Mammary gland nucleic acid content and litter weight gains in lactating rats: effects of ovarian hormones, hydrocortisone and prolactin

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Mammary gland nucleic acid content and litter weight gains in lactating rats: Effects of ovarian hormones, hydrocortisone and prolactin

by

Linden C. Smith Haynes

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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>LITERATURE REVIEW</td>
<td>4</td>
</tr>
<tr>
<td>Mammary Gland Development and Growth</td>
<td>4</td>
</tr>
<tr>
<td>The Hormonal Control of Lactogenesis</td>
<td>15</td>
</tr>
<tr>
<td>Hormonal Control of Galactopoiesis</td>
<td>18</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>26</td>
</tr>
<tr>
<td>Treatment of Animals</td>
<td>26</td>
</tr>
<tr>
<td>Hormone Preparation</td>
<td>27</td>
</tr>
<tr>
<td>Mammary Gland Nucleic Acid Analysis</td>
<td>29</td>
</tr>
<tr>
<td>Statistical Analysis</td>
<td>32</td>
</tr>
<tr>
<td>RESULTS</td>
<td>33</td>
</tr>
<tr>
<td>Final Body and Organ Weights</td>
<td>33</td>
</tr>
<tr>
<td>Mammary Gland DNA</td>
<td>35</td>
</tr>
<tr>
<td>Mammary Gland RNA</td>
<td>38</td>
</tr>
<tr>
<td>Litter Weight Gains</td>
<td>48</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>58</td>
</tr>
<tr>
<td>SUMMARY</td>
<td>64</td>
</tr>
<tr>
<td>LITERATURE CITED</td>
<td>67</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>84</td>
</tr>
</tbody>
</table>
INTRODUCTION

Hormones are foremost among the many factors controlling mammary gland growth. It is well-established that estrogens stimulate growth of the mammary duct system, and the combination of estrogen and progesterone evoke full lobulo-alveolar development. These effects are, however, dependent on an intact pituitary. Large doses of prolactin and growth hormone will also stimulate full mammary lobulo-alveolar development. The ovaries are not required for this effect (Talwalker and Meites, 1961).

Lactogenesis, the appearance of secretory activity, is induced by a hormonal combination that is different from that which gives rise to mammary gland growth. The estrogen-progesterone equilibrium during most of pregnancy is thought to make the mammary glands resistant to the stimulatory effects on the lactogenic complex. This estrogen-progesterone ratio inhibits the secretion of prolactin by the pituitary (Meites, 1966) and also probably other constituents of the lactogenic complex (Ford and Melampy, 1973).

At parturition the fall in plasma progesterone together with a temporary rise in estrogen secretion renders the mammary gland receptive to the lactogenic actions of prolactin and adrenal steroids. Plasma concentrations of prolactin and glucocorticoids rise during late pregnancy. Increased secretory activity of the mammary epithelium is complementary with this rise in lactogenic hormones.
Lactation can be initiated during early, mid- or late pregnancy in the rat with glucocorticoid injections (Talwalker et al., 1961). Better milk yields are obtained with the combination of prolactin and adrenal corticoids. Prolactin alone is ineffective in inducing lactation in pregnant rats.

Plasma progesterone is inhibitory to lactogenesis during most of pregnancy partly due to its inhibitory effect on alpha-lactalbumin synthesis. Exogenous progesterone does not affect established lactation unless massive doses are administered (Barsantini and Masson, 1947).

Endogenous estrogen does not inhibit lactogenesis or galactopoiesis in rats. However, physiological or pharmacological doses of estrogenic compounds suppress lactation in rats with intact ovaries. Only pharmacological doses of estrogen are inhibitory to lactation in ovariectomized rats. Physiological doses have no effect (Griffith and Turner, 1962).

Estrogen-progesterone treatments inhibit lactational performance in both intact and ovariectomized rats. This hormonal combination is effective in both physiological and pharmacological doses.

This investigation was conducted to determine whether glucocorticoids and prolactin, singly and in combination, would override the suppression of lactation caused by estrogen-progesterone treatments. Mammary gland proliferation was determined by DNA content and secretory activity by RNA content, RNA/DNA ratio and litter weight
gains. Attempts were made to relate changes in mammary development and synthetic activity with hormonal treatments.
Biochemical method of determining growth

The most commonly used index of mammary gland growth is the determination of the DNA content of the gland. This procedure was first used on the mammary glands by Kirkham and Turner (1953). It was based on the concept set forth by Boivin and colleagues (1948) and Mirsky and Ris (1949) that the DNA content of cell nuclei is constant for all somatic cells of an organism. Subsequent studies (Griffith and Turner, 1959; Tucker and Reece, 1962) supported this basic premise. Nevertheless, others have suggested that DNA is not constant per cell nuclei (Sod-Moriah and Schmidt, 1968; Simpson and Schmidt, 1970).

Turner (1970) re-examined the DNA procedures and concluded that the estimation of mammary gland growth by this method may be subject to some error involving the possible growth of connective tissue during pregnancy and lactation, but it is the only true quantitative estimate of mammary gland growth. Comparisons of the mammary gland area and DNA methods revealed that the two procedures correlate well (Munford, 1963; Nagasawa et al., 1966). Significant positive correlations were obtained between the two procedures in mice 50-210 days old. This coincided with results of Sinha and Tucker (1966) who observed high correlation between area and DNA
Embryonic mammary development

The mammary band is the first recognizable structure in the developing mammary gland of the rat and mouse. It appears on either side of the midline in 11 day old fetuses. By the 12th day the tissue near the mammary band has further thickened to form the mammary crest. Immediately five ectodermal thickenings appear in each mammary crest. These are the individual mammary anlagen, the mammary hillocks. Between the 13th and 14th days the mammary hillocks increase in volume and assume an almost spherical shape becoming the mammary buds, the primordia of the future mammary gland. In female fetuses, on day 15 the mammary bud sinks into the mesenchyme and elongates into a cord which begins to branch at the distal ends near term (Raynaud, 1961, 1971).

Prepuberal growth

At birth, the mammary gland of the rat is fairly well-developed. Its ducts are dilated and ultrastructurally cells appear to be secretory (Ceriani et al., 1970). The total mammary gland area increases isometrically with the body surface until about the 22nd day when a phase of allometric growth ensues (Cowie, 1949).

Using DNA content as a measure of gland growth, Sinha and Tucker (1966) found that the most rapid proliferation of ducts into the fatty pads occurred between days 23-40 of age. These results
concur with those of Myers (1916) and Nagasawa et al. (1967) who found an increase in the rudimentary duct system at four weeks after birth. This is about the same time that the rat ovary starts to secrete estrogen (Price, 1947). The adipose and connective tissue portions of the gland increase also at this time, and continue to increase whether or not ovariectomy is performed (Paape and Sinha, 1971).

Ovariectomy on the 22nd day can prevent the onset of the allometric growth phase (Cowie, 1949). Normal allometric growth can be restored in the ovariectomized rat and mouse by suitable injections of estrogen (Silver, 1953; Flux, 1954). Estrogen then appeared to be the normal stimulus for the growth of the mammary ducts in the adolescent rat and mouse (Folly, 1956).

Postpuberal growth

After puberty, when the hormonal activities of the ovaries are established, the tissues of the mammary gland grow markedly. As a result of numerous studies kindled by Turner and Schultze (1931) and Astwood et al. (1937), estrogens are thought to stimulate growth of the duct system, whereas lobulo-alveolar development depends on progesterone.

Ancel and Bouin (1911) showed that the corpus luteum was responsible for lobulo-alveolar growth in the pseudopregnant rabbit. Later studies (Turner and Schultze, 1931) revealed that
physiological doses of progesterone had little or no mammogenic capability, but pharmacological doses would stimulate mammary growth in rats (Reece and Bivins, 1942).

Estradiol benzoate treatment causes little mammary gland growth (Moon et al., 1959), even if treatments are extended for periods of up to 60 days (Panda and Turner, 1966). Estrogen treatments increase the rate of mammary gland DNA synthesis and increase the number of cells undergoing DNA replication (Bresciani, 1971). After some time, ovarian hormones may become inhibitory on the mammary epithelium (Panda and Turner, 1966).

It is well established that both estrogen and progesterone together are more effective than either alone in promoting growth of all mammary gland tissues (Jacobsohn, 1961). The ratio of estrogen to progesterone and the absolute amount of each hormone present are important in determining the quantity and quality of mammary development. In the mouse and rat, the optimum ratios appear to be of the order of 1 part of estrogen to 1000 parts or more of progesterone (Elliot and Turner, 1953).

Studies in hypophysectomized rats and guinea pigs showed that estrogens and progesterone were not effective in stimulating mammary growth in the absence of the pituitary gland (Gomez and Turner, 1937). Major elucidation of the roles of the ovarian and pituitary hormones in mammogenesis came from studies on rats by Lyons and his colleagues (1958) and on mice by Nandi (1959).
They studied the effects of various combinations of ovarian and hypophysial hormones on mammary growth in young animals from which the pituitary, pituitary and ovaries or pituitary, ovaries and adrenals (triply operated) had been removed. Small variations in mammogenic responses were observed between the rat and mice, but basically these studies demonstrated that the hormones required for duct growth are estrogen, progesterone and adrenal corticoid. When progesterone and prolactin are added to this combination full lobulo-alveolar growth is stimulated. In subsequent studies, Talwalker and Meites (1961, 1964) demonstrated that frequent large doses of prolactin and growth hormone could induce lobulo-alveolar development in the triply operated rat. This observation did not imply that the ovarian hormones are of no importance in normal mammary growth, but that the pituitary hormones must be regarded as the more essential since the ovarian hormones in the absence of pituitary hormones have little or no mammogenic activity.

Estrogen and progesterone act synergistically with prolactin in stimulating DNA synthesis in rat mammary epithelium. The synergistic effects between prolactin and estrogen are exerted primarily on the ductal epithelium, whereas the effects of progesterone and prolactin are seen in both ductal and alveolar structures (Stoudemire et al., 1975).

Hypophysectomy does not result in a simple deficiency of
only one hormone, and the chronic absence of the anterior pituitary leads to extreme metabolic changes in many if not most body tissues. In addition to removing the trophic hormones which stimulate the ovaries, thyroid and adrenals, hypophysectomy retards body growth and homeostasis (Jacobsohn, 1961). The mammary glands of intact animals that are starved do not respond to ovarian hormones (Engel, 1954). So, the consequences of hypophysectomy may be due in part to reduced food intake.

Small doses of insulin, if given together with ovarian hormones, can induce some development of the mammary ducts in hypophysectomized rats. It is thought that insulin corrects some of the metabolic deficiencies which result from pituitary ablation (Ahrens and Jacobsohn, 1956). However, it has not been demonstrated that insulin alone can elicit mammary growth in either intact or hypophysectomized animals.

The 11-oxygenated corticoids exert no mammogenic effect on the ovariectomized mouse and they block the duct-developing effects of estrogen. Conversely, 11-desoxycorticosterone synergizes the duct-developing action of estrogen in the ovariectomized mouse (Flux, 1954). ACTH has no mammogenic effect in intact mice (Flux and Munford, 1957), while hydrocortisone acetate is mammogenic at low doses and without effect at higher doses (Munford, 1957).

The effect of corticoids on the rat mammary gland is
conditioned by the endocrine state of the animal. Ahrens and Jacobsohn (1957) found that cortisone was unable to induce normal growth in the hypophysectomized-ovariectomized rat even with the addition of ovarian hormones. In the pituitary intact ovariectomized rat cortisone was lactogenic, its mammogenic effect becoming apparent only after addition of ovarian hormones.

The thyroid does not seem to be essential for mammogenesis. A certain degree of hypothyroidism enhances alveolar development in the rat, but it has an inhibitory effect on mammogenesis in the mouse (Folley, 1956). Treating rats with propylthiouracil stimulates mammary growth (Ben-David et al., 1966). Mammogenesis can be elicited in the hypophysectomized-adrenalectomized-thyroidectomized rat by giving ovarian hormones, prolactin, growth hormone and hydrocortisone without the addition of thyroid hormones (Chen et al., 1955).

**Growth during pregnancy**

Early studies concerning the extent of mammary growth during pregnancy have been reviewed by Turner (1939, 1952) and Jacobsohn (1961). Basically, the mammary gland comprises an advance duct system at the onset of gestation, and continues its growth throughout pregnancy. This growth is due to increases in cell size (Mayer and Klein, 1961), cell number and area (Cowie, 1949; Munford, 1963).
Mammary gland DNA increases throughout pregnancy and, in fact, into lactation in the rat (Greenbaum and Slater, 1957; Griffith and Turner, 1961; Tucker and Reece, 1963). This increase in DNA has been observed in guinea pigs (Nelson et al., 1962), mice (Brookreson and Turner, 1959) and hamsters (Sinha et al., 1970). The increase in DNA values applies mostly to the epithelial cells since other cells of the fat pad do not proliferate during pregnancy. Also, the increase in parenchymal cells is accompanied by increased synthesis of collagen around them whereas the collagen content in the rest of the fat pad remains constant (Harkness and Harkness, 1956; Paape and Sinha, 1971).

The mammary RNA content of the rat increases throughout pregnancy, with the greatest increase being in the second half (Tucker and Reece, 1963; Denamur, 1974; Paape and Sinha, 1971). The RNA/DNA ratios increase as pregnancy progresses, but usually do not exceed unity (Tucker and Reece, 1963; Ferreri and Griffith, 1969).

The placenta, which begins to actively maintain pregnancy about day 11 (Amoroso and Porter, 1966), seems to contribute to mammary growth in the rat since ovariectomy on day 12 has no effect on gestational mammary growth (Selye et al., 1933). Normal gestational mammary growth has been observed in mice subjected to ovariectomy, fetus removal, or hypophysectomy on day 12 if the placentae are retained (Newton and Lits, 1938; Newton and Beck, 1939; Nagasawa
and Nandi, 1971).

**Hormonal studies of gestational growth**

Hormones injected into pregnant rats can modify the growth of their mammary glands, either because suboptimal amounts of the hormones are present or because the endogenous hormones cannot attain their full potential (Ceriani, 1974). Thyroxine injections throughout pregnancy increase DNA values by 25 percent (Griffith and Turner, 1961). Injections of 2 mg/day of growth hormone from day 3-19 of pregnancy increased DNA values by 37 percent. The addition of insulin to this treatment increases RNA values even more (Moon, 1965). Prolactin injections into pregnant rabbits for 5 days increase DNA content to values comparable to the normal values of lactation.

**Growth during lactation**

Mammary gland proliferation continues through the secretory phases of lactogenesis and lactation. Increases in cell numbers (Munford, 1963), mitotic activity (Traurig, 1967) and DNA values in rats and mice (Lewin, 1957; Greenbaum and Slater, 1957; Griffith and Turner, 1961) have been observed. DNA content increases approximately 25 percent between the end of pregnancy and the 3rd day of lactation. This increase in DNA content continues and levels off after mid-lactation (Tucker and Reece, 1963; Griffith and Turner, 1961; Greenbaum and Slater, 1957).
This mammary growth occurs at the expense of fat pads which diminish appreciably during lactation.

The maintenance of mammary gland size does not require the presence of the ovaries (Griffith and Turner, 1962), nor does ovariectomy on the 2nd day of lactation affect the increase in mammary gland DNA which accompanies lactation (Griffith and Turner, 1961; Tucker and Reece, 1963).

Pituitary hormones seem to be responsible for promoting postpartum mammary gland growth. Hypophysectomy prevents postpartum mammary growth. Exogenous prolactin stimulates mammary gland DNA synthesis and will increase $^{32}$P incorporation rates to levels near those of pituitary-intact lactating rats (Baldwin and Martin, 1960).

Summary of mammary growth

In the triply operated rat, the ovarian and adrenal steroids are only mammogenic in the presence of anterior pituitary hormones. The minimal hormonal requirements for duct growth are estrogen, adrenal steroids and growth hormone. The addition of progesterone and prolactin induce lobulo-alveolar growth. It is likely that during pregnancy hormones secreted by the placenta are also important in mammogenesis. The situation appears to be similar in mice despite small variations in mammogenic responses.

The hormonal control of mammary development in rats (Lyons et al., 1958) and mice (Nandi, 1959; Nandi and Bern, 1961) are summarized
in Figure 1.

Figure 1. The hormonal control of mammary development in rats and mice. E = estrogen; Prog = progesterone; GH = growth hormone; PL = prolactin; C = corticosteroids.
The Hormonal Control of Lactogenesis

The control of lactation is usually considered in relation to two phases: (1) lactogenesis, the initiation of milk secretion, and (2) galactopoiesis, the maintenance of an established lactation. In lactogenesis some signal of the impending act of parturition must be conveyed to the mammary glands so that the biochemical apparatus involved in synthesizing and secreting milk may rapidly be activated. In galactopoiesis signals generated by the offspring must in some way regulate the activity of the glands to produce milk sufficient to satisfy their needs (Mepham, 1976).

Lactogenesis

Lactogenesis involves the transformation of the mammary gland from a nonsecretory to a secretory state, and the copious flow of milk which follows parturition. The epithelial cells of the alveoli of mammary glands from rats pregnant 12-19 days have a relatively uniform appearance. They are cuboidal to columnar in shape and contain many fat droplets. On the 20th day of pregnancy the alveoli are distended with secretion, and 9 to 10 hours before parturition alveolar distention is still greater and the amount of fat in the lumina has increased (Jefrers, 1935).

Lactase (Shinde et al., 1962; Kuhn, 1968) and \(a\)-lactalbumin (Kuhn, 1968) appear in the mammary gland of the rat between days 19 and 20 of pregnancy. RNA synthesis, measured by the
incorporation of $^{14}$C-glycine and $^{14}$C-orotic acid for 24 hours, increases significantly between day 18 of pregnancy and the day following parturition (Wang and Greenbaum, 1962). RNA synthesis seems to be necessary for milk secretion since actinomycin-D inhibits casein synthesis in ovariectomized pregnant rats (Lui and Davis, 1967). Cytoplasmic glucocorticoid receptors within the mammary epithelium increase significantly by day 16 of pregnancy in mice (Chomczynski and Zwierzchowski, 1976).

DNA increases during late pregnancy and early lactation accompanied by rapid increases in more than 30 enzymes. This suggests that secretory cell development during lactogenesis is a general process involving the synthesis of a large number of different proteins (Baldwin, 1969).

Ovariectomy performed in the rat during mid-pregnancy induces the secretory phenomena (Shinde et al., 1965). A significant increase in mammary RNA occurs 8-16 hours after surgery on day 15 (Liu and Davis, 1967). Adrenalectomy (Davis and Liu, 1969) or injection of progesterone (Wickerman and Davis, 1968) inhibit the nucleic acid and secretory changes caused by ovariectomy. Injections of progesterone during late pregnancy in rats block the induction of $\alpha$-lactalbumin (Turkington and Hill, 1969), lactase (Kuhn, 1969) and the histological appearance of milk (Herrenkohl, 1971).
In vitro studies

The hormonal events that prevail at and immediately prior to parturition with respect to cell structure have been studied methodically by some investigators using in vitro organ culture techniques. This work was reviewed by Topper and Green (1971), Turkington et al. (1973), and Topper and Oka (1974).

The great majority of alveolar cells present in the mammary glands of the mouse at mid-pregnancy are nonsecretory (Mills and Topper, 1970). They have a paucity of rough endoplasmic reticulum and primitive Golgi apparatus. After several days of culture in the presence of insulin, hydrocortisone and prolactin most of the alveolar cells present in the explants appear to be secretory (Mills and Topper, 1970). The sequence by which the hormones induce this transformation has been elucidated. Insulin promotes the formation of a pair of daughter cells, I-cells, from virtually every alveolar cell. Hydrocortisone, in the presence of insulin, promotes the formation of an extensive rough endoplasmic reticulum system. These are termed I-F-cells which assume a secretory appearance and synthesize milk proteins when exposed to both insulin and prolactin.
Hormonal Control of Galactopoiesis

Prolactin

Grueter (1923) and Stricker and Grueter (1923) were first to observe that aqueous extracts of the anterior pituitary would induce lactation in pseudopregnant rabbits. Subsequently, anterior pituitary extracts were shown to induce milk secretion in pregnant rats, guinea pigs, rabbits and goats. The exact role of these hormones in lactogenesis could not be determined because death of the fetuses always preceded lactogenesis (Nelson, 1934; Meites and Turner, 1947). Later, in experiments utilizing injections of purified prolactin, lactation was not initiated in rats (Talwalker et al., 1961), mice (Nandi and Bern, 1961), or ewes (Delouis and Denamur, 1967) although pregnancy was not affected. Prolactin did initiate lactation in pregnant rabbits (Meites et al., 1963).

Hypophysectomy during lactation causes a rapid decline in milk yield (Selye et al., 1933). In the rat milk secretion is completely suppressed within 1-3 days (Cowie, 1957). Mammary cell numbers, DNA, RNA and several enzymes essential for milk synthesis are reduced following hypophysectomy (Baldwin and Martin, 1968; Jones, 1967). Cowie (1957) maintained lactation for a few days in hypophysectomized rats with injections of 50 IU of prolactin twice daily. Better milk yields were obtained, however, when ACTH or growth hormone was given concomitantly with 25 IUs of prolactin
twice daily. Later studies revealed that the minimum requirements for maintaining lactation at a level sufficient to rear litters were an adrenal corticoid or ACTH and prolactin (Bintarningsih et al., 1957, 1958). Lyons et al. (1958) found that the addition of growth hormone improved this milk yield, but did not return it to pre-hypophysectomy levels.

Crude extracts of the anterior pituitary were shown to be lactogenic in hypophysectomized rats also, but purified prolactin was not lactogenic unless combined with ACTH (Gomez and Turner, 1937) or cortisone (Nelson et al., 1943). Using triply operated rats (Lyons et al., 1958) and mice (Nandi, 1959; Nandi and Bern, 1961), prolactin and adrenal glucocorticoids were demonstrated to be the minimum requirements for lactation. In some strains of mice growth hormone can completely replace prolactin (Nandi and Bern, 1961).

Prolactin secretion occurs at a high rate throughout the major part of the 21 day lactation period in the rat (Neill, 1974). The maintenance of prolactin secretion during lactation is sustained by the frequent application of the suckling stimulus. The suspension of nursing is accompanied by an increase in pituitary prolactin levels (Meites, Sar and Voogt, 1969) and suckling increases plasma prolactin concentrations (Ratner and Meites, 1964).

Daily injections of prolactin to lactating rats produced little, if any, increase in the litter growth rate (Masson, 1947).
Declin (1952) observed that prolactin was not luteotrophic and lactogenic at the same time. A definite galactopoietic effect was observed in lactating rats receiving daily injections of 1 mg of prolactin (Johnson and Meites, 1957). Later, Talwalker et al. (1960) found that daily injections of 1 mg of prolactin did stimulate litter weight gains of rats during early lactation. Doses of 3 mg/day have been found to be stimulatory to milk secretion primarily through increased metabolism rather than increases in the size of the mammary gland (Kumaresan et al., 1966). Lactational performance in the rabbit is greatly enhanced by exogenous prolactin during late lactation (Cowie, 1969).

Prolactin appears to increase the synthetic rate of all types of RNA in a general fashion. This ultimately leads to increases in tissue levels of RNA and rates of protein synthesis (Baldwin et al., 1969; Baldwin and Martin, 1968).

**Ovarian hormones**

Ovarian hormone administration during lactation inhibits lactational performance. Parkes and Bellerby (1927) showed that ovarian extracts depressed litter growth when administered to lactating mice. Later, chorionic gonadotropin was found to inhibit lactational performance in mice (De Johng, 1933).

Ovariectomy prevents the inhibitory effects of exogenous estrogen (Anselmino and Hoffman, 1936) and chorionic gonadotropin
(Edelmann and Gaunt, 1941) on lactational performance.

The detrimental effects of ovarian hormones on lactation and litter growth rate have been confirmed in numerous studies. These investigations have been summarized in the reviews of Nelson (1936), Turner (1939), Folley and Malpress (1948), Cowie (1961) and Cowie and Folley (1961).

Both physiological and pharmacological doses of estrogenic compounds suppress lactation in intact rats. The degree of inhibition seems to be related to the amount of hormone administered (Barsantini and Masson, 1947; Walker and Matthews, 1949; Ben-David et al., 1965; Gala and Reece, 1962). Physiological doses of estrogen do not affect lactation in ovariectomized rats, only pharmacological doses are effective (Griffith and Turner, 1962).

Progesterone treatments in the normal ranges do not affect lactational performance in rats (Anselmino and Hoffmann, 1936; Folley, 1942; Walker and Matthews, 1949; Griffith and Turner, 1962; Ben-David et al., 1965). Barsantini and Masson (1947) reported a 25 percent depression in litter weights on rats receiving 25 mg of progesterone daily for 16 days.

Fauvet (1941) demonstrated that the inhibition of milk secretion was due to the combined effects of estrogen and progesterone. This observation was subsequently confirmed (Barsantini et al., 1946; Masson, 1948; Walker and Matthews, 1949). Depression of milk secretion observed in the intact rat after
Estrogen treatment is thought to be due to the combined effect of the exogenous estrogen and of the progesterone from the resulting corpus luteum (Cowie, 1961).

Estrogen-progesterone treatment inhibits lactational performance in both intact and ovariectomized rats. The hormones are effective in physiological doses.

Mammary gland DNA content is not affected by estrogen-progesterone treatments (Griffith and Turner, 1962; Hodson, 1971). A decrease in RNA content was observed by Hodson (1971).

The mammary glands of estrogen-progesterone treated rats contain milk (Griffith and Turner, 1962; Knox and Griffith, 1970; Bruce and Ramirez, 1970). Milk was also seen in the stomachs of the pups of these dams (Hodson, 1975). Reduced growth of litters of estrogen-progesterone treated animals then is not a clear indication of an inhibition of milk secretion.

Weichert and Kerrigan (1942) theorized that pups of estrogen treated rats grow at a reduced rate due to changes in maternal behavior rather than failure of the mother's milk supply. However, no changes in maternal behavior were observed in dams treated with progesterone during pregnancy (Herrenkohl, 1971) or ergocornine during lactation (Numan et al., 1972).

Since lactation is suppressed by doses of estrogen in the range which stimulates prolactin release, inadequate amounts of prolactin do not seem to explain the inhibition of lactational performance.
during ovarian hormone treatment.

Estrogen treatments ranging from .1 ug to 500 ug per day increased prolactin production and secretion (Chen and Meites, 1970). Kalra et al. (1973) observed increased plasma prolactin levels after single doses of estrogen, progesterone or estrogen-progesterone. Prolactin was shown to override the inhibitory effects of stilbesterol administered during lactation (Noble, 1939).

An antagonistic relationship between prolactin and estrogen-progesterone was detected by Meites and Sgouris (1953, 1954). Estrogen-progesterone administration blocked the lactogenic effects of intraductal prolactin injections in rabbits. If larger dosages of prolactin were given, the inhibitory effects of estrogen-progesterone were overridden. Both estrogen and progesterone were required to block the lactogenic effect of prolactin.

**Adrenal steroids**

Lactation can be initiated during gestation in the rat (Talwalker et al., 1961), mouse (Nandi and Bern, 1961) and rabbit (Meites et al., 1963) with glucocorticoid injections. The combination of prolactin and glucocorticoid will induce milk secretion similar to normal lactation (Reece, 1939; Meites et al., 1963; Friesen, 1966).

A small increase in mammary gland RNA in response to hydrocortisone acetate was observed in pregnant rats (Ferreri and
Griffith, 1969). Nevertheless, the increase was not as great as the normal increase at parturition.

Lactation is impaired when the lactating animal is adrenalectomized (Cowie and Tindal, 1958; Anderson and Turner, 1962, 1963). Lactation ceases even sooner if the animal is simultaneously ovariectomized and adrenalectomized (Flux, 1955; Anderson and Turner, 1963).

Either glucocorticoids or mineralocorticoids will partially restore milk secretion, but a combination of the two is most effective (Cowie and Tindal, 1958).

Lactational performance has been increased 12-27 percent in lactating rats with injections of optimum doses of corticosterone (Hahn and Turner, 1966), cortisol acetate (Talwalker et al., 1960), cortisone acetate or ACTH (Johnson and Meites, 1958). Pharmacological doses of corticosteroids are inhibitory to lactation (Kowalewski, 1969).

Cortisol appears to act specifically by increasing the synthetic rate of nonribosomal RNA (Baldwin et al., 1969). Thatcher and Tucker (1970) found the adrenal corticosterone content to be highly correlated with mammary gland nucleic acid content during lactation. ACTH and adrenal functions decrease simultaneously with decreases in milk synthesis. Glucocorticoid supplementation prevents the reduction in milk synthesis strongly suggesting that adrenal secretions are rate limiting to milk synthesis during
prolonged lactation in the rat.

When high fat diets are administered in conjunction with glucocorticoids, both mammary gland nucleic acid content and lactational performance can be maintained at maximum values for at least 32 days (Emery et al., 1971).

**Brain amines and prolactin**

In rats systemic injection of a wide variety of amines and other substances thought to be involved in neural transmission including adrenaline, noradrenaline, acetylcholine and serotonin can elevate prolactin secretion (Mittler and Meites, 1967; Kamberi et al., 1971a). Monoamine oxidase and catechoi-0-methyi transferase inhibitors, which interfere with normal amine inactivation, both depress serum prolactin levels while at the same time elevate hypothalamic PIF content. Conversely, drugs which in some way interfere with amine function including reserpine, chlorpromazine, fluphenazine and perphenazine all increase plasma prolactin levels and reduce hypothalamic PIF content (Ben-David et al., 1971; Meites et al., 1972; Sulman and Winnik, 1956).

Serotonin and melatonin injected into the 3rd ventricle in rats stimulate prolactin and inhibit FSH secretion (Kamberi et al., 1971b). Systemic injections of the serotonin precursors tryptophan and 5-hydroxytryptophan both elevate serum prolactin levels significantly (Lu and Meites, 1971; Meites and Clemens, 1972).
MATERIALS AND METHODS

Treatment of Animals

Six female rats weighing at least 180 grams and 1 mature male of the Sprague-Dawley strain were placed in large cages for breeding. These animals remained together until the females were visibly pregnant. Animals in treatment groups L, EPL, and EPLF were purchased pregnant from ARS/Sprague-Dawley of Madison, Wisconsin.

Pregnant animals were caged individually and provided with shredded newspaper for nest material. A standard diet (Teklad) and water were provided ad libitum throughout the investigation. A light cycle of 14:10 with lights on from 8:00 A.M. to 10:00 P.M. and temperature of 25 ± 3°C were the standard animal room conditions.

The day following parturition was designated day 1 of lactation. On day 1 litter sizes were adjusted to 8 pups; dams were assigned to experimental groups; those in group 0 were ovariectomized, and hormonal treatments were begun.

Subcutaneous injections of the respective hormone(s) were given each morning through day 10 of lactation. Litters were weighed also at this time. Litter size was readjusted to 6 pups on day 4. Dead or injured pups were replaced with pups of equal weight.

All animals were sacrificed by decapitation on the morning
of day 11. The abdominal-inguinal mammary glands were excised, minced and defatted. Adrenals and pituitaries were removed and weighed.

Treatment groups and symbols used to identify them are listed in Table 1.

Hormone Preparation

**Estrogen-Progesterone (EP)**

A stock estrogen solution was prepared by combining 0.1 gm of estradiol benzoate, 5 ml of benzyl alcohol and 95 ml of Mazola corn oil.

The estrogen-progesterone solution was prepared by combining 3 grams of progesterone, 1 ml of stock estrogen, 5 ml of benzyl alcohol and enough Mazola corn oil to make 100 ml of solution. This solution was heated in a water bath to 35°C and continuously stirred with a magnetic stirrer for several hours. The quantity injected was 1 \( \mu \)g E + 3 mg P in 0.1 ml corn oil.

**Prolactin**

Ovine prolactin (NIH-P-S12) was dissolved in less than 5 ml of .9% saline adjusted to pH 8.0-8.5 with NaOH, then filled to volume (5 ml). The required quantity was injected in 0.1 ml of alkaline saline.
Table 1. Treatment groups

<table>
<thead>
<tr>
<th>Group Symbol</th>
<th># in Group</th>
<th>Hormonal Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>11</td>
<td>None</td>
</tr>
<tr>
<td>F</td>
<td>11</td>
<td>250 μg Hydrocortisone Acetate (F)</td>
</tr>
<tr>
<td>EP</td>
<td>12</td>
<td>1 μg Estrogen (E) + 3 mg Progesterone (P)</td>
</tr>
<tr>
<td>EPF</td>
<td>11</td>
<td>1 μg E + 3 mg P + 250 μg F</td>
</tr>
<tr>
<td>EPF-1</td>
<td>11</td>
<td>1 μg E + 3 mg P + 125 μg F</td>
</tr>
<tr>
<td>EPF-2</td>
<td>11</td>
<td>1 μg E + 3 mg P + 62.5 μg F</td>
</tr>
<tr>
<td>L</td>
<td>10</td>
<td>1 mg Prolactin (L)</td>
</tr>
<tr>
<td>EPL</td>
<td>10</td>
<td>1 μg E + 3 mg P + 1 mg L</td>
</tr>
<tr>
<td>EPLF</td>
<td>10</td>
<td>1 μg E + 3 mg P + 1 mg L + 250 μg F</td>
</tr>
<tr>
<td>EPL-IF</td>
<td>7</td>
<td>1 μg E + 3 mg P + .25 mg L + 250 μg F</td>
</tr>
<tr>
<td>0 (ovariectomized)</td>
<td>8</td>
<td>1 μg E + 3 mg P + 1 mg L + 250 μg F</td>
</tr>
<tr>
<td>5-HTP</td>
<td>12</td>
<td>3 mg 5-hydroxytryptophan</td>
</tr>
</tbody>
</table>

**Hydrocortisone acetate**

Hydrocortisone was suspended in a carrier solution consisting of 100 ml of saline, 0.9 ml of benzyl alcohol and 1 drop of Tween 80 per 5 ml of saline. The suspension was kept in a storage bottle in the refrigerator. The solution was shaken before each injection to maintain the suspension. The required amount of hormone was given in 0.1 ml of solution.
**5-Hydroxytryptophan**

L-5-hydroxytryptophan (ICN Pharmaceuticals) was dissolved in slightly alkaline saline to which 0.5 N HCl was added a drop at a time to bring the pH to 6.5-7.0. The injected amount was 1.5 mg per 0.1 ml twice daily.

**Mammary Gland Nucleic Acid Analysis**

**Preparation of dried fat free tissue (DFFT)**

Immediately after removal, the 6 abdominal-inguinal mammary glands were defatted in approximately 50 ml of chloroform-methanol (2:1, V/V) for 2 12-hour periods, and in ether for 2 subsequent 12-hour periods. Vials were continuously agitated during the extraction periods. After the final solvent was decanted, the tissues were allowed to air dry under a fume hood. The DFFT was weighed and ground to a fine powder in a Wiley mill with a number 20 sieve.

**Nucleic acid extraction**

Nucleic acid was extracted from the DFFT by the method of Schneider (1945) as modified by Ferreri (1971). A 25 mg sample of DFFT was suspended in 5 ml of 5% trichloroacetic acetic acid (TCA) in a thick walled 15 ml volumetric centrifuge tube. This was allowed to stand at room temperature until the DFFT became saturated and settled to the bottom of the tubes with gentle tapping.
Samples were then covered with glass marbles to prevent evaporation and placed in a hot water bath (90-95°C) for 15 minutes and then placed in a cold water bath. Samples were centrifuged for 30 minutes, and the supernatant decanted into 12 ml volumetric centrifuge tubes.

A second extraction in 5 ml of hot TCA was made on the centrifugate, and the supernatant added to that recovered from the first extraction. The total volume was brought up to 10 ml with cold 5% TCA, transferred to screw capped glass tubes and stored at 4°C.

**Colorimetric determination of DNA content**

DNA concentrations were measured by the diphenylamine reaction (Burton, 1956; Ferreri, 1971). One ml of the nucleic acid extract was combined with 1 ml of 1 N perchloric acid and 4 ml of diphenylamine reagent. Diphenylamine reagent was prepared immediately before use, and consisted of 1.5 g of reagent grade diphenylamine in 100 ml of A.R. acetic acid, 1.5 ml of concentrated sulfuric acid and 0.5 ml of acetaldehyde (16 mg/ml).

A blank was prepared with 1 ml of 5% TCA, 4 ml of diphenylamine reagent and 1 ml of perchloric acid. Samples were kept for 16-20 hours at room temperature, and absorbance was measured against the blank at 600 nm.

The concentration of DNA in the sample was calculated from a
least squares estimate of the slope and Y intercept of the standard curve, plotting absorbance vs. concentration.

RNA concentration was determined by the orcinol reaction outlined by Schneider (1957).

Half a milliliter of nucleic acid extract, DNA or RNA standard was combined with 2.5 ml of orcinol reagent and 2 ml of 5% TCA. Orcinol reagent consisted of 0.5 g of FeCl$_3$ in 100 ml of concentrated HCl and 1 g of orcinol prepared immediately prior to use. Samples were heated for 20 minutes in hot water (90-95°C).

A blank was prepared with 2.5 ml TCA and 2.5 ml of orcinol reagent. Absorbance was measured against the blank at 660 nm.

Both DNA and RNA standards were used in this procedure so that corrections for absorbance due to DNA could be subtracted from the total optical density. Remaining optical density was due to RNA, and the concentration was calculated from a least squares estimate of the slope and Y intercept of the standard curve, plotting corrected absorbance vs. concentration.

**Preparation of nucleic acid standards**

Stock solutions of hydrolyzed DNA and RNA were prepared by dissolving 100 mg of nucleic acid in 80 ml of hot (90°C) 5% TCA. This temperature was maintained for 15 minutes while the solution was constantly stirred with a magnetic stirrer. The samples were then allowed to cool and total volume brought up to
100 ml with 5% TCA. From these, dilutions were made for determination of standard curves.

**Statistical Analysis**

Separate analyses of variance (ANOVA) were conducted for all variables. The total variation of treatments was subdivided into orthogonal comparisons. A split-plot analysis of variance was used to test the effects of hormonal treatments over time on litter weight gains.
RESULTS

Final Body and Organ Weights

Body weights (Table 2)

The average body weights of rats used in these experiments ranged from 256.4 grams for EPLF to 365.36 grams for EPF-2. All groups except C, L and O gained weight during the treatment period. C, L and O animals lost an average of 12.9, 0.4 and 9.85 grams, respectively.

Dried fat free tissue (DFFT) weights

Hormonal treatments affected DFFT weights (p < .0001). The difference between the DFFT weights of EP vs. EPF-2 approached significance (p < .05). However, there were no significant differences in DFFT weights between the pooled mean of EP and EPF-2 and EPF-1 or EPF. C vs. F and EPL vs. EPLF exhibited similar weights. DFFT of 5HTP animals was significantly greater (p < .05) than that of L animals.

Pituitary weights

Pituitary weights were similar in all groups and did not differ significantly in any of the planned comparisons. When the mean pituitary weights of groups administered prolactin were pooled and compared with groups which did not receive prolactin, the groups without prolactin exhibited significantly higher (p < .0001)
Table 2. Effect of hormonal treatments on final body weight, mammary gland dried fat free tissue, pituitary and adrenal weights

<table>
<thead>
<tr>
<th>Group</th>
<th>B.W. (gm)</th>
<th>DFFT (gm)</th>
<th>Pituitary (mg)</th>
<th>Adrenals (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>260.72 ± 39.60</td>
<td>1.053 ± 0.142</td>
<td>11.72 ± 1.88</td>
<td>65.52 ± 4.57</td>
</tr>
<tr>
<td>F</td>
<td>289.18 ± 31.69</td>
<td>1.205 ± 0.163</td>
<td>10.18 ± 1.96</td>
<td>62.44 ± 9.06</td>
</tr>
<tr>
<td>EP</td>
<td>274.42 ± 28.17</td>
<td>0.904 ± 0.245</td>
<td>14.35 ± 2.92</td>
<td>83.22 ± 15.13</td>
</tr>
<tr>
<td>EPF</td>
<td>268.02 ± 26.45</td>
<td>1.067 ± 0.134</td>
<td>13.14 ± 2.03</td>
<td>85.98 ± 16.56</td>
</tr>
<tr>
<td>EPF-1</td>
<td>364.36 ± 24.95</td>
<td>1.569 ± 0.259</td>
<td>17.74 ± 4.53</td>
<td>92.69 ± 10.37</td>
</tr>
<tr>
<td>EPF-2</td>
<td>325.08 ± 33.66</td>
<td>1.068 ± 0.231</td>
<td>13.15 ± 2.23</td>
<td>70.73 ± 12.63</td>
</tr>
<tr>
<td>L</td>
<td>267.90 ± 21.44</td>
<td>1.142 ± 0.114</td>
<td>9.90 ± 1.20</td>
<td>71.80 ± 8.94</td>
</tr>
<tr>
<td>EPL</td>
<td>259.90 ± 21.64</td>
<td>0.922 ± 0.089</td>
<td>9.95 ± 2.51</td>
<td>68.79 ± 9.32</td>
</tr>
<tr>
<td>EPLF</td>
<td>256.40 ± 13.25</td>
<td>1.070 ± 0.131</td>
<td>9.69 ± 0.59</td>
<td>61.30 ± 6.14</td>
</tr>
<tr>
<td>EPL-IF</td>
<td>337.57 ± 38.06</td>
<td>1.603 ± 0.186</td>
<td>--</td>
<td>115.56 ± 9.20</td>
</tr>
<tr>
<td>O</td>
<td>282.85 ± 30.52</td>
<td>1.065 ± 0.104</td>
<td>11.40 ± 1.20</td>
<td>69.21 ± 5.27</td>
</tr>
<tr>
<td>5-HTP</td>
<td>343.41 ± 25.95</td>
<td>1.356 ± 0.235</td>
<td>11.74 ± 1.93</td>
<td>63.31 ± 8.72</td>
</tr>
</tbody>
</table>
pituitary weights.

Adrenal weights

The adrenals of animals receiving 125 and 62.5 μg of hydrocortisone were significantly larger (p < .01) than those of animals administered 250 μg of hydrocortisone. The animals with the largest adrenals were EPL-1F (115.56 mg) and EPF-1 (92.69 mg), both of which were markedly different (p < .001) from controls (62.52 mg).

Mammary Gland DNA

DNA content - μg DNA/mg DFFT (Tables 3 and 4)

The average μg DNA/mg DFFT of EP animals was 34.74. Graded levels of hydrocortisone, 250 μg, 125 μg or 62.5 μg, administered simultaneously with estrogen-progesterone did not significantly affect DNA content. The presence of hydrocortisone did slightly reduce the DNA values. The pooled mean of groups EP, EPF, EPF-1 and EPF-2 was significantly higher (p < .001) than the average μg DNA/mg DFFT value of control animals.

DNA values of F (23.35) vs. C (25.38), L (27.95) vs. 5HTP (26.19) and EPL (28.94) vs. EPLF (27.19) did not differ significantly. Animals in groups given estrogen-progesterone alone or in combination with hydrocortisone had higher (p < .0001) μg DNA/mg DFFT values than animals which received prolactin alone or in combination
<table>
<thead>
<tr>
<th>Group</th>
<th>µg DNA/mg DFFT</th>
<th>Total DNA</th>
<th>DNA/100 g B.W.</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>25.38 ± 2.36</td>
<td>26.57 ± 3.14</td>
<td>10.34 ± 1.48</td>
</tr>
<tr>
<td>F</td>
<td>23.35 ± 3.43</td>
<td>27.80 ± 3.02</td>
<td>9.76 ± 1.82</td>
</tr>
<tr>
<td>EP</td>
<td>34.78 ± 5.21</td>
<td>30.90 ± 7.58</td>
<td>11.26 ± 2.46</td>
</tr>
<tr>
<td>EPF</td>
<td>32.41 ± 5.26</td>
<td>34.42 ± 6.37</td>
<td>12.90 ± 2.54</td>
</tr>
<tr>
<td>EPF-1</td>
<td>32.00 ± 4.64</td>
<td>49.28 ± 4.51</td>
<td>13.60 ± 1.61</td>
</tr>
<tr>
<td>EPF-2</td>
<td>32.33 ± 4.77</td>
<td>33.79 ± 5.77</td>
<td>10.51 ± 2.09</td>
</tr>
<tr>
<td>L</td>
<td>27.95 ± 3.49</td>
<td>31.72 ± 3.33</td>
<td>11.90 ± 1.56</td>
</tr>
<tr>
<td>EPL</td>
<td>28.94 ± 2.46</td>
<td>26.80 ± 4.25</td>
<td>10.32 ± 1.42</td>
</tr>
<tr>
<td>EPLF</td>
<td>27.17 ± 1.17</td>
<td>28.96 ± 3.05</td>
<td>11.29 ± 0.55</td>
</tr>
<tr>
<td>EPL-1F</td>
<td>25.08 ± 2.814</td>
<td>39.86 ± 2.636</td>
<td>11.88 ± 0.917</td>
</tr>
<tr>
<td>O</td>
<td>27.62 ± 2.785</td>
<td>29.39 ± 3.57</td>
<td>10.83 ± 1.42</td>
</tr>
<tr>
<td>5-HTP</td>
<td>26.19 ± 3.43</td>
<td>35.09 ± 3.03</td>
<td>10.20 ± 1.82</td>
</tr>
</tbody>
</table>
Table 4. Analysis of variance of DNA concentration

<table>
<thead>
<tr>
<th>Source</th>
<th>Degrees Freedom</th>
<th>Sum of Squares</th>
<th>Mean Squares</th>
<th>F</th>
<th>Level of Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatments</td>
<td>9</td>
<td>1394.4</td>
<td>154</td>
<td>9.79</td>
<td>.0001</td>
</tr>
<tr>
<td>EP vs EPF-2</td>
<td>1</td>
<td>36.3</td>
<td>36.3</td>
<td>2.3</td>
<td>Not significant</td>
</tr>
<tr>
<td>EP + EPF-2 vs EPF-1</td>
<td>1</td>
<td>27.1</td>
<td>27.1</td>
<td>1.7</td>
<td>Not significant</td>
</tr>
<tr>
<td>EP + EPF-2 vs EPF</td>
<td>1</td>
<td>6.0</td>
<td>6.0</td>
<td>&lt; 1</td>
<td>Not significant</td>
</tr>
<tr>
<td>C vs F</td>
<td>1</td>
<td>22.7</td>
<td>22.7</td>
<td>1.4</td>
<td>Not significant</td>
</tr>
<tr>
<td>L vs 5HTP</td>
<td>1</td>
<td>24.9</td>
<td>24.9</td>
<td>1.6</td>
<td>Not significant</td>
</tr>
<tr>
<td>EPL vs EPLF</td>
<td>1</td>
<td>15.7</td>
<td>15.7</td>
<td>&lt; 1</td>
<td>Not significant</td>
</tr>
<tr>
<td>EP, EPF, EPF-1, EPF-2 vs C, F, L, EPL, EPL-1</td>
<td>1</td>
<td>1105.9</td>
<td>1105.9</td>
<td>69.99</td>
<td>.005</td>
</tr>
<tr>
<td>Remainder</td>
<td>2</td>
<td>155.8</td>
<td>77.9</td>
<td>5.0</td>
<td></td>
</tr>
<tr>
<td>Error</td>
<td>100</td>
<td>1583.2</td>
<td>15.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>109</td>
<td>2977.6</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
with estrogen-progesterone, F alone and controls.

Total DNA and μg DNA per 100 g body weight (Table 3. Figs. 2 and 3)

The total DNA of EP (30.90) and EPF-2 (32.33) were not significantly different. The total DNA of EPF-1 was significantly different (p < .01) from EP and EPF-2. The combined mean of EP, EPF-2 and EPF-1 was similar to that of EPF.

No statistically significant differences were observed between C (26.57) and F (27.80), L (31.72) and 5HTP (35.09) or EPL (26.80) and EPLF (28.90). The average total DNA of animals in groups EP, EPF, EPF-2, and EPF-2 was significantly (p < .001) higher than animals which received prolactin alone or in combination with estrogen-progesterone, F alone and controls.

There were no significant differences in the DNA/100 g body weight values between any groups. The lowest value, 9.76, was found in group F but it was not markedly different from controls.

Mammary Gland RNA

RNA per mg DFFT and mg RNA per 100 g body weight (Tables 5 and 6. Figs. 4 and 5)

The overall effects of hormonal treatments on mammary gland RNA content were highly significant (p < .0001). When the treatment effects were partitioned out into single degree of freedom comparisons, EP (54.32) and EPF-2 (55.42) did not differ significantly.
Fig. 2. DNA on a per 100 g body weight basis
(Mean ± S.D.)
Fig. 3. DNA on a per 100 g body weight basis (Mean ± S.D.)
Table 5. Effect of hormonal treatment on mammary gland RNA content

<table>
<thead>
<tr>
<th>Group</th>
<th>μg RNA/mg DFFT</th>
<th>Total RNA</th>
<th>RNA/100 g B.W.</th>
<th>RNA/DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>70.73 ± 6.60</td>
<td>74.28</td>
<td>29.14 ± 6.52</td>
<td>2.80 ± 0.34</td>
</tr>
<tr>
<td>F</td>
<td>89.36 ± 14.33</td>
<td>106.97</td>
<td>37.44 ± 8.28</td>
<td>3.86 ± 0.66</td>
</tr>
<tr>
<td>EP</td>
<td>54.32 ± 13.13</td>
<td>49.74</td>
<td>18.21 ± 7.73</td>
<td>1.61 ± 0.50</td>
</tr>
<tr>
<td>EPF</td>
<td>68.00 ± 6.60</td>
<td>72.42</td>
<td>27.21 ± 4.88</td>
<td>2.15 ± 0.41</td>
</tr>
<tr>
<td>EPF-1</td>
<td>64.65 ± 12.71</td>
<td>102.19</td>
<td>28.14 ± 7.72</td>
<td>2.05 ± 0.44</td>
</tr>
<tr>
<td>EPF-2</td>
<td>55.42 ± 7.32</td>
<td>60.18</td>
<td>18.69 ± 5.92</td>
<td>1.76 ± 0.42</td>
</tr>
<tr>
<td>L</td>
<td>74.30 ± 7.62</td>
<td>84.46</td>
<td>31.71 ± 4.17</td>
<td>2.67 ± 0.25</td>
</tr>
<tr>
<td>EPL</td>
<td>77.65 ± 14.08</td>
<td>71.75</td>
<td>27.47 ± 4.72</td>
<td>2.71 ± 0.62</td>
</tr>
<tr>
<td>EPLF</td>
<td>95.53 ± 6.47</td>
<td>109.79</td>
<td>39.67 ± 3.34</td>
<td>3.52 ± 0.26</td>
</tr>
<tr>
<td>EPL-1F</td>
<td>77.43 ± 2.156</td>
<td>124.05</td>
<td>36.86 ± 3.401</td>
<td>3.11 ± 0.314</td>
</tr>
<tr>
<td>0</td>
<td>97.09 ± 3.37</td>
<td>105.77</td>
<td>37.26 ± 4.37</td>
<td>3.44 ± 0.75</td>
</tr>
<tr>
<td>5-HTP</td>
<td>81.71 ± 10.19</td>
<td>112.45</td>
<td>32.50 ± 7.59</td>
<td>3.21 ± 0.76</td>
</tr>
</tbody>
</table>
### Table 6. Analysis of variance of RNA content (µg RNA/mg DFFT)

<table>
<thead>
<tr>
<th>Source</th>
<th>Degrees Freedom</th>
<th>Sum of Squares</th>
<th>Mean Squares</th>
<th>F</th>
<th>Level of Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatments</td>
<td>9</td>
<td>18148</td>
<td>2016</td>
<td>18.58</td>
<td>.0001</td>
</tr>
<tr>
<td>EP vs EPF-2</td>
<td>1</td>
<td>7</td>
<td>7</td>
<td>&lt; 1</td>
<td>Not significant</td>
</tr>
<tr>
<td>EP + EPF-2 vs EPF-1</td>
<td>1</td>
<td>926</td>
<td>926</td>
<td>8.53</td>
<td>.005</td>
</tr>
<tr>
<td>EP + EPF-2 vs EPF</td>
<td>1</td>
<td>936</td>
<td>936</td>
<td>8.63</td>
<td>.005</td>
</tr>
<tr>
<td>C vs F</td>
<td>1</td>
<td>1911</td>
<td>1911</td>
<td>17.6</td>
<td>.005</td>
</tr>
<tr>
<td>L vs 5HTP</td>
<td>1</td>
<td>385</td>
<td>385</td>
<td>3.55</td>
<td>.05</td>
</tr>
<tr>
<td>EPL vs EPLF</td>
<td>1</td>
<td>1599</td>
<td>1599</td>
<td>14.7</td>
<td>.005</td>
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<td>EP, EPF, EPF-1, EPF-2 vs C, F, L, EPL, EPL-1, EPLF</td>
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Fig. 4. RNA on a per 100 g body weight basis (Mean ± S.D.)
Fig. 5. RNA on a per 100 g body weight basis (Mean ± S.D.)
Therefore the means of these two groups were pooled for further comparisons. EP + EPF-2 were significantly lower (p < .005) than EPF-1 (64.65) and EPF (68.00).

All RNA parameters of F treated animals were significantly higher (p < .005) than corresponding control parameters. Estrogen-progesterone treatments suppressed mammary gland RNA. Mean of any RNA parameter for EP, EPF, EPF-1 and EPF-2 was significantly less (p < .001) than the mean RNA value for controls.

Prolactin in combination with estrogen-progesterone slightly elevated μg RNA/mg DFFT values (77.65) when compared to prolactin alone animals (L, 74.30). This increase was not statistically significant. When F was added to the estrogen-progesterone-prolactin (EPLF) complex, all RNA parameters were significantly (p < .005) increased.

5HTP animals exhibited higher (p < .05) μg RNA/mg DFFT values than group L animals. RNA/100 g body weight values of the two groups were similar.

RNA to DNA ratio (Table 5. Figs. 6 and 7)

The RNA to DNA ratio in the mammary glands of EP (1.61) and EPF-2 (1.76) animals did not differ significantly. They were lower (p < .05) than EPF (2.15) and EPF-1 (2.05). All combinations of estrogen-progesterone and hydrocortisone, EP, EPF, EPF-1 and EPF-2, were significantly (p < .001) lower than controls (2.80).

Hydrocortisone acetate alone (F, 3.86) increased the RNA:DNA
Fig. 6. RNA:DNA ratio
(Mean ± S.D.)
Fig. 7. RNA:DNA ratio
(Mean ± S.D.)
ratio ($p < .0001$) above controls (C, 2.80). In the case of the animals receiving prolactin in combination with EP, RNA:DNA was increased (2.71) to near controls. When F was added to this combination, the RNA:DNA ratio (3.52) exceeded ($p < .005$) controls.

5HTP animals showed an increased ($p < .01$) RNA:DNA ratio (3.21) when compared to group L animals (2.67).

Litter Weight Gains

Litter growth curves (Tables 7 and 8. Figures 8, 9, 10 and 11)

Hormonal treatment of the lactating dams had profound effects on litter weight gains ($p < .0001$). Litter weights of EPF-2 animals were suppressed equivalent to EP litters. Pups of EPF-2 animals were slightly larger than EP + EPF-2, with the difference approaching significance ($p < .05$). EPF litters were significantly larger ($p < .01$) than either EP, EPF-1 or EPF-2. When the average litter gains of EP, EPF, EPF-1 and EPF-2 were compared to controls, they were smaller ($p < .05$).

Two hundred-fifty µg of hydrocortisone had a positive effect on litter weights. Litters of F treated animals were significantly ($p < .05$) larger than controls. Litters of L treated animals were larger ($p < .05$) than 5HTP litters. Litters of EPL and EPLF were not significantly different when treatment only was considered.

Litters of O animals were the largest of all experimental groups. They were significantly larger than controls, F and
Table 7. Analyses of variance of litter weight gain, whole plot and split-plot

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<th>Source</th>
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<th>F</th>
<th>Level of Significance</th>
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<tr>
<td>Treatment (TRT)</td>
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<tr>
<td>EP + EPF-2 vs EPF-1</td>
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<td>86.8</td>
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<tr>
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Table 8. Average daily pup weights (gm)

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Fig. 8. Litter growth curves
Fig. 9. Litter growth curves
Fig. 10. Litter growth curves

Day of Lactation

Mean Pup Weight (gm)

Fig. 10. Litter growth curves
Fig. 11. Litter growth curves
When the effects of treatments were analyzed over time, the differences in effects became even more apparent (Table 8, split-plot ANOVA). There was a linear relationship between treatment and time in 90 percent of the growth curves. EP and EPF-2 were still essentially the same. Significant differences (p < .0001) between the growth curves of EP + EPF-2 and EPF-1 became evident in this analysis. The pooled slope of EP, EPF-2 and EPF-1 growth curves was much less (p < .0001) than that of EPF. The slope of the growth curve of control animals was larger (p < .001) than the mean of the combined slopes of EP, EPF, EPF-1 and EPF-2.

The differences between C, L and F were not as extreme as the EP groups, but F and L did exhibit greater (p < .01) slopes than C. There was no difference between slopes of F and L. The presence of F enhanced the growth rate of EPLF litters producing a significantly (p < .005) larger slope than EPL litters. This effect could not be seen when treatment effects were analyzed ignoring time. When combined with hydrocortisone, smaller doses of prolactin (EPL-F) overcame the suppressive effects of EP on litter growth.

5HTP treatments diminished litter growth rates. The slope of the 5HTP litter growth curve was less (p < .0001) than that of L litters.
The principal difference between the complex of hormones required for full mammary gland development and those required for lactation appears to be the ovarian steroids (Lyons et al., 1958). This has led to the view that the estrogen-progesterone equilibrium during pregnancy inhibits the initiation of lactation. They inhibit prolactin secretion and its effects on mammary cells (Meites, 1966).

At parturition the fall in plasma progesterone, coupled with the temporary maintenance of high levels of estrogen, renders the mammary gland receptive to the lactogenic actions of prolactin and glucocorticoids.

The biological actions of glucocorticoids may be minimized during pregnancy due to increased binding to corticoid binding globulin (CBG). Gala and Westphal (1965) observed a marked fall in CBG values and a coincidental rise in free corticosteroids during late pregnancy and early lactation. Exogenous hydrocortisone during late pregnancy increased RNA content (Ferreri and Griffith, 1969) and induced mammary secretions (Talwalker et al., 1961). This suggests that the availability of active glucocorticoids could be the limiting factor for lactogenesis during pregnancy.

The inhibitory effects of estrogen-progesterone on lactation have been previously established (Cowie, 1961; Griffith and Turner, 1962, Hodson, 1975). The results of this investigation agree with
these earlier studies. Estrogen-progesterone treatments depressed RNA content, RNA/DNA ratio and litter weight gains, all of which are estimates of mammary cell secretory activity. Conversely, μg DNA/mg DFFT and total DNA values of these animals were increased significantly above controls.

The ability of hydrocortisone to overcome the suppressive effects of estrogen-progesterone on lactation is dependent on the quantity administered. The lowest quantity used in this study, 62.5 μg, was unable to override the estrogen-progesterone effects. In fact, litter growth was further depressed and nucleic acid parameters unaffected. The adrenals of these animals were significantly smaller than estrogen-progesterone animals probably due to decreased endogenous adrenal steroid secretion. This low level of hydrocortisone could possibly have synergized with estrogen-progesterone in their mammogenic actions or could have been converted to progesterone. The latter would explain the increased depression of litter weight by this quantity of hydrocortisone.

Ahrens and Jacobsohn (1957) found normal mammary gland growth to be inhibited or impeded by estrogen-progesterone plus 1 mg of cortisone when the metabolic actions of cortisone could not be counteracted efficiently. The animals in the estrogen-progesterone plus 62.5 μg hydrocortisone group all gained weight during the experimental period; therefore, increased metabolic activity then would not seem to explain the detrimental effects of this hormonal
combination on litter growth.

Estrogen-progesterone plus 125 µg hydrocortisone treated animals exhibited significant increases in RNA content and RNA/DNA ratio. The RNA/DNA ratio of this group was significantly less than controls and higher than estrogen-progesterone alone. The RNA/DNA ratio of this group was equal to ratios reported by Tucker and Reece (1963) for normal lactating rats. This group exhibited the highest total DNA and mg DNA/100 g body weight values of all experimental animals. One hundred twenty-five µg of hydrocortisone then appears to be optimum for maximum mammogenesis. This action could be through synergism with estrogen-progesterone or through mimicry of their effects.

Litter weights of animals receiving 250 µg of hydrocortisone plus estrogen-progesterone were equal to controls. RNA content and RNA/DNA ratio of these animals were in the range of normal lactating animals in this and other studies (Griffith and Turner, 1962; Tucker and Reece, 1963). DNA content was slightly increased above controls indicating increased mammogenesis.

The effect of hydrocortisone in combination with estrogen-progesterone is dependent on the quantity administered. The principal effect of 62.5 µg of hydrocortisone was mammogenesis. Both mammogenic and secretory effects, e.g., increases in DNA, RNA and litter weight, were induced by 125 µg of hydrocortisone. The mammary glands of 250 µg hydrocortisone treated animals were highly secretory.
Thyroxine, estrogens and progesterone have been shown to enhance the binding capacity of CBG (Gala and Westphal, 1965). This could partly explain the depression of milk secretion by estrogen-progesterone. If larger quantities of glucocorticoids are bound to CBG decreasing the amount of free glucocorticoids, lactational performance could then be hindered. Lyons et al. (1958) demonstrated that prolactin and glucocorticoids are the minimal requirements for the maintenance of lactation in the rat. Yet, there must be a minimum level of glucocorticoid which is sufficient. It is conceivably then that the amount of free glucocorticoid could be reduced to near or below this critical level depressing milk secretion.

This could possibly explain how 250 µg of hydrocortisone was able to override the suppressive effects of estrogen-progesterone on lactation and lower quantities were not. This quantity of hydrocortisone could saturate the binding sites of CBG, while simultaneously increasing the amount of free glucocorticoid. Also, exogenous glucocorticoids have been shown to depress the binding capacity of rat plasma (Gala and Westphal, 1967). The antagonistic actions of estrogen-progesterone and glucocorticoids on binding capacity of rat plasma could be shifted in favor of the glucocorticoid effects by 250 µg of hydrocortisone acetate.

Estrogen-progesterone plus prolactin treatments induced changes in litter weights, nucleic acid content and RNA/DNA ratio similar to EP plus 125 µg hydrocortisone. Inadequate quantities of glucocorticoid somehow prevent normal milk secretion. When 250 µg
hydrocortisone is combined with EPL, litter weights are normal and RNA/DNA ratio is significantly elevated above controls or estrogen-progesterone prolactin treated animals.

Litter growth was, in fact, enhanced by reducing the amount of prolactin to .25 mg given in combination with EP plus hydrocortisone. This is not to say that prolactin is not important in the initiation and maintenance of milk secretion. Meites et al. (1969) showed that progesterone, testosterone propionate and cortisol acetate injections into rats increased pituitary prolactin concentrations and promoted mammary development. This reduced quantity of prolactin could be insufficient to inhibit pituitary prolactin release stimulated by the other hormones. The concentrations and ratios of prolactin-glucocorticoid may then become optimum for maximal milk secretion. In support of this idea is the high mortality rate of pups of dams treated with estrogen-progesterone plus 2 mg of prolactin (unpublished research of L. Haynes and D. R. Griffith). This quantity of prolactin was not able to override the inhibitory effects of estrogen-progesterone.

Chen and Meites (1970) and Kalra et al. (1973) observed increased prolactin secretion after treatment of rats with estrogen, progesterone or estrogen-progesterone. Treatment of animals with estrogen-progesterone plus quantities of prolactin greater than 1 mg may increase plasma prolactin levels to the point that prolactin-glucocorticoid ratios or absolute quantities are upset thus
depressing lactation. More research is needed to determine optimum prolactin-glucocorticoid ratios for normal lactation.

Lactational performance was increased by injections of 250 µg hydrocortisone acetate. Litter weights, RNA and RNA/DNA ratio were significantly increased above controls. These results were obtained without the loss in weight of the dams reported by Johnson and Meites (1958) with 1 mg of cortisol acetate.

DNA content was unaffected by 5-hydroxytryptophan treatments. RNA content and RNA/DNA ratio were significantly increased but litter weights were below normal. The mechanism(s) by which 5HTP exerts its effects is unknown.
SUMMARY

The ability of hydrocortisone acetate and/or prolactin to override the suppressive effects of estrogen-progesterone treatments on lactation was studied. Mammary gland growth was measured by DNA content and secretory activity was estimated from RNA content, RNA/DNA ratio and litter weight gains.

Estrogen-progesterone treatments suppressed litter weight gains, RNA content and RNA/DNA ratio while simultaneously elevating total DNA content.

A dose-response relationship was observed between the amount of hydrocortisone administered in combination with estrogen-progesterone and litter weight gains. Litters of dams receiving the largest dose of hydrocortisone were equal in size to controls.

Administration of 125 μg and 250 μg of hydrocortisone acetate increased RNA content and RNA/DNA ratio above that of animals receiving estrogen-progesterone alone. Increased litter weights coincided with these increases in nucleic acid parameters. The low dose of hydrocortisone, 62.5 μg, was not able to override the estrogen-progesterone effects on DNA, RNA, RNA/DNA ratio or litter size.

Two hundred-fifty μg of hydrocortisone alone decreased DNA and increased RNA, RNA/DNA ratio and litter weight gains. The slope of litter growth curves of 250 μg hydrocortisone treated animals was higher than those of all experimental groups except those of
animals receiving 1 mg of prolactin alone.

Prolactin alone increased DNA content above that of control animals and those receiving 250 μg of hydrocortisone alone. However, hydrocortisone was a more effective stimulator of RNA, RNA/DNA ratio and litter growth.

When prolactin or hydrocortisone was given in conjunction with estrogen-progesterone, their effects were similar: slight increase in DNA and significant increase in RNA, RNA/DNA ratio and slopes of litter growth curves. When both prolactin and hydrocortisone were given simultaneously with estrogen-progesterone, there was a negligible increase in DNA but highly significant increases in RNA, RNA/DNA ratio and slopes of litter growth curves. Litter weights in this group were equal to controls. These effects were potentiated by ovariectomizing dams.

5HTP elevated RNA content and RNA/DNA ratio above controls and of animals receiving prolactin. Litter weights of 5HTP animals were below both control and prolactin animals. The mechanism(s) by which 5HTP exerted its effects is unknown. Its effects on RNA and RNA/DNA ratio could possibly be mediated by increased prolactin release, and its detrimental effects on litter growth could be due to the chemical itself occurring in the milk.

The general observation from this study is that either 250 μg of hydrocortisone acetate or 1 mg of prolactin is sufficient to overcome the suppressive effects of estrogen-progesterone treatments.
However, a combination of the two induces a greater response in terms of nucleic acid synthesis, RNA/DNA ratio and litter growth rates. Ovariectomy enhances these effects.

The specific observations are:

1. Estrogen-progesterone treatment of lactating rats reverts the lactating mammary gland to the pregnant or non-secretory state.

2. The effects of hydrocortisone acetate are dependent on the quantity administered: 62.5 μg induced mammogenesis, 125 μg induced both mammogenesis and galactopoiesis and 250 μg induced galactopoiesis.

3. The mechanism by which glucocorticoids override the inhibitory effects of estrogen-progesterone on lactation is probably through changes in the binding capacity of plasma.
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