread in autoradiographs of tritium-labeled cells. Int. J. appl. Radiat., 7:33-37, 1959.

Rowe, W.P., Bryan, W.R., Lennette, E.H., and MacMahon, B.: A survey of the tumor virus problem from an epidemiologic standpoint. A Symposium. In: Cancer Research, 25:1277-1291, 1965.

Shay, H., et al.: Development of adenocarcinoma of the breast in the Wistar rat following the gastric instillation of MCA. J. nat. Cancer Inst., 10:255-266, 1949.

Shay, H., Gruenstein, M., and Kessler, W.B.: Experimental mammary adenocarcinoma of rats: Some considerations of methylcholanthrene dosage and hormonal treatment. J. nat. Cancer Inst., 27:503-513, 1961.

Shellabarger, C.J., et al.: The occurrence of mammary tumors in the rat after sublethal whole-body irradiation. Radiat. Res., 6:501-512, 1957.

Silver, I.P.: Myoepithelial cells in the mammary and parotid glands. J. Physiol., 125:8-9, 1954.

Silver, Marian: The onset of allometric mammary growth in the female hooded Norway rat. J. Endocr., 10:35-45, 1953a.

.....: A quantitative analysis of the role of oestrogen in mammary development in the rat. J. Endocr., 10:17-34, 1953b.

Slater, T.F.: Studies on the mammary involution. I. Chemical changes. Arch. int. Physiol., 70:167-178, 1962.

Stevens-Hooper, C.E.: Use of colchicine for the measurement of mitotic rate in the intestinal epithelium. Amer. J. Anat., 108:231-244, 1961.

Stevens, L., Stevens, E., and Currie, A.R.: Histological studies and measurement of nucleic acid synthesis in rat mammary tumors induced by 9, 10 dimethyl-1,2-benzanthracene (DMBA). J. Path. Bact., 89: 581-589, 1965.

Sutter, Monroe: Cyclic changes in mammary gland of rat associated with oestrus cycle. Anat. Rec., 21:59, 1921.

Sydnor, K.L.: Race-strain factor related to hydrocarbon-induced mammary cancer in rats. J. nat. Cancer Inst., 29:805-814, 1962.

Tucker, H. Allen, and Reece, R.P.: Nucleic acid content of mammary glands of pregnant rats. Proc. Soc. exp. Biol. Med., 112:370-371, 1963a.

.....: Nucleic acid content of mammary glands of lactating rats. Proc. Soc. exp. Biol. Med., 112:409-412, 1963b.

Tucker, H.A., and Reece, R.P.: Nucleic acid content of rat mammary glands during post-lactational involution. Proc. Soc. exp. Biol. Med., 112:1002-1004, 1963c.

Traurig, Harold H., and Morgan, Chas. F.: Audioradiographic studies of the epithelium of mammary gland as influenced by ovarian hormones. Proc. Soc. exp. Biol. Med., 115:1076-1080, 1964a.

Turner, C.W.: The Mammary Glands. In: Allen, Danforth and Doisy: Sex and Internal Secretions, ed. 2, Baltimore, Williams and Wilkins, (1939), pp. 740.

Vendrely, R.: The Desoxyribonucleic Acid Content of the Nucleus. In: Chargaff and Davidson: The Nucleic Acids, Vol. II, New York, Academic Press, Inc., (1955), pp. 155-180.

Weatherford, Harold L.: A cytological study of the mammary gland. Amer. J. Anat., 44:199-281, 1929.

Williams, W. Lane: Normal and experimental mammary involution in the mouse as related to the inception and cessation of lactation. Amer. J. Anat., 71:1-41, 1942.

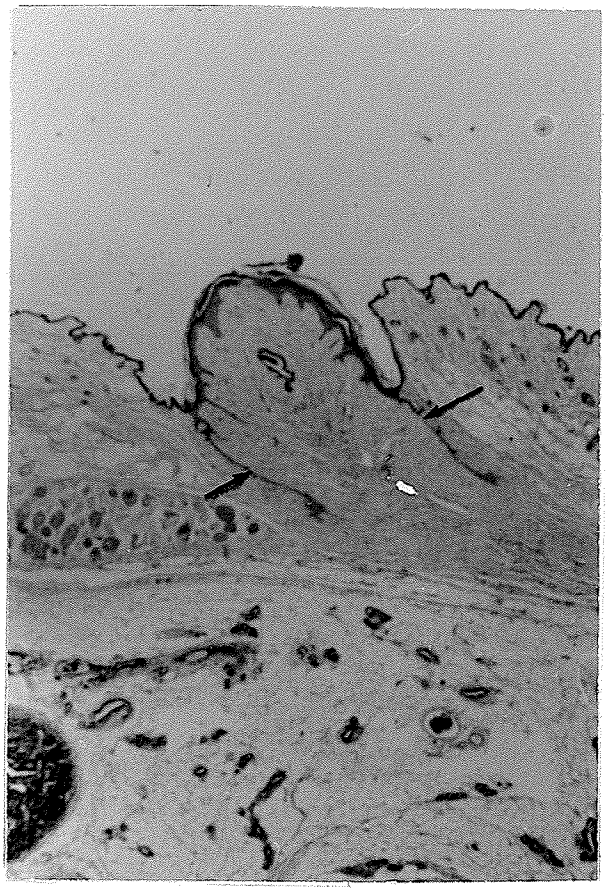
Young, Stretton, and Cowan, Dorothea M.: Spontaneous regression of induced mammary tumors in rats. Brit. J. Cancer, 17:85-89, 1963.

Young, S., Cowan, D.M., and Sutherland, L.E.: The histology of induced mammary tumors in rats. J. Path. Bact., 85:331-340, 1963.

ILLUSTRATIONS

FIGURE

1. Nipple of virgin rat showing epithelial ingrowth or hood, remnant of the embryonic mammary pit. H. and E., x 40.

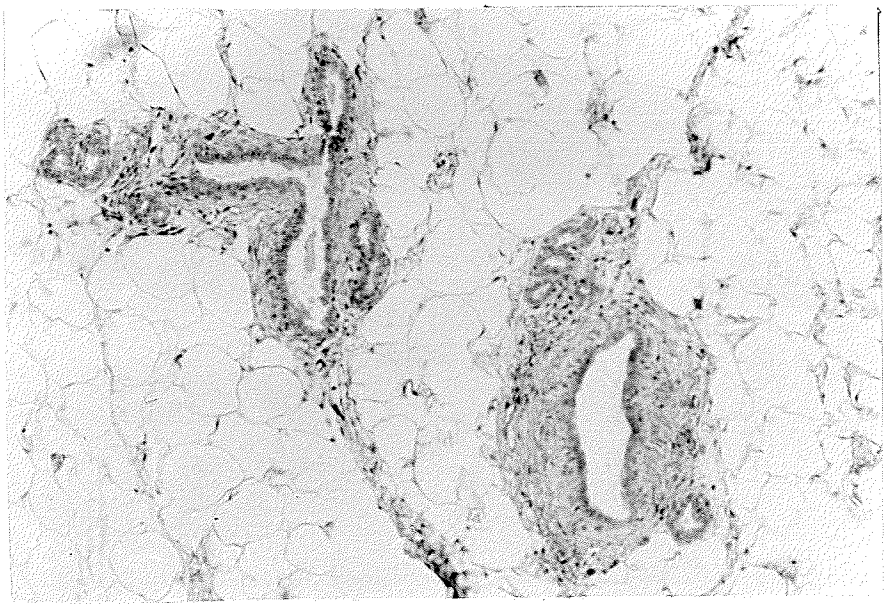


①

FIGURE

2. Mammary gland of virgin rat in stage I of estrus. Ducts and alveolar end-buds are embedded in adipose tissue. H. and E., x 160.

3. Higher magnification of ducts and alveolar end-buds in virgin mammary gland. H. and E., x 800.



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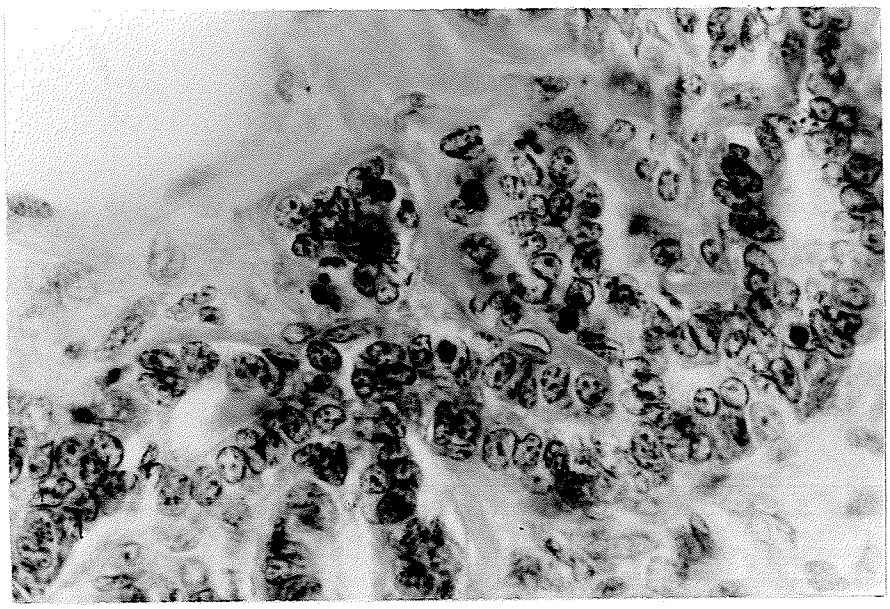
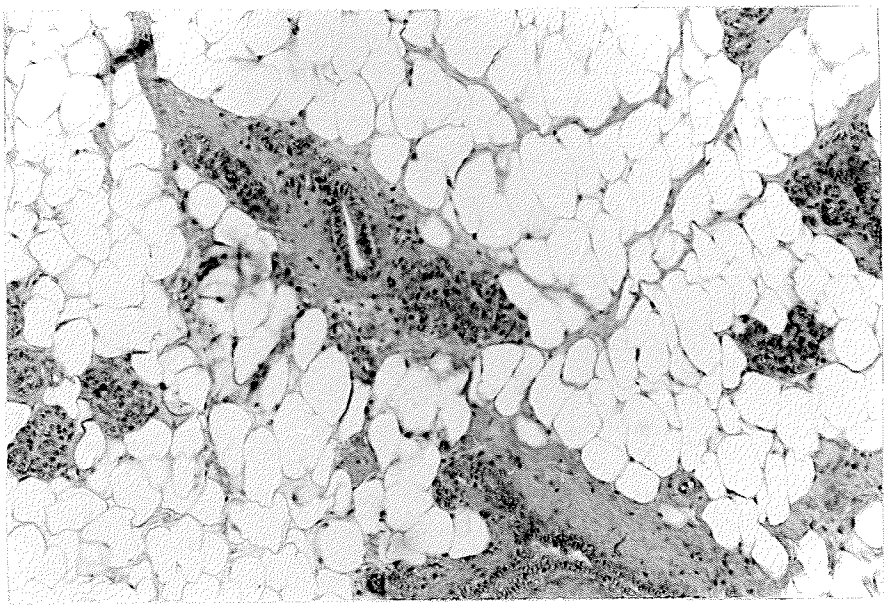


(3)

FIGURE

4. Mammary gland of rat at day four of pregnancy. H. and E., x 160.

5. Higher magnification of ducts and end-buds at day four of pregnancy. A "colchicine metaphase" is visible at arrow. H. and E., x 800.



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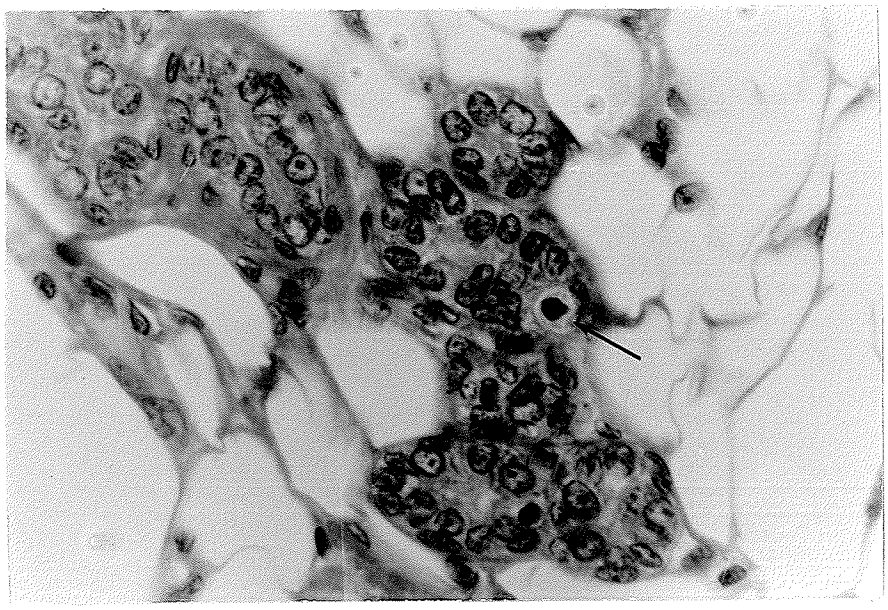
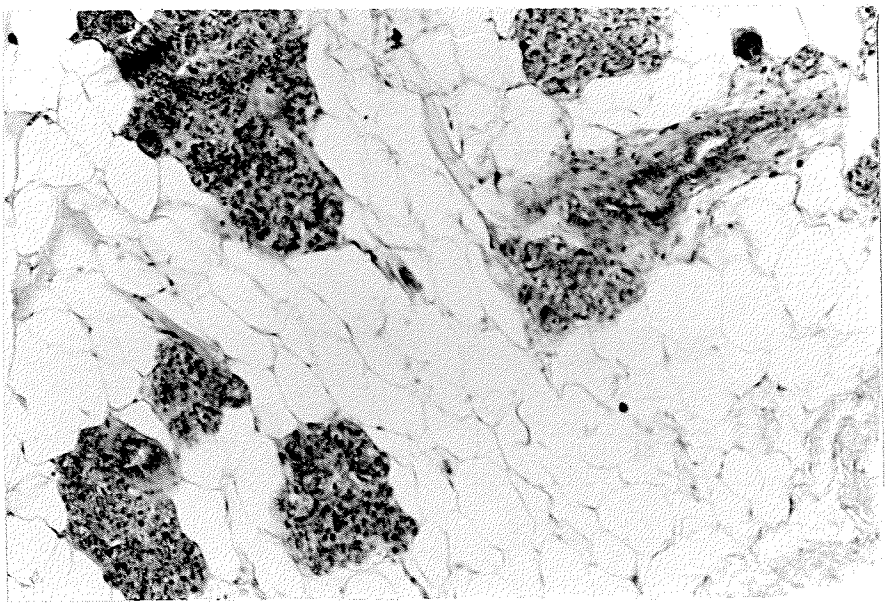


5

FIGURE

6. Mammary gland of rat at day seven of pregnancy. An increase in parenchyma over that of virgin animal (Fig. 2) can be seen. H. and E., x 160.

7. Higher magnification of mammary gland at day seven of pregnancy. Several "colchicine metaphases" are visible. H. and E., x 800.



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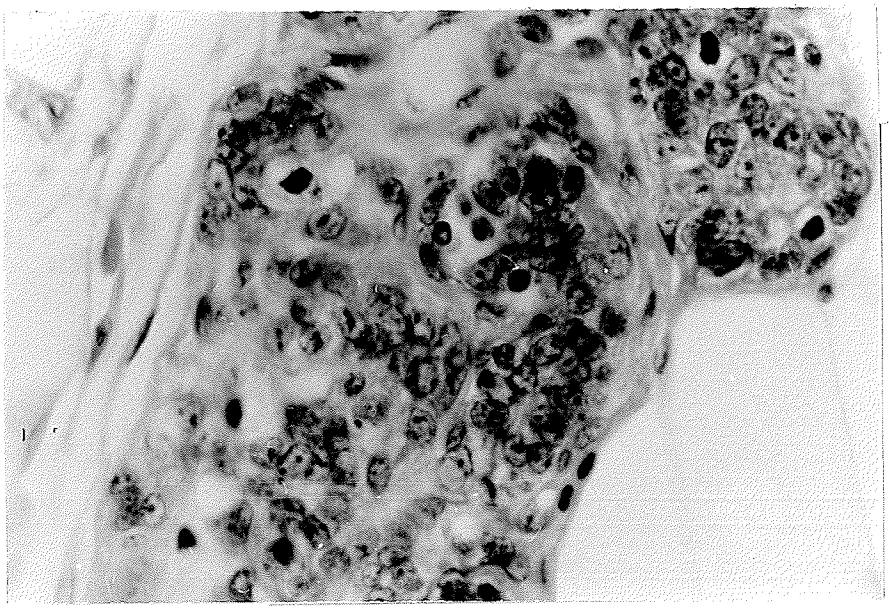


7

FIGURE

8. Mammary gland of rat at day twelve of pregnancy. Note increase in parenchyma over day seven (Fig. 6). H. and E., x 160.

9. Numerous "colchicine metaphases" are visible in mammary gland of rat at day twelve of pregnancy. H. and E., x 800.

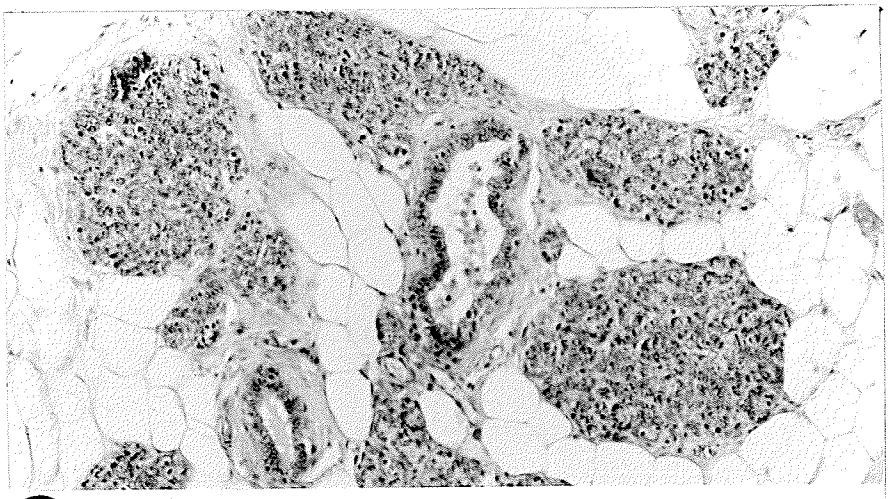
10. Higher magnification showing typical "colchicine metaphases" at day twelve of pregnancy. H. and E., x 1040.

FIGURE

8. Mammary gland of rat at day twelve of pregnancy. Note increase in parenchyma over day seven (Fig. 6). H. and E., x 160.

9. Numerous "colchicine metaphases" are visible in mammary gland of rat at day twelve of pregnancy. H. and E., x 800.

10. Higher magnification showing typical "colchicine metaphases" at day twelve of pregnancy. H. and E., x 1040.



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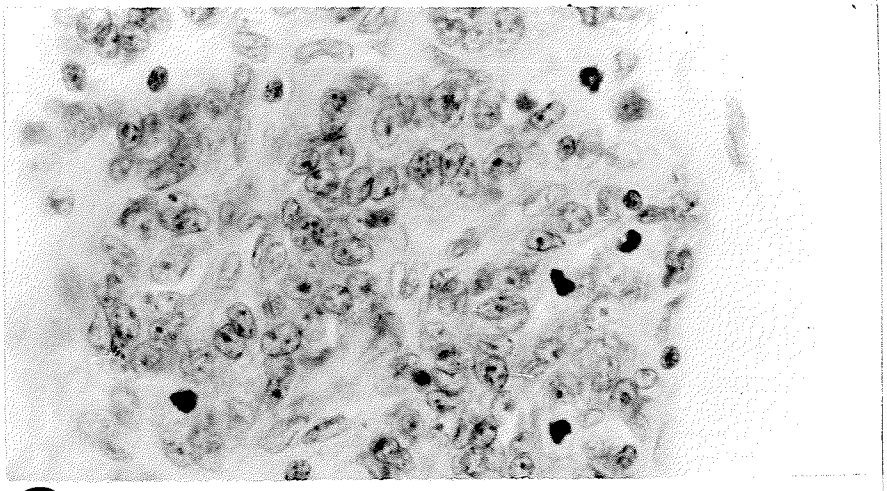

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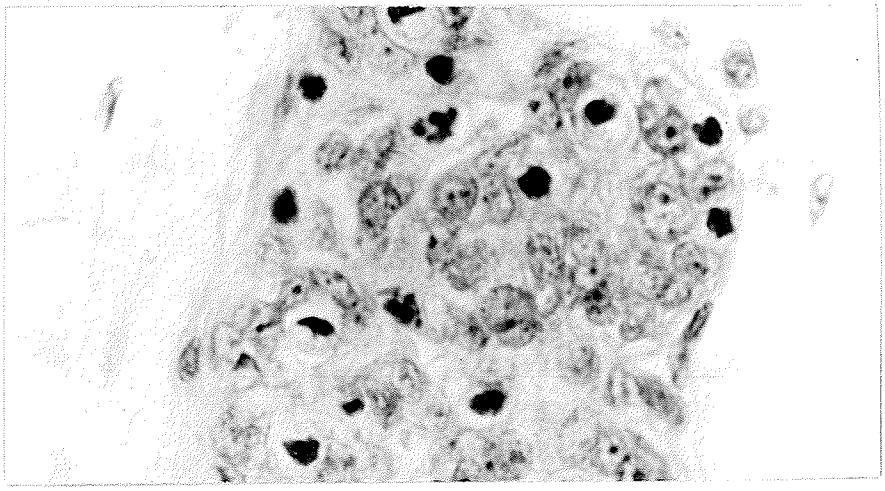
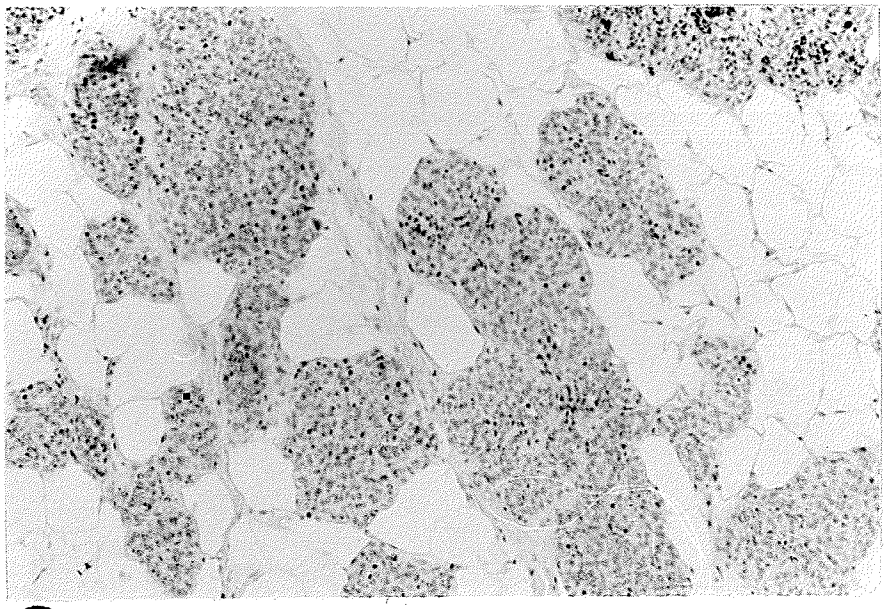


10

FIGURE

11. Mammary gland of rat at day fourteen of pregnancy. H. and E., x 160.

12. Higher magnification showing alveoli of mammary gland at day fourteen of pregnancy. Several "colchicine metaphases" are visible. H. and E., x 800.



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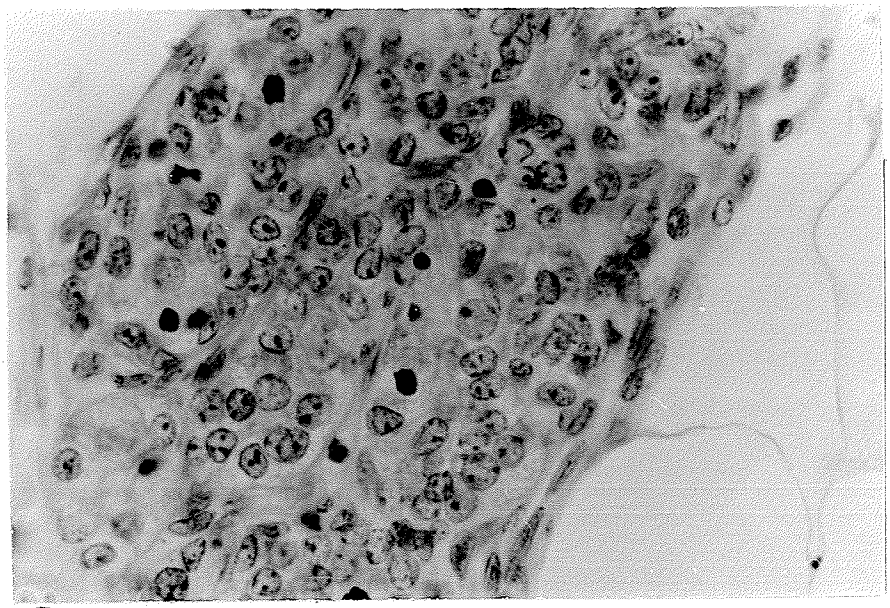
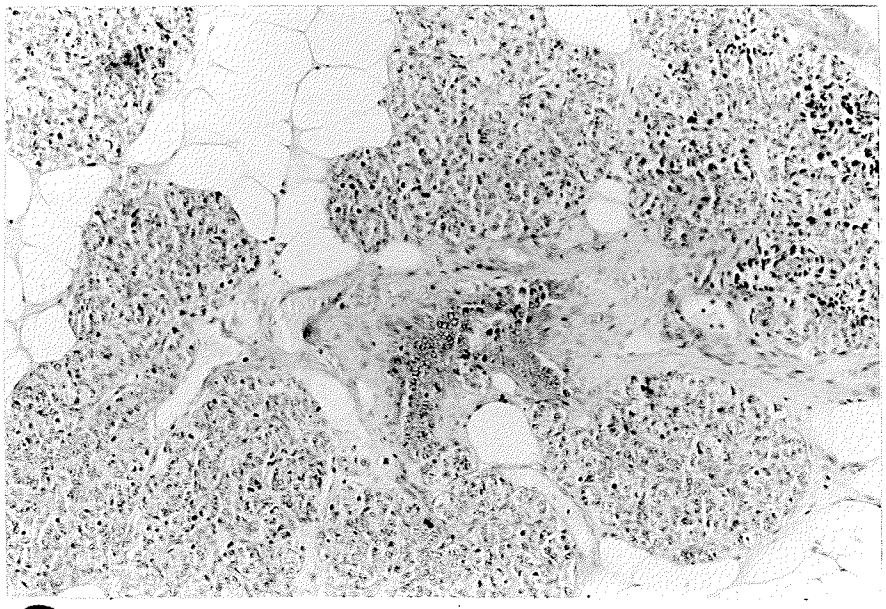


(12)

FIGURE

13. Mammary gland of rat at day eighteen of pregnancy. Note increase in amount of parenchyma over day fourteen (Fig. 11).
H. and E. x 160.

14. Higher magnification of mammary gland at day eighteen of pregnancy. Epithelial cell cytoplasm is vacuolated, indicating secretory activity. H. and E., x 800.



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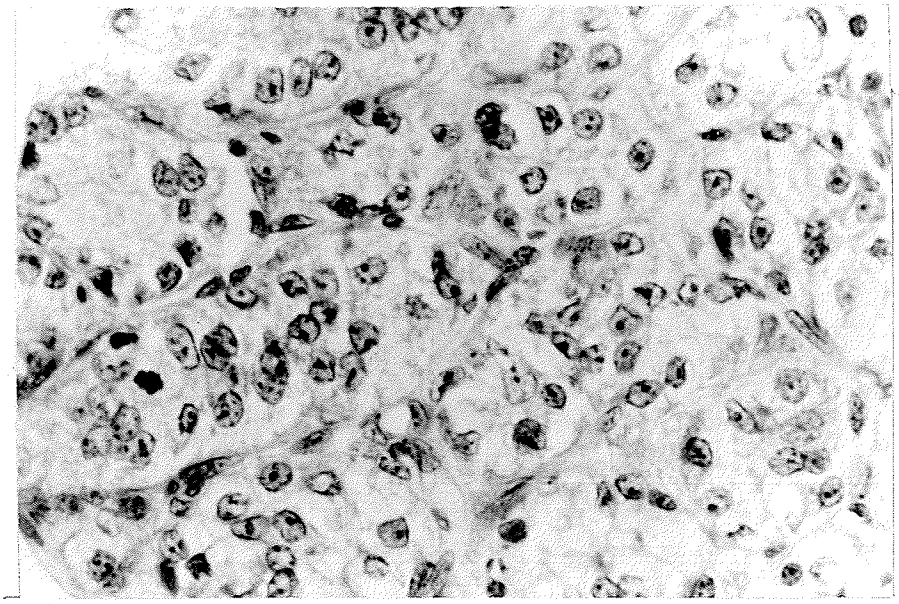
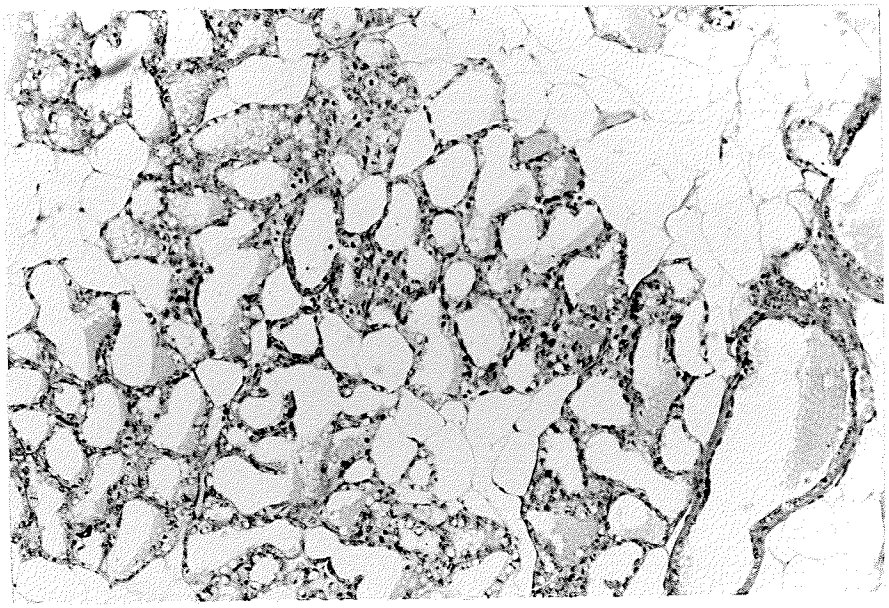


14

FIGURE

15. Mammary gland of rat in lactation. Alveoli are distended with secretion. H. and E., x 160.

16. Higher magnification to show several alveoli in lactating mammary gland. H. and E., x 800.



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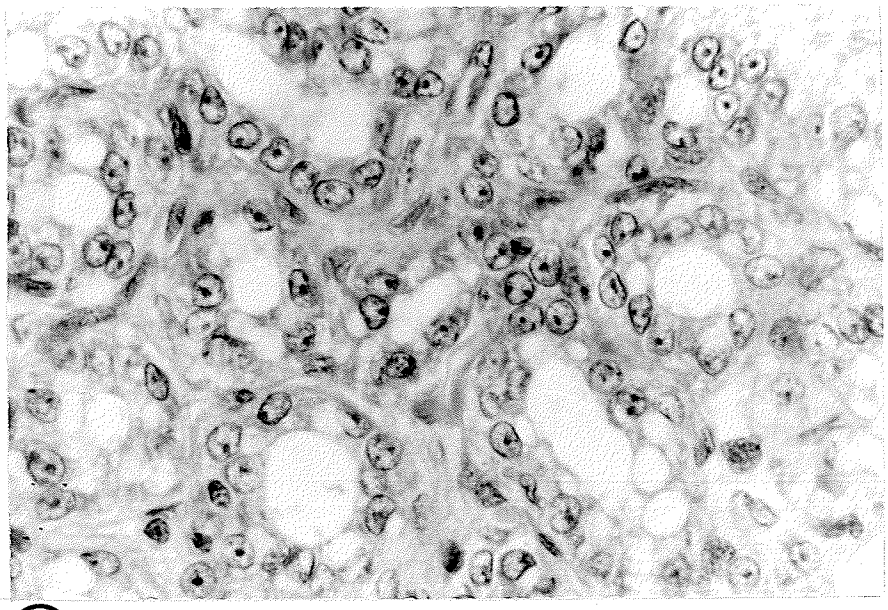
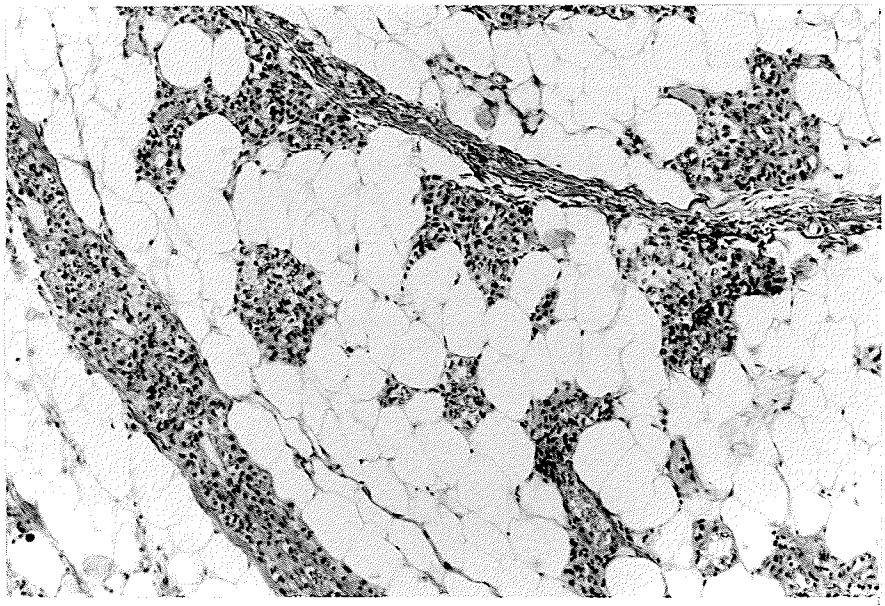


16

FIGURE

17. Low power photo-micrograph of mammary gland, seven days after weaning. Parenchyma has regressed, with a concurrent increase of connective tissue. H. and E., x 160.

18. Higher power photo-micrograph of involuting mammary gland. Lymphocytic infiltration is apparent. H. and E., x 800.



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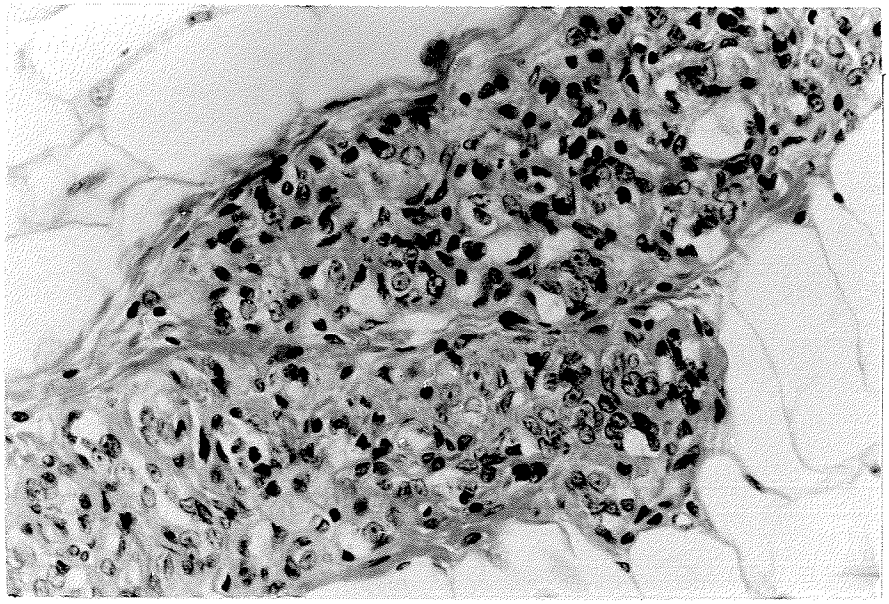
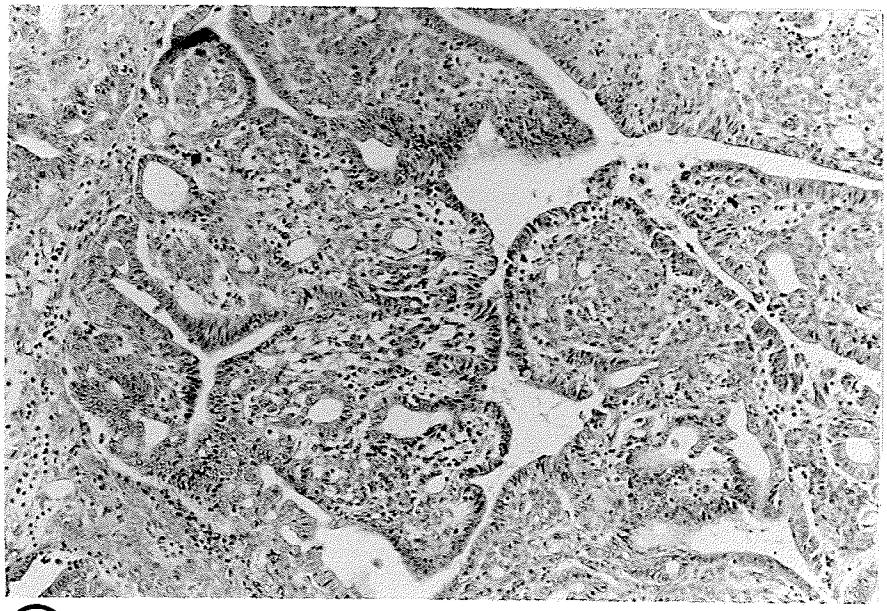


18

FIGURE

19. Medullary carcinoma with lymphoid stroma. Mitoses uncommon. Stromal reaction vigorous. H. and E., x 160.

20. Higher magnification of Figure 19. H. and E., x 800.



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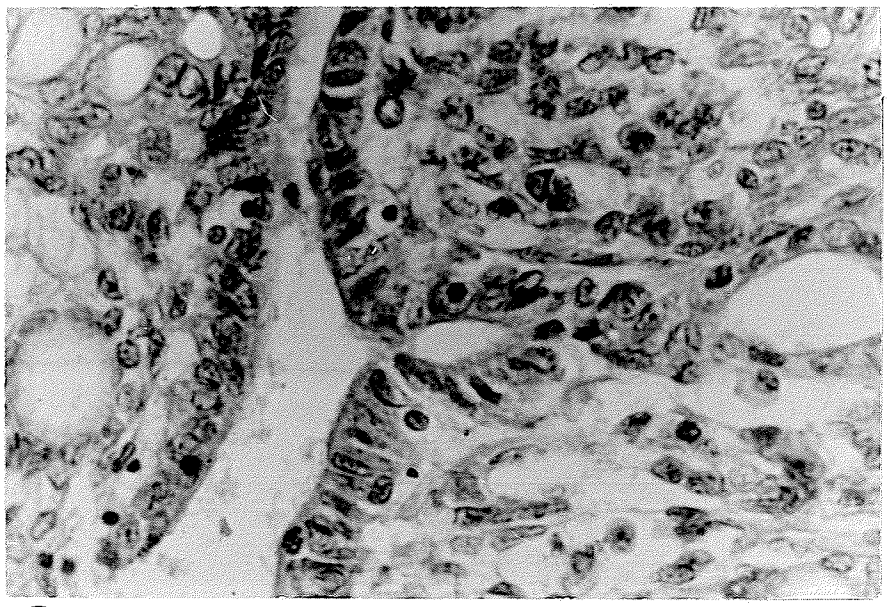
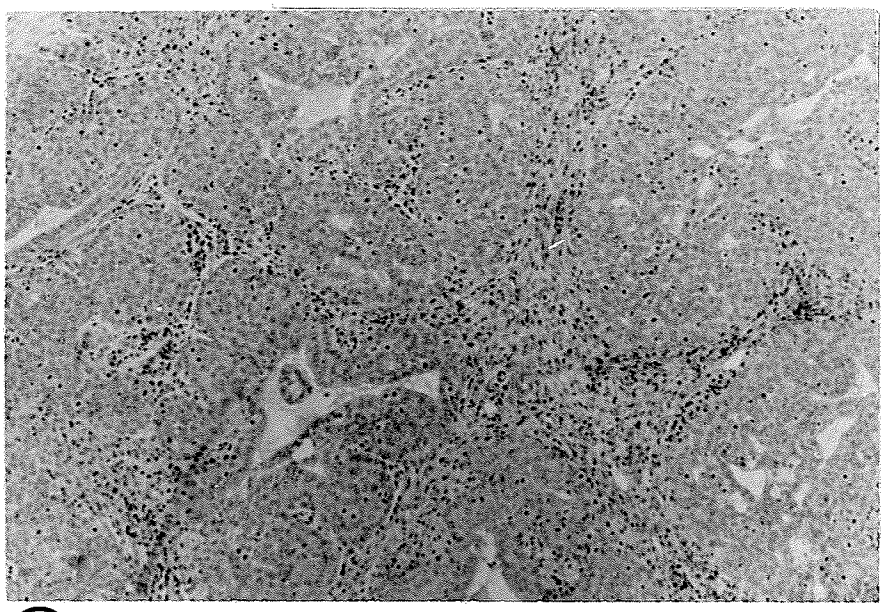


20

FIGURE

21. Infiltrating medullary carcinoma with lymphoid stroma. Grade II - III. H. and E., x 160.

22. Higher magnification of Figure 21. Note "colchicine metaphases". H. and E., x 800.



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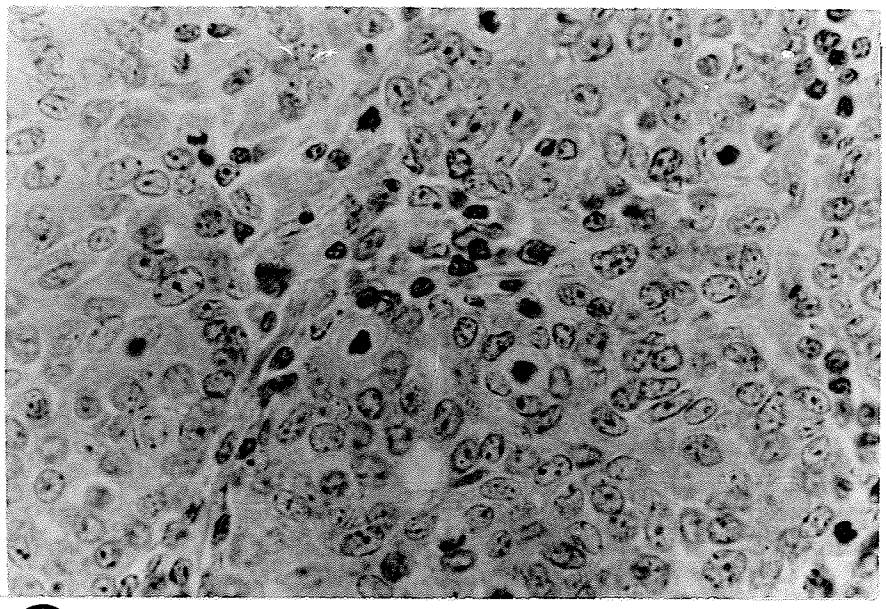
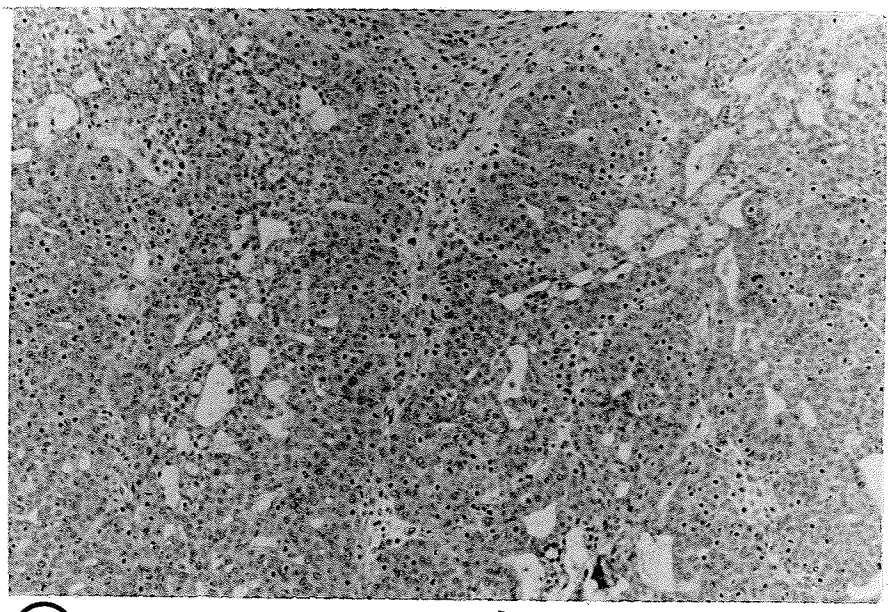


22

FIGURE

23. Infiltrating adenocarcinoma, bordering on anaplastic (Grade III - IV). Mitoses very frequent. No stromal reaction. H. and E., x 160.

24. Higher magnification of Figure 23. Numerous "colchicine metaphases" are visible. H. and E., x 800.



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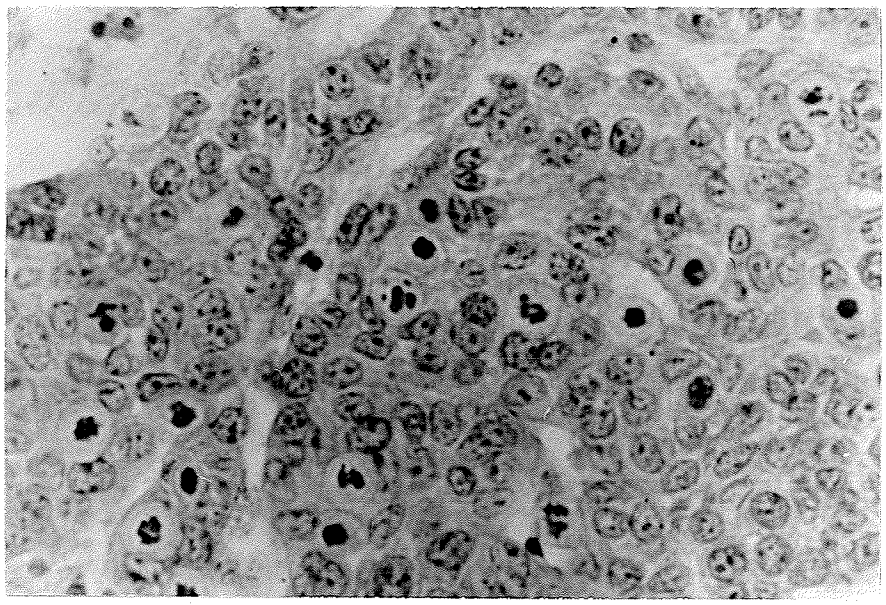

(24)