THE MAINTENANCE OF MILK SYNTHESIS DURING EXTENDED LACTATION

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This is to certify that the

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ABSTRACT

THE MAINTENANCE OF MILK SYNTHESIS
DURING EXTENDED LACTATION

by William W. Thatcher

The primary objective of these studies was to determine some of the physiological and endocrine factors that may limit lactational performance in rats.

Intensive nursing, which was maintained by replacing all 16-day-old litters every 4 days with 12-day-old foster litters and supplying at least one pup per mammary gland, failed to prevent declines in mammary DNA, RNA and litter weight gains (LWG) between days 20 and 36 of lactation. Intense nursing maintained pituitary prolactin during extended lactation but pituitary ACTH decreased 68%.

Adrenal corticosterone content and concentration decreased significantly from day 16 to 32 of lactation. Peripheral plasma concentrations of corticosterone in lactating rats were higher than the resting levels measured in virgin rats. However, corticosterone concentrations in plasma did not change from day 16 to 32 of lactation. Measurements of corticoid binding globulin activity in pooled plasma samples indicated

that binding activity of rats killed at day 32 of lactation was greater than the activity found in rats killed at day 16 and 24. Adrenal corticosterone content of rats at day 16 of lactation was significantly correlated with mammary gland DNA ($r=0.59$, $P<0.01$), RNA ($r=0.60$, $P<0.01$) and litter weight gain ($r=0.44$, $P<0.05$). The decrease in adrenal corticosterone content from day 16 to 32 of lactation was correlated significantly with the decrease in mammary gland DNA ($r=0.35$, $P<0.01$) and RNA ($r=0.45$, $P<0.01$) content.

Oxytocin injections during advanced lactation caused increases in DNA and RNA content of the mammary gland but did not significantly increase litter weight gain or synthetic activity per cell (RNA/DNA).

Hydrocortisone acetate, prolactin and growth hormone were given singularly and in all possible combinations to determine if these treatments would maintain mammary nucleic acid content and lactational performance during extended lactation. Hydrocortisone acetate maintained mammary DNA and RNA content and reduced the normal decline in litter weight gain. Prolactin given with hydrocortisone acetate reduced the DNA response, but stimulated synthetic activity of the mammary cells (RNA/DNA). Prolactin and growth hormone did not exert a galactopoietic influence on milk yield.

The chronic secretion of rat prolactin from five isotransplanted pituitaries under the kidney capsule of lactating rats did not increase litter weight gain

although the mammary gland DNA and RNA content and RNA/DNA ratio were significantly increased. Prolactin is probably not rate limiting to milk synthesis in rats because pituitary prolactin content did not decrease during extended lactation and neither ovine prolactin injections nor rat prolactin from isotransplanted pituitaries increased milk yield.

A daily dose of 50 µg of 9-fluoroprednisolone acetate (Predef) from day 16 to 32 of lactation maintained mammary gland DNA and RNA content and increased milk yield. A delay in the onset of injections until day 24, or injection of 50 µg of Predef daily from day 16 to 23 and 100 µg of Predef daily from 24 to 32 days of lactation maintained LWG and increased mammary gland nucleic acid content. Results of in vitro incubations indicated that Predef injections for 16 days increased the ability of rat livers to metabolize cortisol into polar metabolites while the formation of non-polar metabolites was inhibited.

Adrenocortical secretions may be rate limiting to milk synthesis during advanced lactation in rats because pituitary ACTH and adrenal corticosterone content decrease during this time, corticosteroid binding globulin activity of plasma increases (although total circulating levels of corticosterone did not change) whereas the declines in mammary gland nucleic acid content and litter weight gain can be prevented with exogenous hydrocortisone acetate or Predef.

THE MAINTENANCE OF MILK SYNTHESIS DURING EXTENDED LACTATION

By

William W. Thatcher

A THESIS

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Dedicated to My Parents

Muriel and William Thatcher

BIOGRAPHICAL SKETCH

William Watters Thatcher was born on January 12, 1942, in Baltimore, Maryland. He attended public schools in Baltimore County and graduated from Kenwood High School in June, 1959. In September, 1959, he enrolled at the University of Maryland, majoring in Animal Husbandry, and received a Bachelor of Science degree in June, 1963. In June, 1963, he enrolled in the graduate school of the University of Maryland. He received the Master of Science degree in September, 1965, majoring in Dairy Science.

In September, 1965, he was awarded a graduate research assistantship by the Department of Dairy, Michigan State University. He was granted a NIH Predoctoral Fellowship in March, 1968 and completed the requirements for the Ph.D. degree on December 12, 1968.

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INTRODUCTION

Milk secretion is initiated immediately after parturition in the bovine at a relatively high level. Milk yield normally increases during the initial 4 to 6 weeks of lactation and then decreases progressively until the end of lactation. Any means of minimizing this normal decline in milk production would represent a sizeable contribution of animal protein for human consumption as well as a marked gain in operational efficiency to dairy farmers.

The primary objective of these studies was to determine some of the physiological and endocrine factors that may limit lactational performance. The rats were the experimental animals. Changes in milk yield and biochemical composition of the mammary gland were measured in response to both nursing intensity and exogenous administration of hormones believed to be intimately associated with milk production. Based on the results of these experiments, a subsequent study was conducted to determine if adrenal function became limiting as lactation advanced.

REVIEW OF LITERATURE

Terminology

Milk secretion involves both intracellular synthesis of milk in alveolar epithelium of the mammary gland and subsequent passage of milk from the cytoplasm into the alveolar lumen. The term lactogenic describes those factors responsible for the initiation of milk secretion whereas galactopoietic indicates the ability of factors to enhance an already established milk secretion (Cowie, 1961). Galactopoiesis is used in a general sense to infer the maintenance of milk secretion. The act of suckling influences milk secretion through the synthesis and release of certain anterior pituitary hormones that are in turn either directly or indirectly involved in the stimulation of mammary alveoli to produce milk.

Milk removal from the mammary gland involves both active and passive withdrawal of milk. The suckling process initiates active milk expulsion, the so-called milk ejection reflex. During this reflex suckling elicits sensory afferent nerve impulses which travel from the teats to the central nervous system to cause a release of oxytocin from the posterior pituitary. Oxytocin is

subsequently transported in the blood to the mammary gland. At the mammary gland oxytocin stimulates contraction of the myoepthelial cells which surround the alveoli and small milk ducts and thus forces the milk into the large milk ducts, gland cisterns and sinuses. The external physical force of suckling causes the passive withdrawal of milk from the large milk ducts, cisterns and sinuses of the mammary gland to the exterior of the body.

Measurements of Lactational Performance

It is more difficult to measure accurately lactational response in small laboratory animals than in larger animals such as the cow and goat. In the latter species milk can be weighed gravimetrically over a long period of time whereas in laboratory animals milk production is usually measured indirectly as some function of litter weight gain. Cowie and Folley (1946) used a logistic equation. They plotted the mean growth curve for litters of lactating rats from the time of birth to 16 days of age and obtained a sigmoid curve. From day 6 to day 11 of lactation, daily litter weight gain was maximum. The gain during this five-day period was termed "litter growth index" and has been used as a quantative measure for lactational responses in rats. The two extremes of the sigmoid curve were defined as

the initial and declining phases of the lactation curve. However, it should be pointed out that a litter weight gain is not an absolute but a relative index of lactational response. In litter growth curves, the daily milk yield is not directly calculable, since the daily weight loss due to excreta and perspiration are not measured. In addition there is some question as to whether each pup is able to convert milk to body weight at the same rate.

Grosvenor and Turner (1959a) used as an index of lactational performance the amount of milk obtained (stomach content) in a litter of six during 30 minutes nursing. Each litter was isolated from its mother for 10 hours. During this interval any milk present in stomachs of the young was digested and the mammary glands of the mother rat became turgid with milk. At the end of isolation, mother and young were reunited and the litter permitted to nurse 30 minutes. After the nursing period, the litter was weighed, killed by decapitation and stomach contents weighed. The weight of milk obtained was then expressed as a percent of litter body weight.

Tucker and Reece (1963a) determined DNA and RNA content of the mammary gland throughout lactation and observed marked changes in these parameters that paralleled changes in functional activity. Later Tucker (1966) correlated litter weight gain with total DNA,

RNA and RNA/DNA, and observed the highest correlation coefficient between litter weight gain and total RNA (r=0.93). He suggested, that at a given time in a lactation, total RNA could be used as an index of lactational performance.

Factors Affecting Lactational Performance

In Hypophysectomized and Adrenalectomized Animals

To evaluate the hormones required to maintain lactation, one test system would be experimental animals devoid of endogenous hormones that might participate in the lactational process. However, attempts to maintain full lactation in these types of animals is rather difficult because the process of lactation involves the interaction of several hormones.

Hypophysectomy caused a marked depression of lactation in rats, goats, and sheep (Cowie, 1957), (Lyons et al., 1958), (Cowie and Tindal, 1960), (Cowie et al., 1964), (Denamur and Martinet, 1961). Bintarningsih et al. (1957) and Cowie and Tindal (1961) were able to obtain partial maintenance of milk secretion in hypophysectomized rats with injections of prolactin and either cortisol acetate or prednisolone acetate. In goats the combination of ovine prolactin, bovine growth hormone, triiodo-L-thyroxine, zinc protamine insulin, and tablet

implants of dexamethazone maintained milk secretion at the pre-operative level when therapy was started immediately after hypophysectomy (Cowie et al., 1964).

Early investigations established that rats adrenalectomized early in lactation were unable to lactate sufficiently to maintain their young (Gaunt et al., 1942) (Cowie and Folley, 1947). The predominant secretions of the rat adrenal cortex are aldosterone and corticosterone (Peron, 1960). Supplementation of adrenalectomized lactating rats with either aldosterone (Cowie and Tindal, 1955) or aldosterone and cortiocosterone (Anderson and Turner, 1963) maintained lactational performance at about 70-80% of normal.

In a classical study Lyons (1958) developed in hypophysectomized, ovariectomized, and adrenalectomized rats a mammary lobular-alveolar system with a combination of estrogen, progesterone, adrenal corticoids, prolactin and growth hormone. Milk secretion based upon histological appearance of the mammary gland was subsequently initiated by the injection of adrenal corticoids and prolactin. These studies in animals devoid of the sources of hormones thought to be involved in lactation, showed that it was possible to mimic the mammary growth response of pregnancy and to duplicate lactogenesis associated with parturition.

In Intact Animals

Maintenance of milk secretion is a response to the coordinated actions of several pituitary hormones. Grosvenor and Turner (1959c) suggested that an increase in milk production associated with exogenous administration of a hormone(s) indicates a sub-optimal secretion of that hormone(s). On the other hand such responses to hormones may represent pharmacological responses and be unrelated to the secretion rate of the hormone.

Johnson and Meites (1958) reported that daily injections of cortisone acetate in rats produced a significant rise in lactational performance during the first 18 days post-partum. In an experiment in which injections of hydrocortisone acetate, oxytocin and prolactin were given singularly and in all combinations, Talwalker et al. (1960) found that only hydrocortisone acetate increased milk synthesis significantly. Apparently suckling released optimal levels of oxytocin because administration of this hormone in combination with the corticoid did not increase litter weight gains. Exogenous prolactin was not galactopoietic in the intact rat. This effect of prolactin in the rat was confirmed by Macdonald and Reece (1961).

Several workers (Cotes et al., 1949) (Flux et al., 1954) (Shaw et al., 1955) (Brush, M. G., 1960) (Campbell et al., 1964) reported that adrenalcorticotropic hormone (ACTH) or glucocorticoids caused temporary decreases

rather than increases of milk production in cows. However, Roy (1947) reported that injection of ACTH increased milk production.

Folley and Young (1940) showed that a single injection of partially purified prolactin in cows did not increase milk production during the declining phase of lactation. Similarly Cotes et al. (1949) demonstrated that single injections of 40 mg of purified prolactin had no effect on the milk yield of lactating cows.

Meites (1957) and Macdonald and Reece (1961) reported that injections of growth hormone in rats had no galactopoietic effect although the mothers increased in body weight. Many workers (Cotes et al., 1949) (Donker and Petersen, 1951) (Donker and Petersen, 1952) (Chung et al., 1953) (Wrenn and Sykes, 1953) (Shaw, 1955) (Hutton, 1957) demonstrated that growth hormone is galactopoietic when administered to cows. Shaw (1955) suggested that exogenous growth hormone may increase the availability of milk precursors in the blood, increase the efficiency of milk secretion, and increase the amount of mammary tissue in the cow.

Using milk yield (stomach contents) during a 30-minute nursing period as an estimate of lactational performance, a series of experiments from the laboratory of C. W. Turner over the last decade indicated that growth hormone, thyroxine, prolactin, parathyroid hormone,

corticosterone and aldosterone increased milk secretion in the rat (Grosvenor and Turner, 1959abc) (Djojosoebagio and Turner, 1964ab) (Kumaresan and Turner, 1965) (Hahn and Turner, 1966) (Kumaresan et al., 1966). However, several of these reported stimulatory responses due to hormones such as growth hormone (Grosvenor and Turner, 1959a) and prolactin (Kumaresan et al., 1966) are in direct conflict with the results of others (Meites, 1957) (Talwalker et al., 1960) (Macdonald and Reece, 1961) who used litter weight gain as an estimate of lactational performance. The stomach contents procedure evaluates lactational performance for a half-hour period of time, whereas litter weight gain estimates performance over a much longer period of time. The apparent discrepancy between results from the Turner group and results from others appears to be due to the method of measuring lactation. If growth hormone causes a 40% increase in milk yield as suggested by Grosvenor and Turner (1959a), then the stimulation should be reflected in a sizeable increase in mammary nucleic acid content (a quantative index of functional activity of the target tissue). Such an experiment should resolve the question whether growth hormone exerts a galactopoietic effect on the rat mammary gland.

Milk-ejection Reflex

Ott and Scott (1910) were first to demonstrate the milk ejection reflex after an injection of unfractionated

posterior pituitary extracts. Gaines (1915) described in detail the similarity of response to natural milk ejection and the ability of oxytocin to cause milk let-down when anesthesia blocked normal milk ejection in the bitch. Ely and Peterson (1941) suggested that a neurohormonal reflex mechanism existed whereby afferent stimuli from the teats during suckling or milking caused the release of neurohypophyseal oxytocin. Using the contractile response of the estrogenized uterus to estimate reflex liberation of oxytocin, Fuchs and Wagner (1963) observed that suckling of one or two rabbit pups released only 1-2 mμ of oxytocin. However, suckling of an entire litter of rabbits (four pups) released 50-100 mμ of oxytocin.

The milk ejection reflex is partially dependent on afferent impulses originating from the sensory receptors of the mammary gland. The degree of dependency on these impulses to maintain milk production (synthesis and removal of milk) varies among species. Complete sensory denervation of the mammary gland had no detectable effect upon lactation in sheep (as cited by Grosvenor, 1964a) and goats (Denamur and Martinet, 1959ab, 1960) even when no supplemental oxytocin was given. In fact, Linzell (1960) transplanted entire goat mammary glands to the sides or necks of animals, which completely destroyed the peripheral nerve supply to the gland, and he noted

that milk secretion was 70 to 90 percent of that expected from glands *in situ*. In contrast severance of the spinal cord resulted in almost total cessation of milk synthesis in the cat (Beyer *et al.*, 1962) and rabbit (Mena and Beyer, 1963). However, lactation in spinal cord-transected rabbits is partially maintained if oxytocin is injected periodically, and further improvement occurs if ACTH or prolactin is administered with oxytocin (Mena and Beyer, 1963). Grosvenor (1964a) reported that oxytocin and prolactin injections in the spinal cord-sectioned rat partially maintained lactation.

Release and Synthesis of Hormones

Since prolactin and glucocorticoids are required to maintain milk synthesis in the spinal sectioned and in the hypophysectomized rat, it was of interest to determine how lactation and the suckling stimulus affects release and synthesis of hormones associated with lactation.

Pituitary prolactin concentrations during lactation exceeded those observed during the estrous cycle, pseudo-pregnancy, or pregnancy in rats (Reece and Turner, 1936) (Grosvenor and Turner, 1958) (Minaguchi and Meites, 1967). Hurst and Turner (1942) reported a marked rise in prolactin content of the pituitary glands of post-partum rats. It reached a peak on day 3 of lactation and declined precipitously thereafter. Grosvenor and Turner

(1958a) measured prolactin concentrations in pituitaries from rats which had been deprived of their litter for 10 hours. Prolactin concentration increased from day 2 to day 6 of lactation but it declined toward the end of lactation. These results agreed with the original observations of Reece et al. (1939).

Relative to chronic nursing stimulation, Meites and Turner (1942ab) reported that parturient rats and rabbits which were not suckled for the first week or 10 days post-partum contained less pituitary prolactin than suckled controls. Recently, Tucker et al. (1967b) observed that chronically nursed rats contained significantly more prolactin than non-nursed controls. In addition increasing chronic nursing intensity from two to six pups during lactation increased pituitary prolactin concentration in lactating rats (Tucker et al., 1967a).

Reece and Turner (1937a) provided the first direct evidence that the suckling stimulus caused prolactin release. They reported a decrease in pituitary prolactin concentration in rats when mother and pups were reunited for a 3-hour period following a 12-hour interval of non-suckling. Ligation of the galactophores did not prevent the decrease in prolactin due to suckling, which suggested that the suckling stimulus rather than removal of milk was the factor responsible for prolactin release (Reece and Turner, 1937b). Later, Grosvenor and Turner

(1957ab, 1958b), Grosvenor (1965), Grosvenor et al. (1965) and Moon and Turner (1959) showed that 30 minutes of suckling, following 10 hours isolation, were sufficient to induce a significant decrease in pituitary prolactin if applied during the first two-thirds of lactation, but failed to do so on day 21 of lactation. Grosvenor and Turner (1957ab) determined that one-half of the pre-suckling level of prolactin was restored within 2.5 hours post-suckling. Complete restoration was not accomplished by 9.5 hours post-suckling. However, a more comprehensive inventigation indicated that complete restoration was achieved within 8 hours after suckling (Convey, 1968). Grosvenor et al. (1967) reported that 2 minutes of suckling was as effective as 30 minutes in evoking the discharge of prolactin. Neither 2 nor 30 minutes of suckling by six pups induced a fall in pituitary prolactin if the preceding period of non-suckling was of 16 hours duration.

As previously described suckling can cause a release of prolactin from the anterior pituitary and of oxytocin from the posterior pituitary. It is not surprising that Petersen (1942) postulated a causal link between the two processes. He suggested that oxytocin from the posterior pituitary may be responsible for the rapid decrease in pituitary prolactin observed after suckling. Grosvenor and Turner (1958b) demonstrated that oxytocin injections 30 or 60 times physiological levels did not alter prolactin content in pituitaries of lactating rats. Likewise,

oxytocin did not affect the production of prolactin from anterior pituitaries cultured *in vitro* (Nicoll and Meites, 1962).

Benson and Folley (1956ab, 1957) reported that oxytocin retarded the involution of the secretory epithelium which occurs after removal of the young. They suggested that oxytocin stimulated secretion of prolactin from the anterior pituitary. These observations on maintenance of mammary structure were subsequently confirmed by others including Meites (1958, 1959), Ota and Yokoyama (1958), McCann *et al*. (1959) and Meites and Nicoll (1959). The results of Ota *et al*. (1962, 1965) indicated that administration of oxytocin preserved only the structural integrity of the mammary tissue. On the other hand, prolactin injections not only prevented structural involution but also maintained some functional activity of the tissue.

Since the above experiments were performed in intact rats, the possibility existed that oxytocin may have exerted some effect via the anterior pituitary. When oxytocin was injected with prolactin and ACTH into hypopysectomized lactating rats, milk secretion (based on histological ratings of the mammary tissue) was observed in 92% of the rats (Meites and Hopkins, 1961). In comparison, milk secretion was observed in only 21% of the rats that received only prolactin and ACTH. These results were

interpreted to mean that the favorable effects of oxytocin in retarding mammary involution were exerted directly on the mammary gland.

Using involution of the thymus as an index of increased ACTH secretion, Gregorie (1947) showed that suckling maintained ACTH secretion in ovarietomized rats. The results reported by Tucker et al. (1967a) indicated a trend for pituitary ACTH to decrease with advancing stages of lactation. Various measurements of adrenal corticol function such as adrenal weight, cholesterol and ascorbic acid content, and peripheral plasma levels of corticoids have been used to estimate changes in ACTH secretion of lactating rats. Reports on rats are contradictory as to whether adrenal weights increase or decrease during lactation. Bearn et al. (1960) indicated that during lactation there was a significant decrease in adrenal weight when compared with virgin controls at estrus, but there was no significant change when compared with controls at other stages of the estrous cycle. In contrast, Poulton and Reece (1957) reported that adrenal activity increased during lactation, based on the evidence that adrenal cholesterol concentrations were maintained at a low level throughout lactation. Anderson and Turner (1962) measured adrenal ascorbic acid levels during pregnancy and lactation in rats. Lower adrenal abcorbate levels found during early lactation were interpreted to

represent a high level of adrenocortical function. After day 6 of lactation adrenal function decreased to the level of virgin controls. However, there is some question as to the validity of using adrenal ascorbate measurements as an estimate of increased corticotropin release (Hedner and Rerup, 1962). To my knowledge, direct measurements of adrenal content of corticoids in the lactating rat during advanced lactation are not available.

Chronic suckling stimulation (12 pups/litter) during lactation caused a progressive increase in plasma corticosterone from parturition to day 12 of lactation. And these elevated levels were maintained to day 21 of lactation (Gala and Westphal, 1965). Concurrently, there was a decrease in corticoid binding globulin (CBG) activity of blood plasma from parturition to day 3 of lactation, and this low level was maintained until day 21. Furthermore, rats receiving a lower nursing intensity (4 pups/litter) had smaller plasma levels of corticosterone and greater CBG activity compared with more intensely nursed rats.

The ACTH activity of anterior pituitaries of goats and ewes declined significantly from pre-milking levels to a minimum value 30 minutes after milking (Denamur et al., 1965). Within 90 minutes post-milking, pre-milking levels were regained. In contrast, pituitary ACTH did not change after an 8-hour non-nursing period

followed by a 1-hour suckling period in rats (Taleisnik and Orias, 1966).

A significant depletion of growth hormone and thyroid stimulating hormone from the anterior pituitary, following an overnight non-nursing period with subsequent acute nursing stimulation, occurred in lactating rats (Grosvenor, 1964b, Grosvenor et al., 1968). Furthermore, varying the chronic nursing intensity from 0 to 2, 6, or 12 pups progressively decreased pituitary growth hormone content whereas mammary development and metabolic activity increased (Tucker and Thatcher, 1968). These results suggested that growth hormone and thyroid stimulating hormone secretion rates are probably enhanced during lactation.

Intensity of Nursing Stimuli and the Extension of Lactation

Milk secretion is essentially a continuous process whereas the act of suckling or milking is intermittent. It is therefore possible that the nursing stimulus triggers the release of additional amounts of the galactopoietic hormones to maintain mammary function for several hours. Maintenance of an intense nursing stimulation, which may increase the secretion of galactopoietic hormones, has been used in several studies to extend lactation.

According to Selye and McKeown (1934) regular replacement of young with foster litters prolonged lactation. With the use of foster litters, Nicoll and Meites (1959) partially maintained lactation to 70 days in rats. A steady decline in milk yield (litter weight gain) was evident in these animals. In marked contrast, Bruce (1958, 1961) observed in three rats almost constant lactational performance for periods up to one year when a frequent litter replacement regime was employed. Tucker and Reece (1963b) using litter replacements every 21 days maintained structural integrity but not functional integrity of the mammary gland. However, Moon (1962) noted that by day 28 of lactation, after replacement of original litters with foster litters on day 14 of lactation, there was a significant reduction in cell numbers (DNA) as well as in milk production.

MATERIALS AND METHODS

Experimental Animals and Design

Experimental animals were primiparous rats of the Sprague-Dawley strain. They were maintained at 24±1C and subjected to illumination between 5 AM and 7 PM. All rats received a 21.2% protein diet (Appendix I) and water *ad libitum*.

Experiment 1.--Lactational Performance of Intensely Nursed Rats During Extended Lactation

The purpose of this experiment was to determine if intensive nursing would maintain mammary cell numbers and secretory activity during an extended lactation. A second objective was to relate changes in mammary cell number and synthetic activity with prolactin and ACTH content in the pituitary.

On the third day of lactation thoracic teats of 48 rats were ligated, litter size adjusted to six pups, and mother rats weighed and assigned to one of four groups to be killed either on day 16, 20, 28, or 36 of lactation. Both 16-day-old original litters and foster litters were replaced every 4 days with 12-day-old foster litters. Cumulative litter weight gains were recorded between

days 13 and 16 of age for all litters. Final body weights of lactating rats were recorded at time of killing, and the nucleic acid content determined on six abdominal-inguinal mammary glands.

Twelve anterior pituitaries were collected from each of two additional groups of rats killed on either day 20 or 36 of lactation. Prolactin potency of the 12 pooled pituitaries of each group was estimated by the method of Reece and Turner (1937a). The ACTH potency of the two groups of pituitaries was determined by the method of Saffran and Schally (1965). Litter weight gain and nucleic acid content of mammary tissue were also determined in these two groups. In addition, lactic acid content of mammary glands was determined (Barker and Summerson, 1941) as another index of mammary function (Cowie and Folley, 1961). Results were analyzed by analysis of variance.

Experiment II.--Lactational Performance of Intensely Nursed Rats Injected with Oxytocin During Extended Lactation

The purpose of experiment II was to determine if oxytocin injections would increase lactational performance.

On the third day of lactation thoracic teats of 24 rats were ligated, litter size adjusted to six pups and mother rats weighed and assigned to one of two treatment groups. Oxytocin (Syntocinon Sandoz, Pharmaceuticals)

injections were initiated at day 16 of lactation and continued to day 32 of lactation. One unit of oxytocin was given three times daily at 8:00 AM, 4:00 PM and 12:00 PM. Controls received 0.1 ml of 0.85% sodium chloride. At day 16 of lactation original litters were replaced with 8-day-old foster litters and subsequent 16-day-old litters, at day 24 of lactation, were again replaced with 8-day-old foster litters. This litter replacement regimen was intended to maintain an intense suckling stimulus throughout the treatment periods. Litter weights were recorded daily during lactation. Cumulative litter weight gains were calculated from day 16 to 24 and from day 24 to 32 of lactation. All rats were killed on day 32 of lactation by decapitation and the six abdominal-inguinal glands removed, weighed, trimmed and stored in 0.25 M sucrose at -20C until analyzed for nucleic acid content. Body weights, anterior and posterior pituitary, adrenal, ovary, and uterus weights were recorded at autopsy.

Unless stated otherwise, these experimental details (teat ligations, litter size adjustments, litter replacement regimen, litter weight gains and routine autopsy procedures) were applicable in all subsequent experiments.

Experiment III.--Lactational Performance of Intensely Nursed Rats Injected with Hydrocortisone Acetate (H), Prolactin (P), and Growth Hormone (G) During Extended Lactation

The purpose of Experiment III was to determine if the exogenous administration of hydrocortisone-21-acetate (Sigma Chemical Co.), NIH-P-S8 ovine prolactin, and NIH-GH-S8 ovine growth hormone singularly or in all possible combinations would maintain milk synthesis during extended lactation.

Sixty-three rats were divided among eight treatments (H, P, G, H and G, H and P, P and G, H and P and G, saline) in a 2X2X2 factorial experiment. All hormone injections were initiated at day 16 of lactation and continued daily to day 32 of lactation (autopsy). Injections were given daily at 8:00 AM and 5:00 PM. The total daily doses injected were 0.5 mg of H, 2 mg of P and 1 mg of G. Controls received 0.85% sodium chloride. Due to a limited amount of available prolactin there were unequal numbers of rats in the treatment groups. Consequently, a least squares statistical analysis of the data was performed.

Experiment IV.--Lactational Performance of Intensely Nursed Rats Bearing Isotransplanted Pituitaries During Extended Lactation

Experiment IV was conducted to verify that prolactin was not limiting to lactational performance.

On the third day of lactation, five anterior pituitaries from mature female rats were transplanted under the left kidney capsule of each of 14 lactating recipient rats. Kidneys of sham-operated controls (11) were manipulated surgically in a manner similar to that of rats receiving pituitary transplants. Both groups of rats were killed on day 32 of lactation.

Experiment V.--Lactational Performance of Intensely Nursed Rats Injected with 9-Fluoroprednisolone Acetate (Predef) During Extended Lactation

In an initial experiment, injections of Predef (Upjohn Co.) were given twice daily from day 16 to 32 of lactation at doses of either 10 µg, 50 µg or 100 µg per day. The numbers of rats in the 10 µg, 50 µg, 100 µg and saline injected control groups were 9, 10, 8 and 10, respectively.

A second experiment was conducted in which the doses of Predef were varied during the injection period. In one group of rats, Predef was injected at a daily dose of 50 µg from day 16 to 23, and at 100 µg from day 24 to 32 of lactation. A second group of rats received saline injections (0.85%) between day 16 and 23 and 50 µg of Predef daily between day 24 and 32 of lactation. A third group (controls) received saline injections (0.85%) throughout the 16-32 day interval. At

the time of autopsy the livers of rats from the first and third groups were weighed and prepared for subsequent *in vitro* incubations. *In vitro* incubations were conducted to evaluate the ability of the liver of Predef- and saline-treated rats to metabolize cortisol. The procedural details for liver preparations and *in vitro* metabolism studies will be outlined in a subsequent section (page 34).

Experiment VI.--Measurement of Adrenal Function and Corticoid Binding Globulin (CBG) Activity of Intensely Nursed Rats During Extended Lactation

To determine if adrenal function and CBG activity change during lactation, 20 rats per group were killed on day 16, 24 and 32 of lactation. An additional group of 20 virgin rats was killed in metestrus to determine if the experimental procedures resulted in resting plasma levels of corticosterone comparable to that reported in the literature. Rats were maintained at 24C±1C and grouped (4 per cage) for at least 2 weeks under conditions of controlled lighting (fluorescent illumination from 7:30 AM to 8:30 PM) in an isolated room. After the 2-week conditioning period, female rats were co-habited with male rats. When diagnosed pregnant by palpation rats were housed in individual cages.

Routine rat room maintenance and experimental manipulations were carried out between the hours of

1:00 and 4:00 PM. Vaginal smears were taken from the virgin rats daily. Rats were killed by decapitation within 10 seconds from the time the cage was first handled. In order to reduce plasma corticosterone variations due to circadian rhythm, rats were decapitated between 8:00 and 8:30 AM. Trunk blood was collected in heparinized 50 ml beakers, transferred to 15 ml centrifuge tubes and centrifuged at 12,350xg for 10 minutes at 5C. Plasma was frozen at -20C until analyzed for corticosterone. Adrenal glands were trimmed of accessory fat and connective tissue, and frozen in 2 ml of homogenization fluid (0.85% NaCL in 20% ethanol) for subsequent corticosterone analyses.

Corticosterone concentrations in individual rat plasma samples were measured by the method of Silber et al. (1958). Corticosterone determinations of adrenal glands from each rat were determined by the procedure of Moncloa et al. (1959). Gel filtration was used to measure CBG activity as described by Doe et al. (1964). Estimates of CBG activity were made on plasma samples pooled within treatments.

Biochemical Parameters Measured in Mammary Glands

The six abdominal-inguinal mammary glands were removed, dissected free of extraparenchymal fat and connective tissue and weighed. The glands were covered

with ice-cold 0.25 M sucrose and stored at -20C for subsequent analyses. At the time of analysis the samples were thawed at 4C. The mammary glands were homogenized for 2 minutes in distilled water at 4C to produce final concentrations of 50 mg of mammary tissue per ml for subsequent analysis.

The analytical procedure for nucleic acids was based on modifications of the Schmidt and Thannhauser (1945) procedure described by Tucker (1964):

1. Two ml of the 100 mg mammary gland homogenate was pipetted into a 16 ml polypropylene centrifuge tube. Ten ml of 95 per cent ethyl alcohol was added, the tube capped and shaken at room temperature for 12 hours.
2. The tube was then centrifuged at 35,600xg for 15 minutes at 5C and the supernatant fluid discarded.
3. Two ml of anhydrous ether was added and mixed thoroughly with the tissue residue and centrifuged as above. Supernatant material was discarded and the tissue residue dryed in a fume hood to form a firm tissue pellet.
4. Ten ml of methanol: chloroform (2:1) was added to the sample pellet, shaken for 24 hours, centrifuged and the supernatant fluid discarded.

5. Ten ml of anhydrous ether was added, shaken at room temperature for 12 to 24 hours, centrifuged and the supernatant fluid discarded.
6. Five ml of ice-cold 10% trichloracetic acid (TCA) was added to the residues of the mammary gland, mixed, centrifuged and the supernatant fluid discarded. This step was repeated.
7. Five ml of ice-cold 75% ethanol saturated with sodium acetate was added, mixed, centrifuged and the supernatant fluid discarded. Two ml of 1N potassium hydroxide was pipetted into each sample, the tubes capped with rubber stoppers and placed in a 37C oven for 15 hours.
8. The tubes were cooled in ice water, 0.3 ml of ice-cold 6N hydrochloric acid and 5 ml of ice-cold 10% perchloric acid (PCA) added, mixed, centrifuged and the supernatant fluid (RNA fraction) of the mammary homogenate saved.
9. Five ml of ice-cold 5% PCA was added, mixed, centrifuged and the supernatant fluid saved and pooled with the previous RNA fraction. This step was repeated.
10. The combined supernatant fluids (RNA fraction) of the mammary homogenates were brought up to 20 ml with 5% PCA and mixed. One ml of the RNA fraction was then pipetted into a clean 16 ml test tube containing 2 ml of 5% PCA

and 3 ml of freshly prepared orcinol reagent (Appendix II) was added. The tube was then capped and boiled for 30 minutes. Color development was measured in a Beckman DB spectrophotometer (Beckman Instrument Co.) after 15 minutes at 670 mμ. The RNA content of the sample was calculated from a standard curve derived from pure yeast RNA (Worthington Biochem. Corp.).

11. Five ml of ice-cold 5% PCA was added to the precipitate of the mammary gland sample from step nine, mixed, incubated at 70C for 15 minutes, cooled to 5C, centrifuged and the supernatant fluid (DNA fraction) poured into 25 ml graduated test tube.
12. Five ml of ice-cold 5% PCA was added to the precipitate, mixed, centrifuged and the supernatant fluid poured into the 25 ml graduated test tube. This step was repeated.
13. The supernatant fluid (DNA) from the mammary gland homogenate was brought up to 25 ml with 5% PCA in a graduated test tube. The optical density was read in a Beckman DB spectrophotometer (Beckman Instrument Co.) at 268 mμ, and the DNA content of the sample was calculated from a standard curve derived from pure highly polymerized DNA (Worthington Biochemical Corp.).

The Barker and Summerson (1941) chemical assay was used to measure the lactic acid content of the mammary gland. The following procedure was adopted:

1. Mammary gland homogenate containing 200 mg of tissue (4 ml) was pipetted into a 25 ml test tube containing 2 ml of 20 per cent $ZnSO_4 \cdot 7H_2O$ and 8.32 ml of 0.3N $Ba(OH)_2$. The contents of the tube were mixed and allowed to stand for 30 minutes, re-mixed and centrifuged at 85xg for 15 minutes at room temperature.
2. Eight ml of supernatant fluid were pipetted into a tube containing 1 ml of H_2O and 1 ml of 20 per cent $CuSO_4$. One gram of powdered $Ca(OH)_2$ was added to the tube.
3. The test tube was sealed with parafilm, mixed and allowed to stand for 30 minutes at room temperature with intermittent shaking. After centrifugation 1 ml of supernatant fluid was pipetted into a 25 ml test tube. Six ml of ice-cold H_2SO_4 was added slowly from a buret to the tube which was held in an ice bath.
4. The test tube was boiled 5 minutes and then immediately cooled in ice water. A 0.05 ml volume of 4% $CuSO_4 \cdot 5H_2O$ and 0.10 ml of 1.5% p-hydroxydiphenyl in 0.5% NaOH was added to

the tube and mixed immediately. The contents were then heated at 30C for 30 minutes with shaking at 15-minute intervals. After the 30-minute incubation, the contents were boiled in a water bath for 90 seconds and cooled to room temperature.

Optical density was read in a DB spectrophotometer (Beckman Instrument Co.) at 565 mμ. A standard curve was obtained by taking different concentrations of lactic acid (Sigma Chemical Co.) standard (in a 4 ml volume) through the same steps as the unknown sample starting at step one.

Bioassay of Pituitary Hormones

The 12 rat anterior pituitaries, from each of two groups of rats which were killed on either day 20 or 36 of lactation and stored at -20C, were thawed and homogenized in a Potter-Elvehjem homogenizer in 10 ml of 0.85% NaCl in pyrogen-free distilled water. The volume of the homogenate was adjusted to a final concentration of 4 mg of rat pituitary per ml. Homogenates were centrifuged for 15 minutes at 600xg at 5C and supernatant fluids used in bioassays for prolactin and ACTH.

Prolactin

Prolactin potency was assayed in White King pigeons by the method of Reece and Turner (1937a). Pigeons of both sexes were obtained from Cascade Squab Farm, Grand

Rapids, Michigan, at 5-8 weeks of age and housed in a room artificially illuminated between 6 AM and 8 PM. They were fed mixed grain and water ad libitum. The breast feathers were plucked on the day following arrival and pigeons were used for bioassay 2 days later.

Low doses of pituitary homogenate (0.1 mg/day) and standard NIH-P-S_6 ovine prolactin (0.25 µg/day) were injected into right and left crop sac areas, respectively, of 8 birds. High doses of pituitary homogenates (0.4 mg/day) and standard prolactin 1 µg/day) were injected similarly into 8 additional birds. Total doses injected over a 4-day period for the low and high doses of pituitary homogenate were 0.4 mg and 1.6 mg, respectively. Comparable 4-day total doses for standard prolactin were 1 µg and 4 µg.

The test materials were injected intradermally in a 0.1 ml volume with a 1.0 ml tuberculin syringe and a 27 gauge needle. Twenty-four hours after the last injection pigeons were decapitated, crop sacs removed and the area of the response rated visually in terms of Reece-Turner (R-T) units. A crop sac response of 2.1 cm in diameter constituted one R-T unit. The scale ranged from 0.0 to 4.0 R-T units, increasing in multiples of 0.25.

The prolactin potency of the unknown samples was computed from a parallel line assay comparison with

standard prolactin (Bliss, 1952). Calculation of the potencies and the criteria for the validity of an assay, such as the test for nonparallelism, lambda (λ), standard error and 95% confidence interval were performed based on the unknowns and standards assayed at one time.

ACTH Bioassay

The method of Saffran and Schally (1965) was used to assay ACTH potencies of the two groups of pituitaries.

Eight male adult rats were required per assay. Each rat was anesthetized with sodium pentabarbital at a dose of 5 mg per 100 g of body weight. Adrenals were removed from each rat and carefully freed of adhering fat with fine scissors. Each pair of adrenals was weighed and the glands placed into a petri dish containing some incubation medium (Appendix III). Each adrenal pair was cut into quarters with scissors; thus each donor male rat supplied eight quarters. One quarter from each of eight rats was placed on one of eight sectors of a filter paper circle previously moistened with incubation medium. Thus, each sector contained a quarter of an adrenal from each of eight rats--a procedure which minimized rat to rat variation in subsequent incubations.

The eight adrenal quarters in each sector were weighed together and placed in 25-ml Erlenmeyer flasks containing 1.5 ml of incubation medium. Each flask was flushed with a mixture of 95% O_2-5% CO_2 and shaken in

a water bath at 38C for 1 hour. At the end of the pre-incubation period flasks were removed from the bath and the medium aspirated. An additional 0.5 ml of incubation medium was added to each flask, mixed with the adrenal quarters and the medium discarded. An additional 1.4 ml quantity of incubation medium was added to each flask.

Two flasks were given the low dose (3 mμ/0.1 ml) of standard (ACTH, Mann Research Laboratory, Inc.) and two additional flasks the high dose (12 mμ/0.1 ml) of standard ACTH. The remaining two pairs of flasks received the low and high doses of pituitary homogenates. The low and high doses of pituitary homogenate for the rats lactating 20 days were 0.13 mg/0.1 ml and 0.4 mg/0.1 ml, respectively. The corresponding doses for the day 36 of lactation treatment group was 0.4 mg/0.1 ml and 1.6 mg/0.1 ml, respectively. Flasks were gassed with 95% O_2-5% CO_2 and replaced in the water bath for a 2-hour incubation at 38C. After this incubation the flasks were removed and a 1 ml aliquot of medium transferred from each flask to a corresponding 2 ml volumetric tube containing purified redistilled methylene chloride. Tubes were then stoppered, shaken and centrifuged at 480xg to separate the aqueous and methylene chloride phases. An aliquot of the lower methylene chloride phase was removed and optical density was measured in a Beckman

DB spectrophotometer (Beckman Instrument Co.) at 240 and 255 mμ against a blank which contained methylene chloride that had been extracted with 1 ml of incubation medium. The response at each dose was estimated by:

$$\frac{\text{optical density}_{240} - \text{optical density}_{255}}{\text{mg adrenal}} \times 10$$

ACTH potency and assay validity tests were calculated by the method of Bliss (1952).

In Vitro Metabolism of Cortisol by the Liver

To determine if livers from Predef-treated rats metabolized cortisol to a greater degree than livers from saline-injected controls, the following *in vitro* procedures were developed with the cooperation of Dr. Robert Cook (MSU Dairy Department).

Preparation of Liver Microsomes

At the time of necropsy, each liver was weighed and a 0.5 g section frozen at -20C for subsequent liver protein measurements. The remaining part of the liver was homogenized, for two 3-second intervals with a Waring Blender, in 4 volumes of 0.1 M phosphate buffer containing 20% glycerol (phosphate-glycerol buffer), pH 7.4 (Appendix IV). From the time of necropsy to the time of homogenization, livers were stored in ice-cold phosphate-glycerol buffer.

The homogenate was poured into 40 ml polypropylene tubes and centrifuged at 9000xg for 20 minutes at 5C. Tubes were removed and the supernatant fluid decanted and saved. The 9000xg supernatant fluid was then centrifuged in 10 ml tubes at 105,000xg for 60 minutes at 5C. The subsequent supernatant fluid was decanted and frozen at -20C in plastic capped tubes. Microsome pellets (105,000xg pellet) were rinsed once with the phosphate-glycerol buffer and the pellets transferred to a glass grinding vessel containing a volume of phosphate-glycerol buffer equal to 0.5 ml times the number of microsome pellets. The pellets were homogenized with a Teflon power driven pestle by two movements up and down the vessel. The microsome homogenate was then frozen in a screw cap plastic tube at -20C.

On the day of assay microsome preparations within treatments were pooled by pipetting 2 ml aliquots from each microsome preparation. The 105,000xg supernatant preparations within each treatment were also pooled.

In Vitro Incubation Procedure

Pooled liver microsomes and 105,000xg supernatant fluids from the Predef- and saline-treated rats were each incubated with 1 μM (1.0912 μCi/mg), 2 μM (0.548 μCi/mg) and 5 μM (0.219 μCi/mg) of cortisol (Nuclear-Chicago Corp. and Sigma Chemical Co.). Isocitric dehydrogenase, isocitric acid and NADP were used as a NADPH generating system during the incubation.

Prior to the assay 30 mls of 0.1 M phosphate buffer, pH 7.4 without glycerol (Appendix V), was gassed for 5 minutes with 95% O_2-5% CO_2. The incubations were carried out in 50 ml glass stoppered round bottom tubes.

The treatment flask contained:

2.5 mls of 0.1 M phosphate buffer
0.1 ml of $MgCl_2$ (15 µM)
0.1 ml of NADP (1.00 µM)
0.1 ml of isocitric acid (10 µM)
0.2 ml of isocitric dehydrogenase (3 units)
1, 2 or 5 µM of cortisol-cortisol-^{14}C
1.0 ml of pooled microsomes
1.0 ml of pooled 105,000xg supernatant

5.0 ml Total

The reaction blank flasks contained:

0.1 ml of $MgCl_2$ (15 µM)
0.1 ml of NADP (1.00 µM)
0.1 ml of isocitric acid (10 µM)
0.2 ml of isocitric dehydrogenase (3 units)
1 or 5 µM of cortisol-^{14}C-cortisol
4.5 ml of 0.1 M phosphate buffer

5.0 ml Total

The incubations were initiated by the addition of pooled microsomes and pooled 105,000xg supernatant fluid to the reaction flask. Both the reaction and blank flasks were gassed immediately for 30 seconds with 95% O_2-5% CO_2 and then stoppered. All flasks were incubated for 1 hour at 37C. Reactions were terminated by the addition of 1 ml of 1 N HCl. The incubation media and contents were directly shaken with 30 ml of methylene chloride for 30 minutes, and centrifuged at 1651xg for

15 minutes to separate the aqueous and methylene chloride phases. The aqueous phase was aspirated into a 10 ml graduated test tube and the volume recorded. The radioactivity in 0.2 ml of the aqueous phase was counted in a Model 6725 Nuclear Chicago liquid scintillation spectrometer using the scintillation fluid listed in Appendix VI.

Twenty-four ml of the methylene chloride extract was pipetted into a 50 ml conical centrifuge tube and taken to dryness under nitrogen in a sand bath at 45C in a fume hood. The dried residue was redissolved in 1 ml of ethanol. Fifty µl of the ethanol extract was spotted on Eastman silica gel thin layer chromatography plates (Eastman Chromatogram sheet 6060) and chromatographed in chloroform: methanol: water (90:10:1) for 45 minutes. The cortisol substrate spot was visualized with ultraviolet light and marked on the plate. Excluding the cortisol substrate spot, 1 cm strips were cut from the origin to the solvent front. Each strip, including the substrate spot, was placed directly in scintillation vials and radioactivity was quantified in 10 ml of the scintillation fluid described in Appendix VII.

The distribution of DPM from the origin to the solvent front was a quantative measurement of the amount of cortisol metabolized as well as the amount of polar or non-polar metabolites formed. Since the total amount of cortisol and its specific activity were known, direct

calculations were made of the amount of cortisol metabolites produced per hour. The amount of aqueous cortisol and its metabolites were also calculated by isotope dilution.

The amounts of liver, microsomal, and 105,000xg supernatant proteins were measured by the method of Lowry (1951) and used to calculate the specific activity of the enzyme system.

Fluorometric Assay of Plasma and Adrenal Corticosterone

A modification of the method of Silber et al. (1958) was used to measure corticosterone in rat plasma. The procedure of Moncloa et al. (1959) was followed to measure corticosterone in rat adrenals. A Turner model 111 fluorometer, equipped with filters providing narrow band excitation light at 470 mμ and emission at 530 mμ, was used in all corticosterone measurements. These procedures possessed the degree of sensitivity that was required to measure corticosterone concentrations in plasma of individual rats.

Plasma Corticosterone Procedure

Corticosterone-4-^{14}C (9148 DPM) (Nuclear Chicago Corp.) was added to and mixed with a 0.5 ml plasma sample. The sample was brought to a volume of 1 ml with double distilled water. Each sample was analyzed in duplicate in a heavy walled 15 ml ground glass stoppered centrifuge

tube. Six ml of redistilled methylene chloride was pipetted into the tube and the contents extracted by a gentle rotation for 3 minutes and then centrifuged at 1651xg for 3 minutes.

The upper steroid hormone-free aqueous layer was aspirated and discarded. Partial purification of the steroid extract was accomplished by a vigorous extraction of the methylene chloride for 30 seconds with 0.75 ml of cold 0.1 N NaOH. The samples were centrifuged at 1651xg for 3 minutes and the 0.1 N NaOH layer was discarded. Five ml of the original methylene chloride extract was transferred to a clean 15 ml glass stoppered centrifuge tube from which 1 ml was pipetted into a scintillation vial. Four ml of fluorescing reagent (Appendix VIII) was added to the 4 ml methylene chloride extract. Corticosterone was extracted into the fluorescing reagent by shaking the tubes vigorously for 1 minute. The mixture was then centrifuged at 1651xg for 2 minutes after which the methylene chloride layer was discarded. The reagent-hormone mixture was allowed to develop for 15 minutes from the end of the extraction before fluorometric determinations were recorded. A reagent blank, consisting of 1 ml of distilled water, was carried through the entire assay procedure for each assay. This served as a correction factor for background fluorescence. Plasma corticosterone concentrations were calculated from a

linear regression equation based on the fluorescence of 4 corticosterone standards (0.02 µg, 0.04 µg, 0.08 µg and 0.16 µg dissolved in 20 µl ethanol) which were analyzed with every assay. Extraction efficiency was determined from the radioactivity in the 1 ml of methylene chloride after being dried under nitrogen and counted in the liquid scintillation spectrometer.

To verify the relative accuracy of the fluorometric procedure for corticosterone quantitation in rat plasma, the fluorometric assay and the colorimetric blue tetrazolium reaction of Elliot et al. (1954) were used to determine the corticosterone concentration of a pooled plasma sample. For the blue tetrazolium reaction 50 ml of plasma was mixed with 25,000 DPM of corticosterone-4-^{14}C and extracted with 200 ml of methylene chloride for 6 minutes by gentle rotation in a separatory funnel. The methylene chloride extract was washed vigorously with 50 ml of cold 0.1 N NaOH and reduced to dryness in a vacuum evaporator. Using methylene chloride the residue was spotted on a silica gel thin-layer chromatography plate. The tube was rinsed with ethanol and these washings were also spotted on the plate. After two dimensional chromatography in hexane:ethyl acetate (5:2) and chloroform: methanol:water (188:12:1) the corticosterone spot was identified by autoradiography over night using Kodax Medical X-ray film (Eastman Kodax Co.). The area on the

silica gel plate containing the ^{14}C-corticosterone was scraped into a 12 ml centrifuge tube. Then the corticosterone was eluted from the silica gel with a double washing of chloroform:methanol (2:1). The eluate was evaporated under nitrogen at 45C, and the residue resuspended in 2.0 ml of ethanol, and corticosterone was measured by the blue tetrazolium reaction. Extraction efficiency was determined by measuring the radioactivity present in an aliquot of the 2 ml sample used to quantify the mass. Mass of corticosterone was corrected for losses of radioactivity.

Triplicate corticosterone analyses of 0.5 ml aliquots of the same plasma pool were determined by the fluorometric procedure outlined previously. The level of corticosterone per 100 ml of plasma, estimated by the fluorimetric method, was 21.55±0.11 µg (mean and standard error) and 23.66 µg using the blue tetrazolium method. Since the fluorimetric value was 91% of the blue tetrazolium value (considered 100%), I considered these values close enough to justify the use of the fluorimetric method.

The degree of precision of the fluorimetric assay was determined by repeated analyses of a pooled plasma sample from stressed rats. Six determinations were made on the plasma over a period of 2 weeks. The resultant estimate was 84.52±0.83 µg corticosterone/100 ml of plasma (mean and standard error of the mean).

Adrenal Corticosterone
Procedure

Adrenal glands were thawed and the two glands transferred to a glass grinding vessel for homogenization in 4 ml of 0.85% saline in 20% ethanol (homogenization fluid). The homogenate was transferred to a 20 ml test tube; and diluted to 10 ml with the homogenization fluid. Duplicate aliquots of the homogenate (0.5 ml and 1.0 ml) were assayed. The samples were pipetted into 15 ml glass stoppered centrifuge tubes. Corticosterone-4-^{14}C (9148 DPM) (Nuclear Chicago Corp.) was added to each sample and mixed thoroughly. Each sample was adjusted to 1 ml volume with the homogenization fluid.

Five ml of petroleum ether was added to each sample and the contents extracted vigorously for 1 minute and then centrifuged at 1651xg for 3 minutes. The upper petroleum ether layer was aspirated and discarded. Six ml of redistilled methylene chloride was added to the remaining aqueous phase and the contents extracted gently for 3 minutes by a rotary motion and then centrifuged for 3 minutes. The upper steroid hormone-free aqueous layer was then aspirated and discarded. The methylene chloride extract was extracted vigorously for 30 seconds with 0.75 ml of cold 0.1 N NaOH, centrifuged and the NaOH layer discarded. One ml of the methylene chloride was saved for measurements of radioactivity. Four ml of the methylene chloride extract was extracted with 4 ml of

fluorescing reagent (Appendix VIII) for 1 minute. After centrifugation for 3 minutes and aspiration of the methylene chloride fraction, the fluoresence of the reagent-hormone mixture was measured in the fluorometer 15 minutes after the extraction. A reagent blank of 1 ml of homogenizing fluid was carried through the entire procedure for each assay as a correction for background fluorescence. Adrenal corticosterone contents and concentrations were calculated from a linear regression equation based on the fluoresence of 4 corticosterone standards (0.02 µg, 0.04 µg, 0.08 µg and 0.16 µg) which were included in every assay. Extraction efficiency was determined from the radioactivity in the 1 ml of methylene chloride that was dried under nitrogen and counted in the scintillation counter.

Measurement of Corticosteroid-Binding Globulin (CBG) Activity

The procedure of Doe et al. (1964), used for the measurement of CBG activity in humans, was adopted for use in the rat plasma of the present study. This method has been used in many species for studies of CBG activity (Seal and Doe, 1965). In the present study inadequate plasma was available to measure CBG activity in individual rats. Consequently, plasma samples were combined within treatments by adding 0.5 ml of plasma from each rat to a common pool. The activity of CBG was determined in

duplicate for each plasma pool for rats killed at either day 16, 24 or 32 of lactation.

Prior to use, 35 g of Sephadex (Pharmacia Co.) G-50 medium was mixed with 400 ml of 0.2 M phosphate buffer pH 7.4 (Appendix IX). The suspension of Sephadex was equilibrated at 4C for 16 hours at which time the fines were decanted. Three columns (2x40 cm) were packed at 4C to a height of 28 cm.

Two ml of each plasma sample was mixed with: 40 µl of corticosterone-4-^{14}C solution (0.05 µc with a mass of 0.32 µg) and 40 µl of a unlabelled solution containing 3.24 µg of corticosterone. Each sample was equilibrated by gentle shaking at 37C for 1 hour and then equilibrated at 4C for 1 hour. After the equilibration periods, 1 ml of plasma from each sample was placed on separate Sephadex columns being careful not to allow the columns to run completely dry. To wash any residual plasma into the Sephadex two 1 ml aliquots of buffer were allowed to run into the column. Then the flow of elution buffer (0.1 M phosphate buffer pH 7.4 Appendix IX) was initiated. The 1 ml of equilibrated plasma layered on the column contained a total mass of 1.92±0.03 µg corticosterone. This total mass of corticosterone (measured fluorimetrically) is composed of an endogenous source as well as the mass from the added radioactive and from the added non-radioactive corticosterone. Approximately 3 ml fractions were collected by means of an automatic fraction

collector. When elution was complete, the Sephadex was resuspended in a flask with phosphate buffer and used to pack the columns the following day for duplicate analyses.

All elution fractions were monitored for protein by absorbancy at 280 mμ with a Beckman DB spectrophotometer (Beckman Instrument Co.). Elution of the protein and protein-bound radioactivity was accomplished in four fractions each with an absorbancy greater than 0.10. The fractions in this group were pooled and the volume adjusted to 25 ml with buffer. A 5 ml aliquot of the pooled eluates was extracted with 5 ml of methylene chloride for 15 seconds by vigorous mixing on a Vortex shaker. After a 3-minute centrifugation the aqueous upper phase was aspirated and discarded. An additional 5 ml of methylene chloride was added to the tube and the contents mixed vigorously for 15 seconds on a Vortex mixer. Following centrifugation for 3 minutes, 8 ml of the methylene chloride extract was transferred to a scintillation vial and the contents evaporated under a flow of air at 45C. The radioactivity was counted in a liquid scintillation spectrometer.

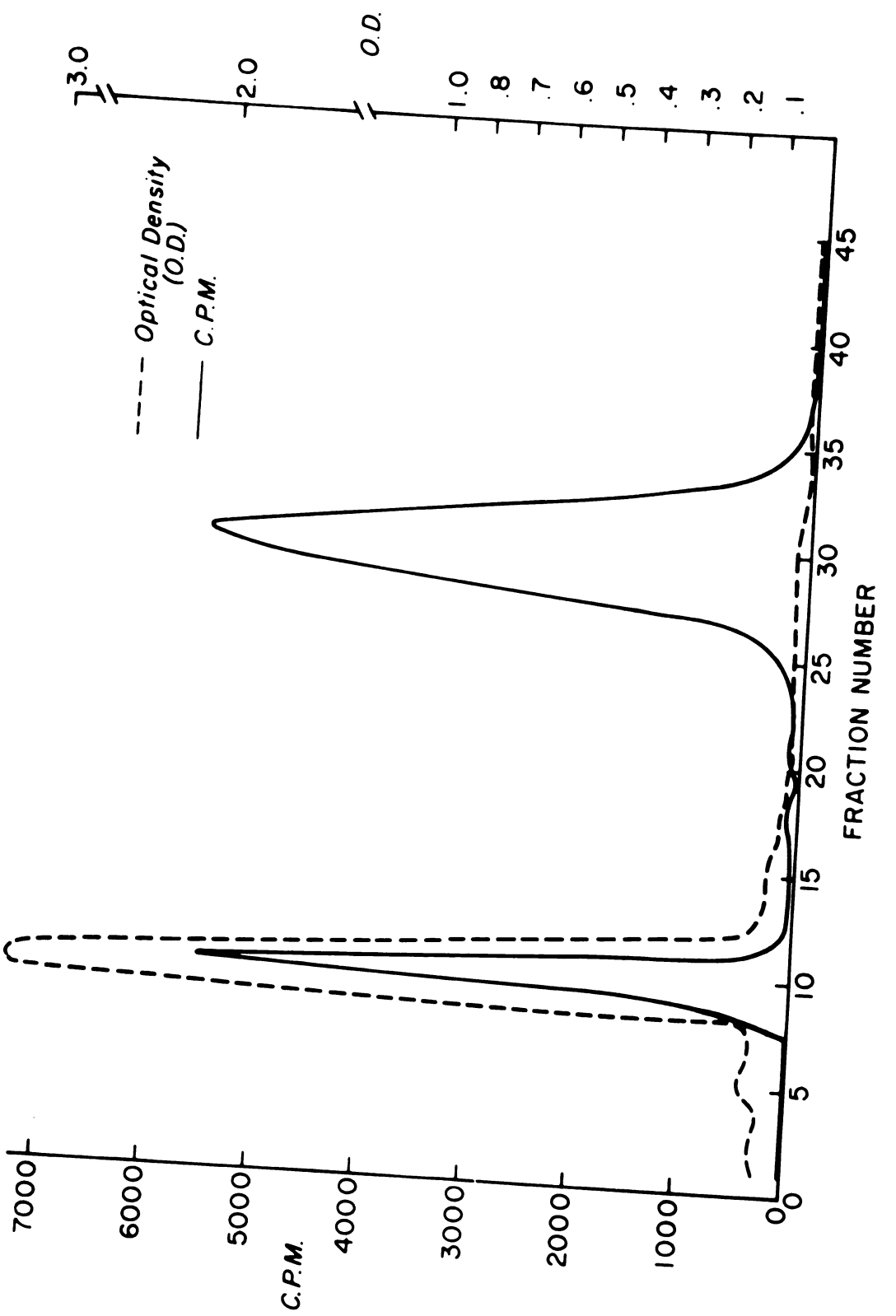
The total amount of radioactivity present in each of the original equilibrated plasma samples was determined by dilution of 0.1 ml with 5 ml of phosphate buffer. This diluted and equilibrated plasma was extracted and counted for radioactivity as described above.

The amount of protein-bound corticosterone per ml of plasma was obtained by multiplying the fraction of the added counts which were protein-bound times the corticosterone content of the sample (added plus endogenous). This value may be converted to µg bound per 100 ml by multiplying by 100. The calculation used is as follows:

$$\text{Total corticosterone } \mu g/ml \times \frac{\text{protein bound CPM}}{\text{total CPM}} \times 100 = \text{protein-bound corticosterone } \mu g/100 \text{ ml}$$

A plot of separation of the protein-bound and non-protein-bound corticosterone-4-^{14}C is shown in Figure 1. The use of gel filtration for assay of CBG content in plasma depends upon the separation of the protein bound fraction from the unbound fraction of corticosterone by molecular exclusion based upon the wide difference in molecular size of the two fractions. The radioactivity was eluted in two peaks. The first peak contained the plasma proteins as measured by ultraviolet absorbancy at 280 mµ and the protein bound hormone. The second peak of radioactivity represents unbound corticosterone-4-^{14}C whose elution from the column was retarded by virtue of its small molecular weight. The low amount of radioactivity between the two peaks demonstrates that there was very little dissociation of the corticosterone-4-^{14}C from the CBG during elution through the column.

Figure 1. Separation of protein-bound and non-protein-bound corticosterone 4-^{14}C during gel filtration.


Optical Density (O.D.)
C.P.M.
O.D.
3.0
2.0
1.0
.8
.7
.6
.5
.4
.3
.2
.1
C.P.M.
7000
6000
5000
4000
3000
2000
1000
0
0
5
10
15
20
25
30
35
40
45
FRACTION NUMBER

RESULTS AND DISCUSSION

Experiment I.--Lactational Performance of Intensely Nursed Rats During Extended Lactation

Final body weight, mammary gland nucleic acid content and litter weight gain (LWG) of intensely suckled rats during extended lactation are presented in Table 1. Final body weights of rats killed at day 28 or 36 of lactation were significantly greater ($P<0.01$) than body weights of rats killed at day 16 or 20. Body weights were not significantly different ($P>0.05$) between day 16 and 20 nor between day 28 and 36.

Total mammary DNA did not change significantly ($P>0.05$) between day 16 (36.2 mg) and 20 (37.2 mg). However, mammary DNA declined linearly ($P<0.01$) between day 20 and 36 (27.0 mg) of lactation. Mammary RNA did not change significantly ($P=0.10$) between day 16 (206.7 mg) and 20 (226.5), but a linear decline ($P<0.01$) of 54% was observed between day 20 and 36. Mammary RNA/DNA ratios were 5.7, 6.1, 4.8, and 3.8 for groups killed on day 16, 20, 28, and 36, respectively. Thus, changes in ratio paralleled very closely total RNA changes. Since body weight of mother rats increased during lactation ($P<0.01$), correcting for body weight merely magnifies the difference among groups for nucleic acid changes.

TABLE 1. Nucleic acid content of mammary glands and litter weight gain of intensely[a] suckled rats during extended lactation.

	Days of Lactation[b]			
	16	20	28	36
Number of rats	12	12	12	12
Body wt. (g)	242 ±5.2	253 ±6.0	259 ±2.8	273 ±3.8
Total DNA (mg)	36.2±1.4	37.2±1.2	32.0±0.9	27.0±0.2
Total RNA (mg)	206.7±8.9	226.5±9.9	154.4±19.9	104.3±6.2
RNA/DNA	5.7±0.1	6.1±0.1	4.8±0.2	3.8±0.2
Litter weight gain (g)[c]	33.5±2.1	39.3±1.8	20.8±2.6	3.9±1.5

[a]Litters 16 days old replaced with 12-day-old foster litters.

[b]Mean and SE of mean.

[c]Cumulative litter weight gains were recorded between days 13 and 16 of age for all litters.

Cumulative LWG for the 3-day period prior to sacrifice at 16, 20, 28, and 36 days of lactation were 33.5, 39.3, 20.8, and 3.9 g, respectively. The percent of litters that had a negative cumulative LWG prior to sacrifice at the four stages of lactation were 0%, 0%, 16%, and 33%, respectively. Thus, the level of milk production for the three days before day 28 or 36 of lactation was not sufficient to meet the minimum maintenance requirements of the litters.

A second experiment was designed to relate changes in mammary cell numbers and synthetic activity with prolactin and ACTH content in the pituitary. Mammary DNA, RNA, RNA/DNA, and 3-day cumulative LWG (Table 2) declined 18, 53, 43, and 114%, respectively, between day 20 and 36 of lactation. These declines are in close agreement with the declines observed in the initial study (Table 1). Total lactic acid content of mammary glands was 5.9 ± 0.3 and 5.0 ± 0.4 mg for the 20- and 36-day treatment groups, respectively. There was no significant difference ($P>0.05$) between mammary gland weights; therefore, lactic acid content per gram of mammary tissue was not significantly different between the two groups ($P>0.05$).

Pituitaries of rats killed at day 20 and 36 of lactation contained 0.018 and 0.015 IU of prolactin/mg of pituitary, respectively, and 22.5 and 7.3 milliunits of ACTH/mg of pituitary, respectively (Table 3).

TABLE 2. Nucleic and lactic acid content of mammary glands and litter weight gain of intensely[a] suckled rats during extended lactation.

	Days of Lactation[b]	
	20	36
Number of rats	12	12
Body wt. (g)	242 ±3	265 ±5
Total DNA (mg)	28.6±1.2	23.6 ±1.4
Total RNA (mg)	172.7±9.8	81.3 ±7.4
RNA/DNA	6.02±0.21	3.39±0.22
Total Lactic Acid (mg)	5.9 ±0.3	5.0 ±0.4
Litter weight gain[c] (g)	29.27±2.54	-4.06±2.51

[a]Litters 16 days old replaced with 12-day-old foster litters.

[b]Mean and SE of mean.

[c]Cumulative litter weight gains were recorded between day 13 and 16 of age for all litters.

TABLE 3. Prolactin and ACTH content of pituitaries of intensely[a] suckled rats during extended lactation.

Days of lactation	Avg. pituitary wt. (mg)	Prolactin (IU/mg)				ACTH (milliunits/mg)			
		Potency	SE	CI 95%	λ	Potency	SE	CI 95%	λ
20	9.0	0.018	0.011	.004-.037	0.40	22.5	9.4	5.7-84.8	0.21
36	9.2	0.015	0.008	.004-.030	0.36	7.3	1.0	5.0-10.8	0.08

[a]Litters 16 days old replaced with 12-day-old foster litters.

The losses of RNA during extended lactation were much greater in magnitude than losses in DNA, and may suggest that factors controlling protein synthesis limited milk production more than factors influencing cell numbers. Nonetheless the significant decline in DNA indicates the mammary gland has undergone a partial cellular involution although a strong sucking stimulus was maintained. The combination of reduced cell numbers and less synthetic activity of the remaining cells at least partially accounted for the marked decline in LWG. In agreement with the present study, Moon (1962) reported a decrease in mammary DNA during advanced lactation. In contrast, Tucker and Reece (1963b) observed that DNA content did not decrease significantly from 21 to 61 days of lactation, but that RNA content decreased significantly and this latter effect probably contributed to the decrease in milk production. The nursing intensity of six pups/six glands used in my experiment has been shown to be sufficient to stimulate mammary growth (DNA) to a maximal value on day 16 of lactation (Tucker, 1966). However, at day 36 mammary cell numbers declined to a level which was less ($P<0.01$) than day 16 of lactation. The lower nursing intensity used by Tucker and Reece (1963b) does not produce as much mammary growth as that obtained in the present study (Tucker, 1966). Perhaps one reason why Tucker and Reece (1963b) did not observe decreased levels of

mammary DNA may be that it is easier to maintain a basal level of cells than to maintain the abnormally large number of cells produced by intense nursing.

Other workers, (Nicoll and Meites, 1959), (Grosvenor, 1961), (Tucker and Reece, 1963b), and (Moon, 1962) using nursing stimuli of less intensity than in the present study, observed a decline in milk production of the rat with advancing lactation. However, Bruce (1961) reported, that lactation was prolonged by foster litters in three rats for 9-12 months, and the litters continued to gain at about the same rate throughout the experiment. The report of Bruce (1961) is in disagreement with the present experiment in which milk production virtually ceased at day 36 of lactation. The nursing intensity in my experiment was of a greater magnitude (six pups/six glands and litters exchanged every four days). Consequently, the great demands of the litters for milk in the current study may have been large enough to deplete the body of certain factors needed for milk production.

Lactic acid production increases in mammary glands when milk is not removed, but no increases occur when milk is removed (Cowie and Folley, 1961). The similar lactic acid content of mammary glands from rats killed at 20 and 36 days of lactation suggest that frequent litter exchange permitted adequate milk removal. These results further suggested that increased anaerobic

oxidation was not a limiting factor to maximal milk synthesis during extended lactation.

The intense nursing stimulus maintained pituitary prolactin content at least until day 36 of lactation. However, intense nursing failed to prevent a decline of 68% in pituitary ACTH between day 20 and 36 of lactation. This decline in ACTH during extended lactation agrees with the observations of Tucker et al. (1967a) in which pituitary ACTH content declined between day 1 and 16 of lactation. Furthermore, in the present experiment, declines in ACTH paralleled declines observed in mammary nucleic acid content and LWG during extended lactation. If reduced pituitary ACTH content reflects an actual decline in synthesis of the hormone, ACTH may be one of the hormonal factors that limits milk synthesis during extended lactation. A decrease in ACTH synthesis and release would cause a decrease in adrenocortical function with advancing lactation.

Experiment II.--Lactational Performance of Intensely Nursed Rats Injected with Oxytocin During Extended Lactation

As shown in Experiment I intensive nursing stimulation failed to maintain maximal milk production during extended lactation in the rat. Therefore, an experiment was conducted to determine if inadequate milk ejection limited milk production during extended lactation.

There were no significant differences ($P>0.05$) between oxytocin- and saline-injected rats in LWG from 16 to 24 or from 24 to 32 days of lactation (Table 4). However, there was a marked decline ($P<0.01$) in LWG from the first 8-day period (day 16 to 24) to the second 8-day period (day 24 to 32) for both treatments (47% for oxytocin- and 72% for saline-treated rats). The oxytocin-treated group contained 37% more DNA and 44% more RNA than the saline-treated group ($P<0.01$), but ratios of RNA/DNA did not differ ($P>0.05$) between treatments.

Oxytocin-treated rats had a greater ($P<0.05$) gain in body weight from day 3 to 32 of lactation than controls. There was no significant difference ($P>0.05$) between treatments with regard to weights of anterior and posterior pituitaries, adrenals or uteri (Table 5).

Although the oxytocin injections retarded mammary cell losses (DNA) and declines in metabolic activity (RNA), synthetic activity per cell (RNA/DNA) remained unchanged and these conditions were not sufficient to prevent a 47% decline in litter weight gain. The maintenance of mammary DNA is in agreement with the results of Ota *et al.* (1965) who reported that the administration of oxytocin preserved the structural integrity of the mammary tissue in weaned rats. However, they were unable to retard decreases in RNA content and RNA/DNA ratio with oxytocin. This contradiction to my results may be due to

TABLE 4. Nucleic acid content of mammary glands and litter weight gain of intensely[a] suckled rats injected with oxytocin between day 16 and 32.

	Oxytocin[b] 3 IU/day	Saline[b]
Number of rats	12	11
Litter weight gain[c] 16-24 days (g)	74.8±6.6	86.7±2.7
Litter weight gain[c] 24-32 days (g)	39.9±3.8	24.0±8.9
Total DNA (mg)	33.2±1.2	24.3±1.6
Total RNA (mg)	142.5±10.1	99.0±9.4
RNA/DNA	4.3±0.2	4.0±0.3

[a]Litters 16 days old replaced with 8-day-old foster litters.

[b]Mean and SE of mean.

[c]Cumulative litter weight gains were recorded between day 8 and 16 of age for all litters.

TABLE 5. Organ weights of intensely[a] suckled rats injected with oxytocin between day 16 and 32.

	Oxytocin[b] 3 IU/day	Saline[b]
Number of rats	12	11
Anterior pituitary (mg)	9.2± 0.6	9.4± 0.5
Posterior pituitary (mg)	2.9± 0.2	2.4± 0.2
Adrenal (mg)	51.0± 0.1	50.7± 1.8
Uterus (mg)	276 ±16	293 ±17
Body weight gain (g) 3-32 days	19.2± 3.4	8.2± 3.5

[a]Litters 16 days old replaced with 8-day old foster litters.

[b]Means and SE of mean.

differences between weaned and lactating rats. Kumaresan and Turner (1966) reported that oxytocin therapy increased LWG 13% between day 7 to 20 of lactation. They postulated that inadequate removal of milk from the gland partially limited lactation. However, my results indicate that oxytocin injections do not increase LWG in advanced lactation (16-32 days). The results of the present study and the observation in Experiment I, that lactic acid content did not change during extended lactation, suggest that the milk-ejection reflex is adequate during advanced lactation.

Experiment III.--Lactational Performance of Intensely Nursed Rats Injected with Hydrocortisone Acetate (H), Prolactin (P) and Growth Hormone (G)

Since neither intense suckling (Experiment I) nor exogenous oxytocin (Experiment II) influenced the decline in milk synthesis during advanced lactation the present experiment was designed to test if adrenal glucocorticoids, prolactin or growth hormone would prevent the declines in milk synthesis.

As shown in Table 6 and Figures 2 and 3 rats receiving any combination of H (H, HG, HP, HGP) had a larger ($P<0.01$) 16-24 day LWG response (95 g) than those rats not receiving H (S, G, P, GP) (80 g). The mean 24- to 32-day LWG response for the corticoid- and non corticoid-injected rats was 68.9 g and 38.7 g, respectively. As

TABLE 6. Nucleic acid content of mammary glands and litter weight gain of intensely[a] suckled rats injected with hydrocortisone (H), prolactin (P) and growth hormone (G) between day 16 and 32.

Treatment	LWG[bd] 16-24 days	LWG[bd] 24-32 days	DNA (mg)[b] per 100 g body wt.	RNA (mg)[b] per 100 g body wt.	RNA/DNA[b]
Saline (10)[c]	71.4±5.9	32.2±4.9	9.2±0.5	51.6±3.2	5.6±.2
H (10)	87.9±4.0	63.8±3.6	15.0±0.6	71.9±3.5	4.8±.2
H, G (11)	102.4±2.2	74.3±5.8	15.1±0.5	78.4±3.9	5.2±.2
H, P (6)	90.6±6.2	61.9±5.7	12.2±0.5	78.0±4.1	6.4±.4
H, G, P (5)	99.0±7.4	75.6±7.8	11.5±0.6	89.2±2.1	7.8±.4
G (10)	77.9±6.4	37.8±4.8	11.4±0.6	50.2±2.6	4.4±.2
P (6)	90.2±4.2	43.9±7.1	12.1±1.0	57.7±5.0	4.8±.4
G, P (5)	80.5±7.4	40.8±4.4	9.4±0.4	57.2±2.1	6.1±.2
16-day control (10)	---	---	14.4±0.8	82.0±5.4	4.7±.2

[a]Litters 16 days old replaced with 8-day-old foster litters.

[b]Mean and SE of mean. [c]Number of rats per group.

[d]Cumulative litter weight gains were recorded between days 8 and 16 of age for all litters.

Figure 2. Litter weight gain of intensely suckled rats injected with hydrocortisone (H), prolactin (P) and growth hormone (G) between day 16 and 24.

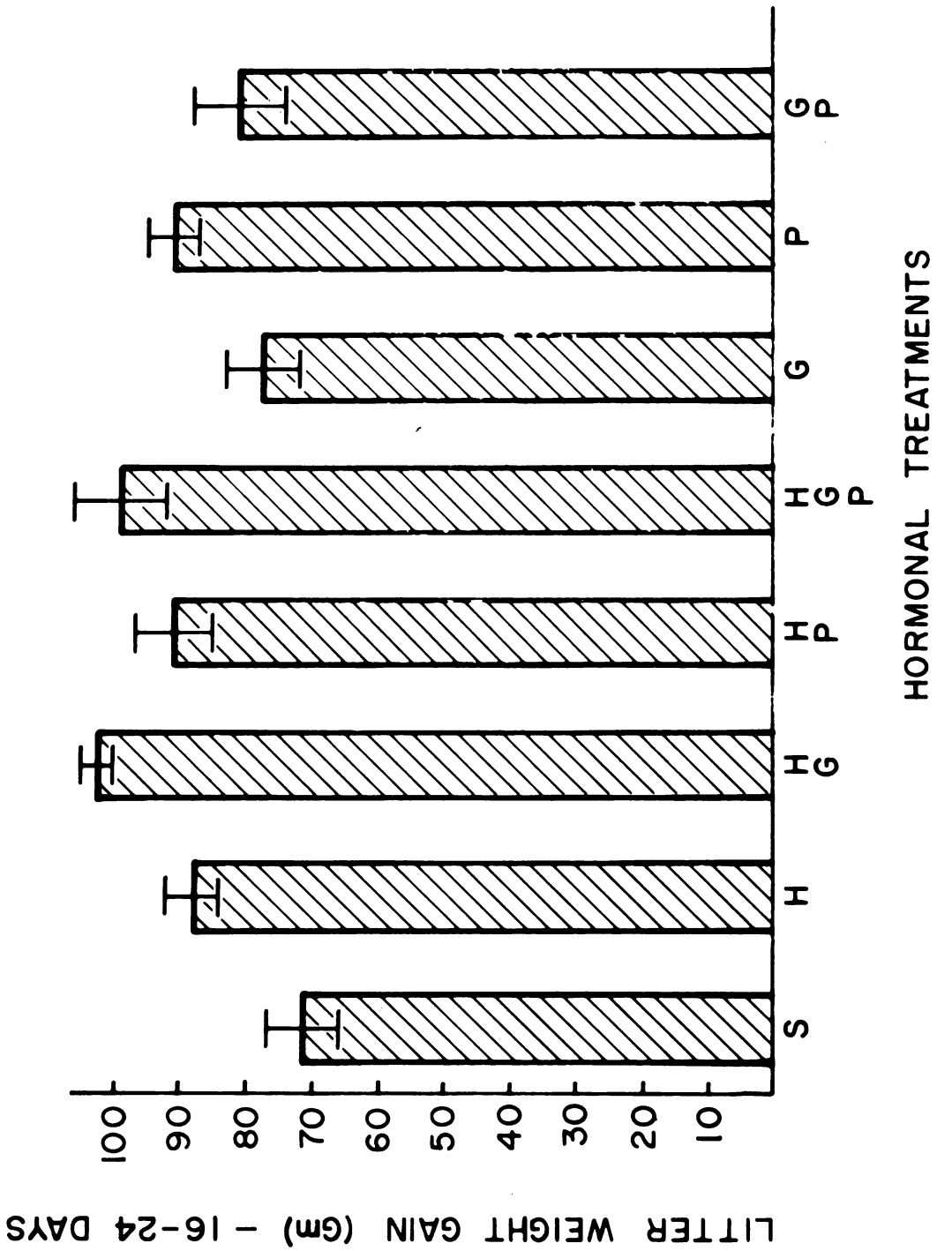
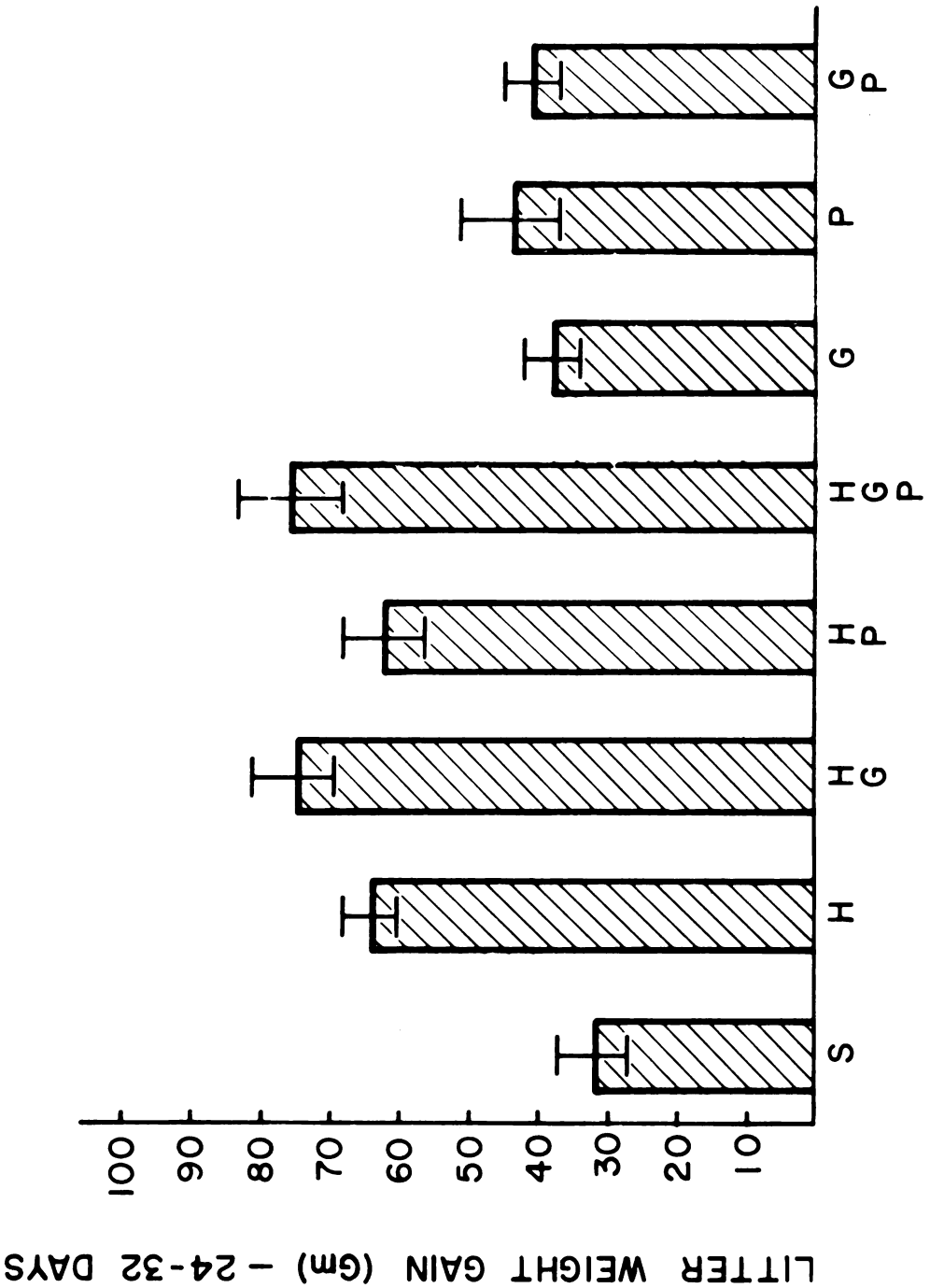

LITTER WEIGHT GAIN (Gm) – 16-24 DAYS
100
90
80
70
60
50
40
30
20
10
S
H
H G
H P
H G P
G
P
G P
HORMONAL TREATMENTS

Figure 3. Litter weight gain of intensely suckled rats injected with hydrocortisone (H), prolactin (P) and growth hormone (G) between day 24 and 32.


LITTER WEIGHT GAIN (Gm) – 24-32 DAYS
100
90
80
70
60
50
40
30
20
10
S
H
H G
H P
H G P
G
P
G P
HORMONAL TREATMENTS

previously stated the average LWG from day 16 to 24 for the H-treated rats was 95 g, whereas the average LWG between day 24 and 32 was 68.9 g. Thus, corticoid therapy increased LWG above saline-injected control values but failed to maintain completely persistent secretory activity throughout the lactation period.

The hormone treatments had a marked effect on the growth of the mother rats from day 18 to 32 of lactation (Table 7). Rats receiving G, or GP gained 30.6 and 36.2 g, respectively. Hydrocortisone-treated rats (H, HG, HP and HPG) exhibited no change or a slight loss in body weight during the treatment period (-4.4, 6.0, -9.5 and 3.0 g, respectively). A significant interaction ($P<0.05$) was detected between H and G in which H reduced the growth response due to the injection of G. Thus, glucocorticoids have the ability to divert nutrients for body growth to the mammary gland for the synthesis of milk.

Since the various hormones markedly influenced body weights, I arbitrarily adjusted the mammary nucleic acid data to 100 g of body weight. In addition to the eight groups of injected rats killed at day 32 of lactation, another group was killed at day 16 of lactation. Mammary DNA (Figure 4) declined from 14.4 mg at day 16 to 9.2 mg at day 32 of lactation for saline-injected control rats. Mammary glands from H and HG treatments contained 15.0 and 15.1 mg of DNA, respectively, comparable to the level

TABLE 7. Organ weights of intensely[a] suckled rats injected with hydrocortisone (H), prolactin (P) and growth hormone (G) between day 16 and 32.

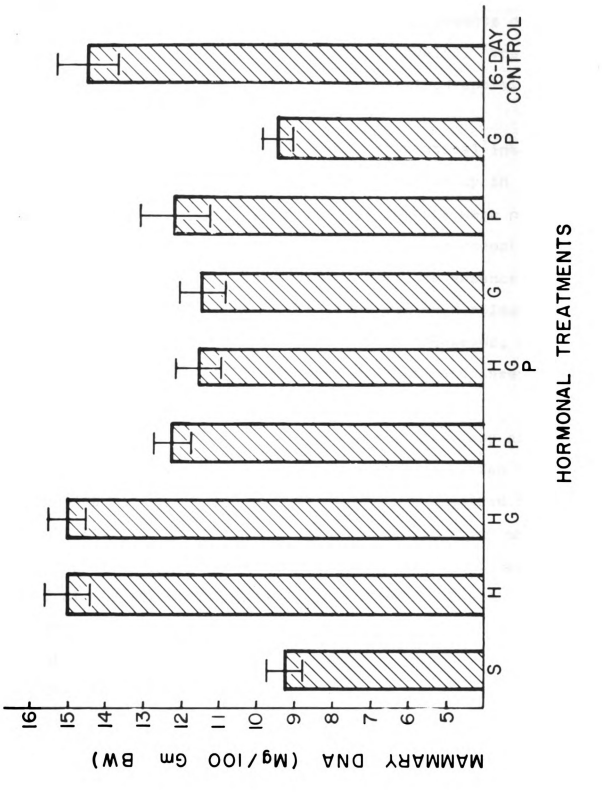
Treatment	Body wt.[b] gain (g) 18-32 days	Anterior[b] Pituitary wt. (mg)	Posterior[b] Pituitary wt. (mg)	Adrenal[b] wt. (mg)	Ovary[b] wt. (mg)	Uterus[b] wt. (mg)
Saline (10)[c]	6.7±3.0	9.3±0.4	3.0±0.4	49.5±2.5	65.0±6.5	256±12
H (10)	-4.4±3.7	9.0±0.4	2.5±0.2	48.0±3.5	62.6±3.3	285±17
H, G (11)	6.0±4.2	9.2±0.6	3.1±0.2	51.3±1.8	66.9±3.9	284±17
H, P (6)	-9.5±8.8	8.3±0.6	2.8±0.3	45.4±2.3	63.4±5.0	285±19
H, P, G (5)	3.0±5.4	8.6±0.5	2.4±0.2	55.4±1.2	64.1±6.8	239±20
G (10)	30.6±6.4	9.7±0.5	2.9±0.2	64.0±3.1	68.0±3.8	300±17
P (6)	6.8±6.5	9.5±0.4	2.7±0.2	54.7±3.6	66.1±2.9	233± 9
G, P (5)	36.2±3.8	8.6±0.6	2.2±0.1	61.5±1.9	46.5±2.6	308±43
16-day control (10)	---	10.2±0.6	2.6±0.2	61.9±2.3	65.2±3.2	229±10

[a]Litters 16 days old replaced with 8-day-old foster litters.

[b]Mean and SE of mean.

[c]Number of rats per group.

Figure 4. DNA content of mammary glands of intensely suckled rats injected with hydrocortisone (H), prolactin (P) and growth hormone (G) between day 16 and 32.

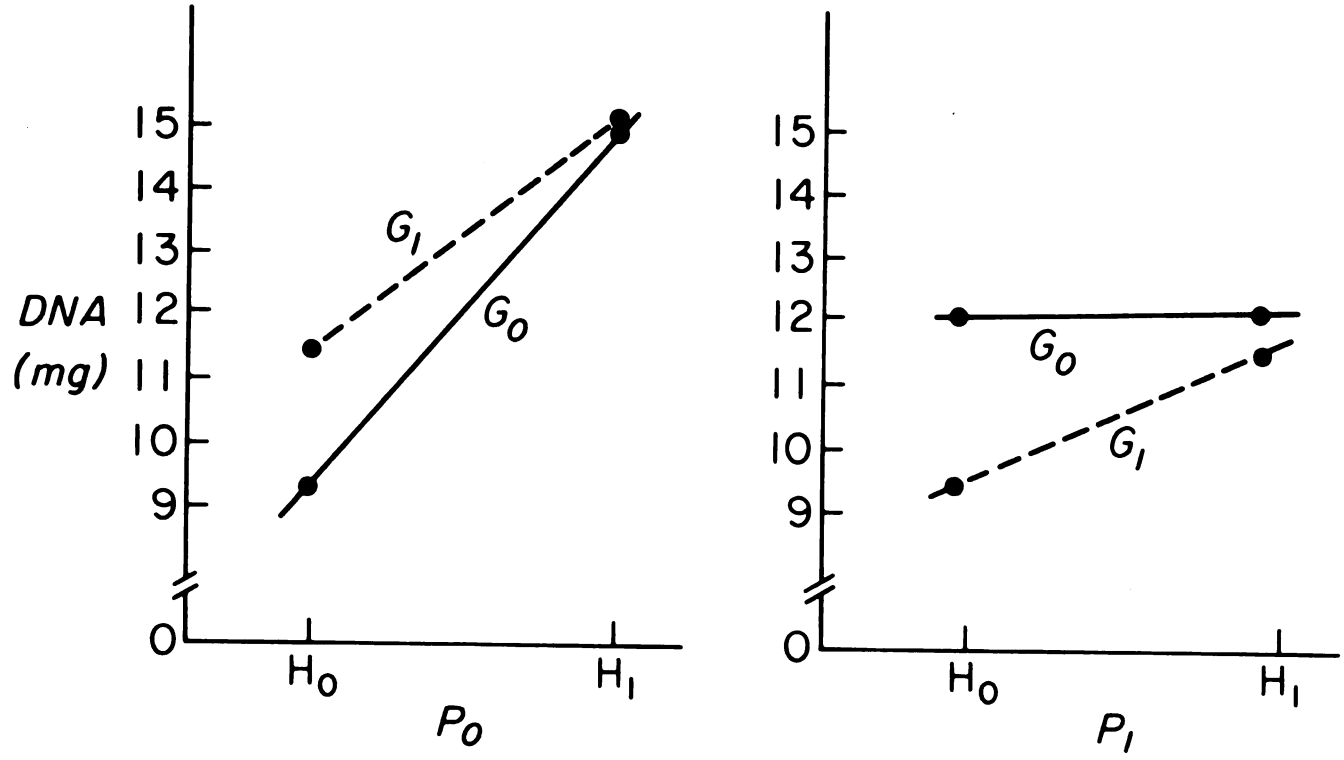

MAMMARY DNA (Mg/100 Gm BW)
16
15
14
13
12
11
10
9
8
7
6
5
S
H
H G
H P
H G P
G
P
G P
16-DAY CONTROL
HORMONAL TREATMENTS

in the 16-day control group. The mammary glands of rats given HP, HGP, and G and P contained similar amounts of DNA (12.2, 11.5, 11.4 and 12.1 mg, respectively). The GP- and the saline-injected rats each contained approximately 9 mg of DNA.

A significant ($P \tilde{=} 0.03$) three-way statistical interaction between H, G and P, represented graphically in Figure 5, showed that the three hormone factors were not independent in their effect on mammary DNA. Hydrocortisone acetate increased DNA with or without G in the absence of P. However, in the presence of P there was no H stimulation of mammary DNA unless G was present. However, the H stimulation of DNA with G was smaller than that observed in the absence of P.

Prolactin inhibited or reduced the stimulatory effects of H and G on DNA (Figure 4). When P was given in combination with H or HG, the DNA content was reduced from 15 mg to approximately 12 mg of DNA. Growth hormone and P given separately caused marginal increases in DNA content from 9 mg (S) to approximately 12 mg of DNA (G and P). However, when G and P were given together the response did not differ from that in saline-injected controls. As with DNA, the mammary RNA content of rats receiving any combination of H was greater ($P<0.01$) then RNA of non-corticoid injected rats (Table 6; Figure 6). In fact mammary RNA content of rats receiving G, P or GP was not

Figure 5. Interaction of hydrocortisone (H), prolactin (P) and growth hormone (G) on DNA content of mammary glands of intensely suckled rats injected between day 16 and 32.



H = HYDROCORTISONE – 21 – ACETATE

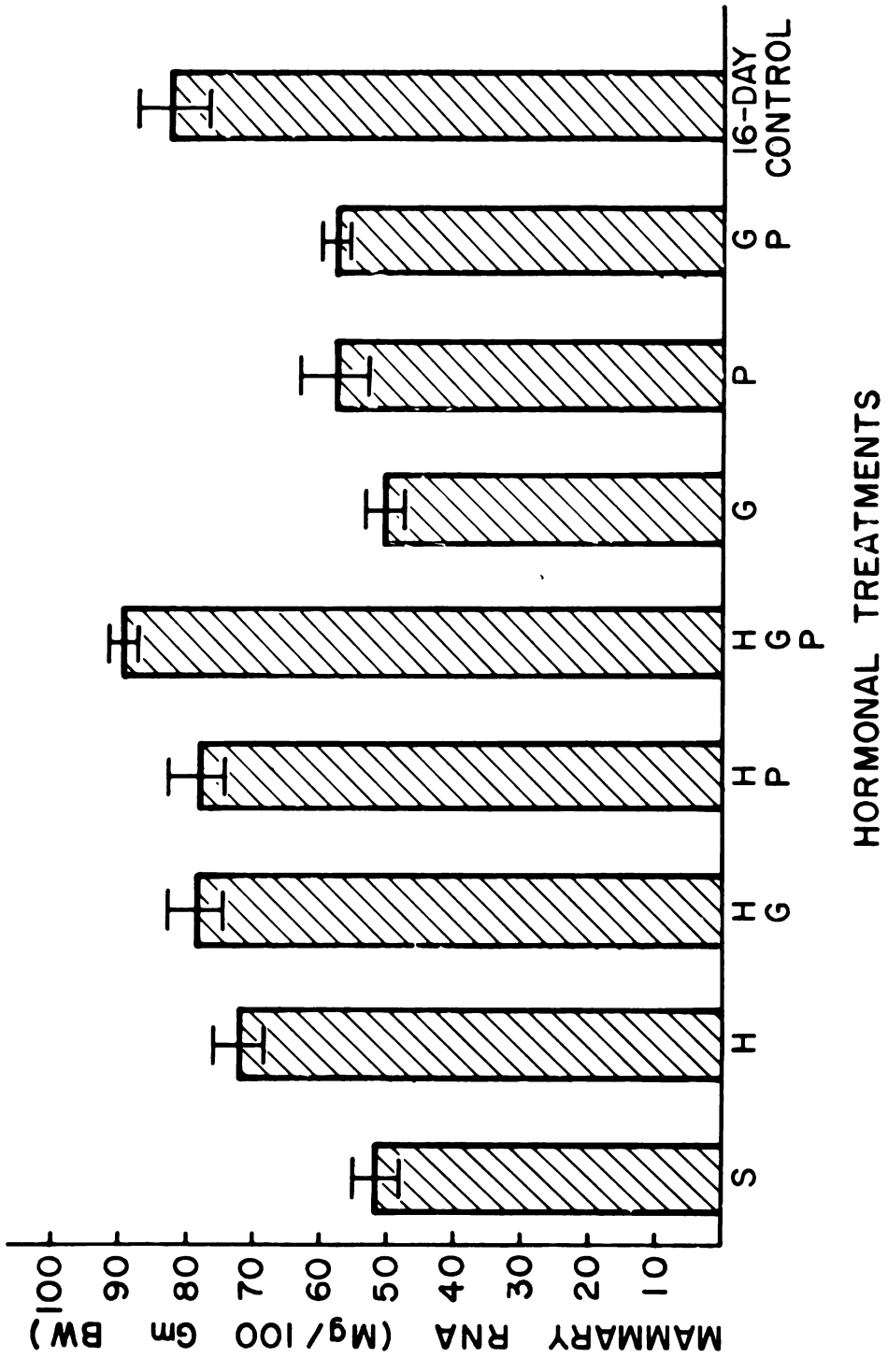
P = PROLACTIN

G = GROWTH HORMONE

1 = PRESENCE OF HORMONE

0 = ABSENCE OF HORMONE

Figure 6. RNA content of mammary glands of intensely suckled rats injected with hydrocortisone (H), prolactin (P) and growth hormone (G) between day 16 and 32.


MAMMARY RNA (Mg/100 Gm BW)
100
90
80
70
60
50
40
30
20
10
S
H
H G
H P
H G P
G
P
G P
16-DAY CONTROL
HORMONAL TREATMENTS

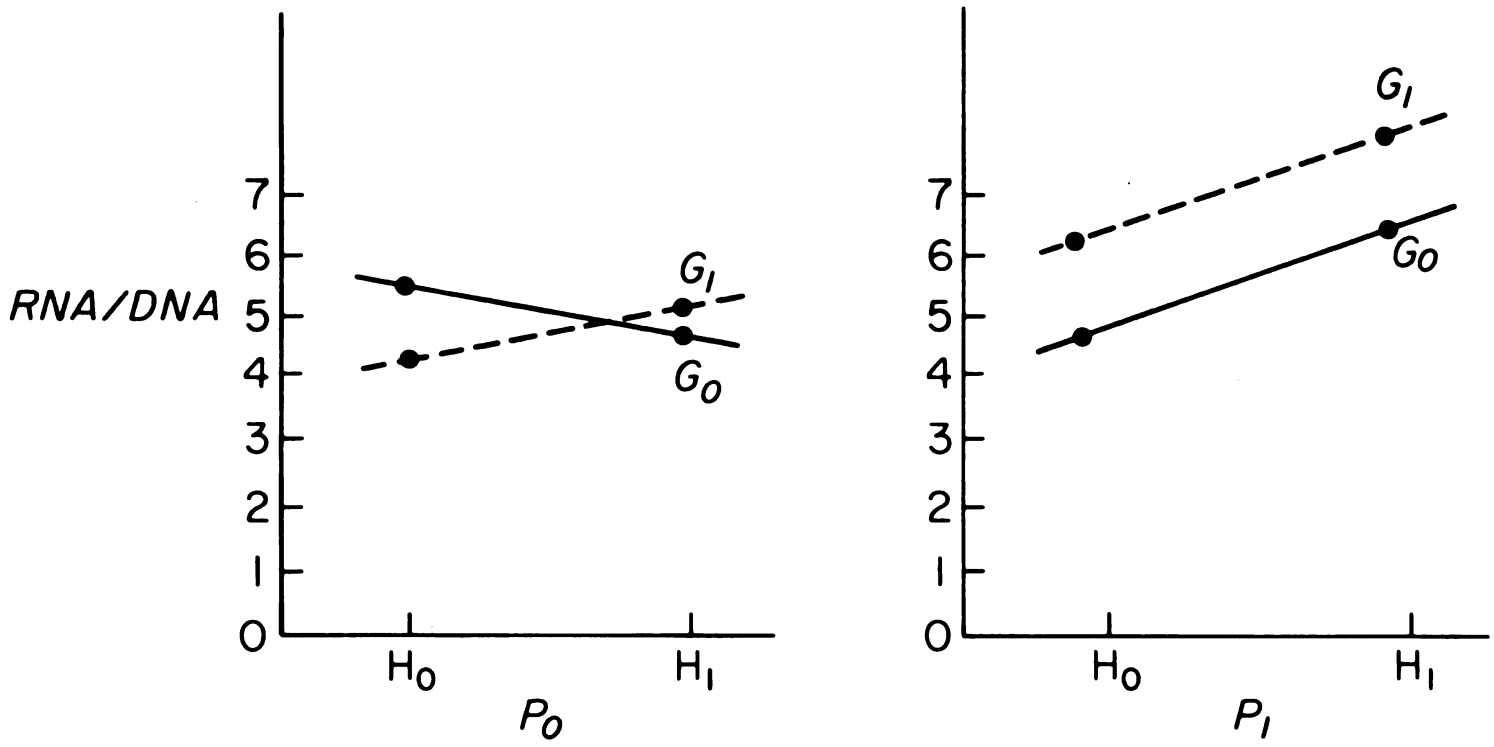
different from saline-injected rats. A two-way interaction which approached statistical significance ($P=0.08$) was detected between H and P. Contrary to its effects on cell numbers (DNA), P in combination with H and HG exerted a stimulatory effect on mammary RNA content.

A three-way interaction which approached significance ($P\tilde{=}0.08$) was detected for the RNA/DNA ratio in response to H, G, and P. Figure 7 is a graphical representation of the three-way interaction. Prolactin increased the RNA/DNA ratio when given with H either in the presence or absence of G. Hydrocortisone decreased the mammary RNA/DNA ratio in the absence of P and G, whereas in the presence of G and the absence of P it caused a slight stimulation of the ratio.

Among the corticoid-treated rats (H, HG, HP, HGP), the RNA/DNA ratios (Table 6) did not increase above saline controls (5.6) unless P was given with H (6.4) or HG (7.8). Consequently, even though the HP- and HPG-treated rats had reduced cell numbers (Figure 4), the synthetic activity per cell (RNA/DNA) was enough to maintain the 24 to 32 LWG (Figure 3) at a level about equal to that of the H- and HG-treated rats.

Adrenal weights (Table 7) of corticoid-treated rats were less ($P<0.01$) than those of non-corticoid treated rats. In addition rats receiving any combination of G had larger ($P<0.01$) adrenal weights than rats not injected

Figure 7. Interaction of hydrocortisone (H), prolactin (P) and growth hormone (G) on RNA/DNA ratio of mammary glands of intensely suckled rats injected between day 16 and 32.



H = HYDROCORTISONE – 21 – ACETATE

P = PROLACTIN

G = GROWTH HORMONE

I = PRESENCE OF HORMONE

O = ABSENCE OF HORMONE

with G. If paired adrenal weights are corrected for body size (mg/100 g body weight) differences in adrenal weights were eliminated. No significant differences ($P>0.05$) among hormone treatments were detected for weights of anterior and posterior pituitaries, ovaries or uteri.

The results of this experiment indicate that injections of glucocorticoids partially retard declines in LWG in the intact, lactating rat during advanced stages of lactation. These results are in agreement with those of Johnson and Meites (1958) and Talwalker et al. (1960) who demonstrated a galactopoietic effect of corticoids in early lactation. Growth hormone and prolactin were not galactopoietic during prolonged lactation in the rat. This is in agreement with other experiments conducted during early lactation (Meites, 1957) (Macdonald and Reece, 1961).

Hydrocortisone acetate maintained mammary DNA content during extended lactation. The interaction between H and P was such that P reduced the DNA response, but P stimulated the synthetic activity of the mammary cells when given with H. In fact, these results suggest that exogenous prolactin when given with a corticoid may reduce mitotic activity in the mammary gland.

Associated with the corticoid stimulation of milk synthesis (LWG) was a reduction in body weight gain during the treatment period. Corticoid injections were able to

reduce the body growth response associated with growth hormone injections. The metabolic effects of adrenal corticoids such as protein catabolism and translocation, mobilization of fatty acids from adipose tissue, and increased blood levels of glucose may be associated with the maintenance of milk precursors in the blood for milk synthesis.

Experiment IV.--Lactational Performance of Intensely Nursed Rats Bearing Isotransplanted Anterior Pituitaries During Extended Lactation

Since the prolactin in Experiment III was of ovine orgin and administered only twice a day, it was of interest to chronically administer rat prolactin to rats through the use of anterior pituitary isotransplants.

Vaginal smears of rats containing five anterior pituitary transplants or sham-operated controls indicated that both groups were in an anestrus state throughout lactation. The 16- to 24- and 24- to 32-day LWG responses for the rats with pituitary transplants were 79.4 and 40.4 g, respectively (Table 8). The comparable responses for the sham-operated rats were 83.3 and 24.5 g, respectively. There were no significant differences ($P>0.05$) in the 16- to 24- or the 24- to 32-LWG responses between the two groups of rats.

TABLE 8. Nucleic acid content of mammary glands and litter weight gain of intensely[a] suckled rats bearing isotransplanted pituitaries between day 3 and 32.

	Pituitary Transplant[b]	Sham Operation[b]
Number of rats	14	11
Litter weight gain[c] 16-24 days (g)	79.4± 5.9	83.3± 4.3
Litter weight gain[c] 24-32 days (g)	40.4± 5.3	24.5± 6.8
Total DNA (mg)	29.2± 1.1	24.0± 1.7
Total RNA (mg)	138.2±10.0	98.2±10.7
RNA/DNA	4.7± 0.2	4.0± 0.2

[a]Litters 16 days old replaced with 8-day-old foster litters.

[b]Mean and SE of mean.

[c]Cumulative litter weight gains were recorded between days 8 and 16 of age for all litters.

Anterior pituitary transplants significantly increased ($P<0.05$) mammary gland DNA content 22%, RNA content 41% and RNA/DNA ratios 18% over non-transplanted control rats. The larger percent increase of total RNA than DNA and the increased synthetic activity per cell indicated that mammary glands of rats bearing anterior pituitary transplants had a greater potential to secrete milk than the controls. But as shown in Table 8 it did not result in increased LWG.

Anterior pituitary and ovarian weights of rats with five anterior pituitary transplants were significantly less ($P<0.05$) than those of sham-operated controls (Table 9). There were no differences in body weight gains, adrenal or uterine weights.

There is substantial evidence that anterior pituitaries transplanted to the kidney capsule synthesize and release copious amounts of prolactin (Meites and Nicoll, 1966). In my experiment the decrease in pituitary weight and the increase in mammary DNA and RNA of rats having five transplanted pituitaries indicated that the transplanted pituitaries were functional during lactation. These data are in agreement with Sinha and Tucker (1968) who observed that prolactin secreted from five anterior transplants in virgin rats depressed the <u>in situ</u> pituitary weight and increased mammary DNA and RNA content. Malven and Sawyer (1966) observed that exogenous prolactin hastened corpora lutea regression in hypophysectomized

TABLE 9. Organ weights of intensely[a] nursed rats bearing isotransplanted pituitaries between day 3 and 32.

	Pituitary Transplant[b]	Sham Operation[b]
Number of rats	14	11
Body wt. gain 18-32 days (g)	6.2± 3.0	-1.0± 2.6
Anterior Pituitary (mg)	8.5± 0.3	9.6± 0.3
Posterior Pituitary (mg)	2.7± 0.2	2.8± 0.2
Adrenal (mg)	51.8± 1.7	54.0± 2.6
Ovary (mg)	64.9± 3.1	78.3± 5.2
Uterus (mg)	266 ±17	316 ±20

[a]Litters 16 days old replaced with 8-day-old foster litters.

[b]Mean and SE of mean.

rats. A decrease in ovarian weight was attributable to induced luteolysis. A possible explanation for the decrease in ovarian weights in my experiment is that prolactin secreted from the pituitary transplants caused luteolysis of the post-partum corpora lutea. Consequently, this regression may account for the decrease in ovarian weights.

In the present experiment, constant secretion of rat prolactin from five anterior pituitary transplants failed to elicit a galactopoietic effect in the lactating rat. This verifies the results of Experiment III in which injections of ovine prolactin were not beneficial to milk yield although mammary nucleic acid content was elevated. Similarly, Cowie et al. (1960) also reported that pituitary grafts under the kidney capsule had no effect on milk yield during early lactation. On the basis of Experiment III and IV, I concluded that the stimulatory effect of prolactin on cell numbers and synthetic activity of the mammary gland is simply not sufficient to maintain persistent milk production during advanced lactation.

Experiment V.--Lactational Performance of Intensely Nursed Rats Injected with 9-Fluoroprednisolone Acetate (Predef)

Although rats receiving hydrocortisone acetate were more persistent in their lactational ability than non-corticoid-treated animals (Experiment III), significant declines in lactational ability still occurred as

lactation advanced. The present experiments were designed to determine if the synthetic glucocorticoid, 9-fluoroprednisolone acetate (Predef) would minimize this decline.

The 16- to 24-day cumulative LWG responses of 105.8 and 115.0 g for rats given daily doses of 50 and 100 µg of Predef, respectively, were larger ($P<0.01$) than the 87.7 and 71.4 g LWG responses to a 10-µg dose of Predef or of saline, respectively (Table 10). The 10 µg dose caused a greater ($P<0.05$) LWG response than the saline injections. During the second 8-day period (24 to 32 days) the stimulatory effect of Predef on lactational performance is even more readily apparent. The 50 and 100 µg doses of Predef caused an approximate 124% increase ($P<0.01$) in LWG above values for saline-injected controls. In addition, the 10 µg dose caused a significant increase ($P<0.05$) in LWG over that of the saline control rats. However, in spite of the Predef there was a decline in LWG from the first to the second 8-day period of lactation.

Rats receiving injections of 50 and 100 µg of Predef had more ($P<0.01$) mammary tissue than either the 10 µg- or saline-treated rats. The 50 µg dose of Predef maintained mammary DNA at a level higher (39.9 mg, $P<0.01$) than that found in either the 10-µg (26 mg) or saline-control (24.1 mg) rats. However, the 100-µg dose

TABLE 10. Nucleic acid content of mammary glands and litter weight gain of intensely[a] nursed rats injected with 9-fluoroprednisolone acetate (Predef) between day 16 and 32.

	µg Predef/day[b]			
	Saline	10	50	100
Number of rats	10	9	10	8
Litter weight gain[c] 16-24 days (g)	71.4±5.9	87.7± 5.0	105.8± 5.6	115.0± 5.4
Litter weight gain[c] 24-32 days (g)	32.2±4.9	54.6± 5.8	71.2± 8.6	73.2± 4.2
Total DNA (mg)	24.1±1.3	26.0± 2.4	39.9± 2.3	28.8± 1.7
Total RNA (mg)	134.6±7.7	169.7±15.8	202.5±13.4	197.1±14.7
RNA/DNA	5.6±0.2	6.6± 0.3	5.1± 0.3	7.0± 0.7

[a]Litters 16 days old replaced with 8-day-old foster litters.

[b]Mean and SE of mean.

[c]Cumulative litter weight gains were recorded between days 8 and 16 of age for all litters.

(28.8 mg DNA) failed to maintain mammary cell numbers comparable to that obtained in the 50-µg treated rats (39.9 mg DNA). Changes in mammary RNA content for the various treatments paralleled the 24 to 32 day LWG responses. Rats receiving saline, 10, 50 or 100 µg of Predef contained 134.6, 169.7, 202.5 and 197.1 mg of mammary RNA, respectively. Rats treated with either 50 or 100 µg of Predef contained more ($P<0.01$) mammary gland RNA than rats receiving 10 µg of Predef or saline. The 10 µg dose of Predef increased ($P<0.08$) RNA content above the value for the saline-injected rats. Although, mammary cell numbers were less for the 100 µg treatment rats, the absolute quantity of RNA was the same for the 50 and 100 µg treatments. This probably contributed to the fact that the LWG responses were equivalent in the two groups of rats. Similarly, the increased quantity of RNA in the 10-µg treated rats at least partially accounted for the significant increase in the 24- to 32-day LWG when compared with saline-control rats. A comparison of the mammary gland nucleic acid changes that occurred among the various treatments gave some insight into the overall mode of action of the hormone at different doses. The 10 µg dose of Predef failed to significantly stimulate mammary gland DNA content ($P>0.05$), but RNA content was stimulated ($P\tilde{=}0.08$). Consequently, the synthetic activity of the remaining mammary gland

cells (RNA/DNA) was increased. In comparison, the 50 µg dose of Predef stimulated cell numbers (DNA) and total synthetic activity (RNA) of the mammary gland. However, the synthetic activity per cell (RNA/DNA) was reduced. These results suggest that the synthetic activity of the mammary cells is stimulated preferentially at the lower dose level, whereas the 50 µg dose maintained the structural integrity in addition to the synthetic activity of the mammary gland. The 100 µg dose of Predef appeared to overstimulate the mammary gland as reflected by a loss of cell numbers (DNA) and a higher synthetic activity of the remaining cells in comparison with the 50 µg treatment group.

Increasing the dose of Predef, from zero before day 16 to 100 µg between day 16 and 32 of lactation, caused a linear decline ($P<0.01$) in body weight gain from day 18 to 32 of lactation (Table 11). As was demonstrated in Experiment III, glucocorticoids have the ability to divert nutrients from body growth to the mammary gland for the synthesis of milk.

Adrenal weights from rats injected with 100 µg of Predef were smaller ($P<0.05$) than the adrenal weights of the 0-, 10- and 50-µg treatment rats. The 50-µg and 100-µg Predef treatment rats had smaller ($P<0.05$) posterior pituitary weights than the 10 µg or saline control rats. Perhaps Predef injections at these doses

TABLE 11. Organ weights of intensely[a] nursed rats injected with 9-fluoroprednisolone acetate (Predef) between day 16 and 32.

	µg Predef/day[b]			
	Saline	10	50	100
Number of rats	10	9	10	8
Body wt. gain 18-32 days (g)	6.7± 3.0	-1.4± 6.8	-13.0± 3.8	-35.0± 5.4
Anterior Pituitary (mg)	9.3± 0.4	10.1± 0.6	9.4± 0.4	9.1± 0.7
Posterior Pituitary (mg)	3.0± 0.4	3.0± 0.2	2.0± 0.1	2.4± 0.2
Adrenal (mg)	49.5± 2.5	56.5± 4.2	49.4± 1.6	41.2± 2.2
Ovary (mg)	65.0± 6.5	73.5± 3.8	71.6± 3.7	68.7± 6.3
Uterus (mg)	256 ±11.8	259 ±15.5	243 ±12	257 ±31

[a]Litters 16 days old replaced with 8-day-old foster litters.

[b]Mean and SE of mean.

exerted a feedback control on secretions from the posterior pituitary. Anterior pituitary, ovarian and uterine weights did not differ significantly ($P>0.05$) among the treatments.

Of the Predef doses administered, the 50 µg dose appeared to be optimum for maximal lactational performance since LWG was near maximum, mammary cell numbers were maintained, adrenal weights were not depressed, and body weight losses were not excessive during the treatment period. Nonetheless, Predef injections only partially retarded declines in milk synthesis. As previously shown, the LWG responses from 24 to 32 days of lactation were less ($P<0.01$) than the LWG from 16 to 24 days regardless of the dose of Predef injected. It became of interest to determine if Predef injections gradually altered corticoid requirements of the mammary gland with time. Perhaps, the initial corticoid injections in the present experiment induced enzymes that metabolized the injected hormone to a form that was less active and/or more readily excreted by the body. The following experiment was designed to determine if the observed declines in litter weight gain could be minimized by increasing the dose of Predef injected during advancing lactation to compensate for any increased losses. In addition, a preliminary *in vitro* study was conducted to measure any differences in cortisol metabolism by the liver which may have been induced by Predef injections.

The first group of rats were injected with saline from day 16 to 32. A second group of rats received saline injections from 16 to 23 days of lactation and 50 µg of Predef daily from 24 to 32 days of lactation. The third group of rats received 50 µg of Predef daily between day 16 and 23 and 100 µg between day 24 and 32. The 24- to 32-day LWG of the second and third groups increased 95% above that of saline controls (Table 12). Injections of 50 µg of Predef daily from day 24 to 32 of lactation resulted in as much LWG during this period as if the 50 µg were injected from day 16 to 23 and then the dose was doubled between day 24 and 32 (group three). Consequently, either a delay in the onset of injections to day 24 of lactation or an increase in the dose during the treatment period, eliminated the decline in LWG as lactation advanced.

The rats receiving Predef had more mammary gland DNA ($P<0.05$), more RNA ($P<0.01$) and greater RNA/DNA ratios than saline-injected controls. Rats in group three, which received the highest dose of Predef for the longest time period, had adrenal weights which were significantly less ($P<0.01$) than either groups one or two (Table 13). The blood level of glucocorticoids regulates the secretion of ACTH which in turn controls the functional status of the adrenal cortex. The depressed adrenal weights of rats in group three probably reflected suppressed ACTH

TABLE 12. Nucleic acid content of mammary glands and litter weight gain of intensely nursed[a] rats injected with 9-fluoroprednisolone acetate (Predef) between day 16 and 32.

Days of Lactation	16-23	24-32[b]	16-23	24-32[b]	16-23	24-32[b]
µg Predef	0	0	0	50	50	100
Number of rats	10		13		10	
Litter weight gain[c] 16-24 days (g)	81.1±4.8		89.0±5.4		92.5± 4.6	
Litter weight gain[c] 24-32 days (g)	42.8±3.5		83.0±5.1		83.7± 7.2	
Total DNA (mg)	28.3±1.2		32.8±1.8		31.2± 1.5	
Total RNA (mg)	140.7±6.6		206.2±9.0		180.4±13.3	
RNA/DNA	5.1±0.4		6.4±0.2		5.8± 0.2	

[a]Litters 16 days old replaced with 8-day-old foster litters.

[b]Mean and SE of mean.

[c]Cumulative litter weight gains were recorded between days 8 and 16 of age for all litters.

TABLE 13. Organ weights of intensely[a] nursed rats injected with 9-fluoroprednisolone acetate (Predef) between day 16 and 32.

Days of Lactation	16-23	24-32[b]	16-23	24-32[b]	16-23	24-32[b]
µg Predef	0	0	0	50	50	100
Number of rats	10		13		10	
Body wt. gain 18-32 days	9.1± 1.5		-18.3± 2.8		-19.7± 4.3	
Anterior Pituitary (mg)	10.0± 0.3		10.3± 0.5		9.6± 0.3	
Posterior Pituitary (mg)	2.5± 0.2		2.4± 0.2		2.2± 0.1	
Adrenal (mg)	49.0± 1.8		49.9± 1.8		42.0± 1.8	
Ovary (mg)	58.0± 4.5		67.9± 3.4		68.5± 4.3	
Uterus (mg)	224 ±13		255 ±13		204 ±11.5	

[a]Litters 16 days old replaced with 8-day-old foster litters.

[b]Mean and SE of mean.

secretion due to an excessive amount of Predef injected. Anterior and posterior pituitary and ovarian weights did not differ significantly ($P>0.05$) among rats in the three groups.

The livers of rats from groups one and three were prepared for *in vitro* incubations as described in materials and methods. The results of a preliminary experiment indicated that an incubation with 1 ml each of microsome and 105,000xg supernatant preparations increased the amount of cortisol metabolized by 144% in comparison with an incubation with only microsomes. Consequently, all subsequent incubations were performed with 1 ml of the microsome fraction plus 1 ml of the 105,000xg supernatant fraction.

The liver preparations of saline- and Predef-treated rats at the various levels of cortisol substrate produced inconsistent results with respect to the total mass of cortisol metabolites produced per hour (Table 14). For example, liver preparations of Predef-treated rats did not consistently produce greater or lesser amounts of cortisol metabolites than livers of saline-treated rats at the 1, 2 and 5 µm substrate levels. However, the percent nonpolar and polar metabolites produced within substrate levels between treatments did show a consistent trend. For example, a larger percentage of polar metabolites for the Predef-treated rats was observed at the 1, 2 and

TABLE 14. Amount of cortisol metabolites produced from liver preparations of intensely nursed rats injected with saline and 9-fluoroprednisolone acetate (Predef) between day 16 and 32.

Pretreatment from 16-32 days of lactation	Cortisol substrate added	Total µg of cortisol metabolites produced per hour	µg of non-polar cortisol metabolites produced per hour	µg of polar cortisol metabolites produced per hour
Saline	1 µm	162	52 (34%)[a]	108 (66%)[a]
Saline	2 µm	201	92 (46%)	109 (54%)
Saline	5 µm	214	126 (59%)	88 (41%)
Predef	1 µm	127	10 (8%)	117 (92%)
Predef	2 µm	243	39 (16%)	204 (84%)
Predef	5 µm	170	31 (18%)	139 (82%)

[a]Percent of total metabolites.

5 µm concentrations in comparison with the saline treatment. Moreover, the Predef preparations consistently produced greater amounts of cortisol metabolites per hour per mg of protein (specific activity) at the 1, 2 and 5 µm concentrations of cortisol (Table 15). Each of the liver preparations from Predef-treated rats produced greater amounts of polar and smaller amounts of non-polar metabolites per mg of protein than the corresponding preparations from control rats. The 2 µm concentration of cortisol appeared to be the optimal substrate level for the incubations. At this substrate level the total amounts of cortisol metabolites produced per hour per mg of protein from the enzyme system of rats pretreated with Predef was two times greater than that for rats given saline. In addition three times as much polar metabolite was formed in the liver preparations of Predef-treated rats than in the saline-treated animals. However, the formation of non-polar metabolites in the Predef system was less than that in the saline system. These results suggested that Predef injections into lactating rats increased the ability of rat livers to metabolize cortisol into polar metabolites while inhibiting the formation of non-polar metabolites. The formation from cortisol of larger proportions of polar metabolites by the livers of Predef-treated rats may indicate that the products are biologically less active and more water soluble. The increased solubility in water may allow the metabolites to be excreted more readily.

TABLE 15. Amount of cortisol metabolites produced per mg of protein from liver preparations of intensely nursed rats injected with saline and 9-fluoroprednisolone acetate (Predef) between day 16 and 32.

Pretreatment from 16-32 days of lactation	Cortisol substrate added	Total µg of cortisol metabolites produced /hour/mg of protein	µg of non-polar cortisol metabolites produced /hour/mg of protein	µg of polar cortisol metabolites produced /hour/mg of protein
Saline	1 µm	6	2 (33%)[a]	4 (67%)[a]
Saline	2 µm	8	4 (50%)	4 (50%)
Saline	5 µm	8	5 (62%)	3 (38%)
Predef	1 µm	9	1 (11%)	8 (89%)
Predef	2 µm	17	3 (18%)	14 (82%)
Predef	5 µm	12	2 (17%)	10 (83%)

[a]Percent of total metabolites.

The total amount of cortisol and its metabolites found in the aqueous extraction phase of the livers was larger for the Predef- than for the saline-treated rats (Table 16). In addition the specific activity for the Predef enzyme system was greater than the saline system at all substrate levels. Since enzyme assays were performed on pooled liver preparations within treatments, there is no estimate of the variation within treatments.

Total liver weights, liver protein content and protein per gram of liver did not differ ($P>0.05$) between Predef-treated and saline control rats (Table 17). Liver weights per 100 grams of body weight were significantly larger ($P<0.025$) in Predef-injected rats than in saline-injected controls. The pooled microsomal protein content of saline-treated rats was 144% higher than the microsomal protein content of Predef-injected rats.

The first series of Predef experiments indicated that relative to saline-injected controls, daily injections of either 10, 50 or 100 µg of Predef for 16 days exerted a galactopoietic effect on milk synthesis. However, significant declines ($P<0.01$) in LWG were observed as lactation advanced. If the injections of Predef were delayed to day 24 of lactation or if the dose of Predef was increased during the treatment period, the declines in LWG during advanced lactation were eliminated. These results *in vivo* suggested that the adrenal corticoid

TABLE 16. Amount of cortisol and cortisol-water-soluble metabolites produced from liver preparations of intensely nursed rats injected with saline and 9-fluoroprednisolone acetate (Predef) between day 16 and 32.

Pretreatment from 16-32 days of lactation	Cortisol substrate added	µg of cortisol and metabolites produced /hour in aqueous phase	µg of cortisol and metabolites produced/hour/mg of protein in aqueous phase
Saline	1 µm	8.3	0.3
Saline	2 µm	13.1	0.5
Saline	5 µm	20.8	0.8
Predef	1 µm	10.7	0.8
Predef	2 µm	17.8	1.3
Predef	5 µm	22.4	1.6

TABLE 17. Liver weights and protein measurements of liver microsomes and 105,000xg supernatant fluid of intensely nursed rats injected with saline and 9-fluoroprednisolone acetate (Predef) between day 16 and 32.

	Predef	Saline
Liver wt. (g)	11.0±0.2 (10)[a]	11.4±0.3 (10)[a]
Liver wt. (g) per 100 (g) of body wt.	4.9±0.1 (10)	4.6±0.1 (10)
Protein (mg) per (g) of liver	173±13 (10)	183±2 (10)
Total liver protein (mg)	1896±139 (10)	1934±55 (10)
Pooled microsomal protein (mg/ml)	6.1	14.9
Pooled 105,000xg pooled supernatant (mg/ml)	8.0	10.6

[a]Number of rats per treatment.

requirements during the Predef treatment period may change. Preliminary *in vitro* incubation studies indicated that Predef-injected rats have a liver enzyme system with a greater ability to metabolize cortisol than that found in saline-injected controls. Consequently, an increased rate of glucocorticoid metabolism induced by adrenal hormone injections may account partially for the increased corticoid requirements to maintain persistent lactation.

Experiment VI.--Measurement of Adrenal Function and Corticoid Binding Globulin (CBG) Activity of Intensely Nursed Rats During Extended Lactation

Since pituitary ACTH and litter weight gains decrease during normal lactation and adrenocorticoid supplementation will prevent these declines, it appears that adrenal secretions may be rate limiting to milk synthesis during advanced lactation in the rat. Consequently, the present experiment was designed to determine directly if adrenocortical function decreased during advanced lactation. Resting levels of plasma and adrenal corticosterone were measured in rats killed at 16, 24 and 32 days of lactation. Changes in adrenal activity were compared with milk production and mammary gland nucleic acid measurements.

Cumulative LWG for an 8-day period prior to sacrifice at 16, 24 and 32 days of lactation were 79.3, 82.7 and 30.6 g, respectively (Table 18). The LWG response for the 32-day treatment group was less ($P<0.01$) than the

TABLE 18. Nucleic acid content of mammary glands and litter weight gain of intensely[a] suckled rats during extended lactation.

	Days of Lactation[b]		
	16	24	32
Litter weight gain (g)[c]	79.3±6.4	82.7±3.9	30.6±2.9
Total DNA (mg)	32.2±1.2	31.0±1.0	25.5±0.9
Total RNA (mg)	172.9±9.4	140.5±8.6	92.7±5.7
RNA/DNA	5.3±0.1	4.5±0.2	3.6±0.2

[a]Litters 16 days old replaced with 8-day-old foster litters.

[b]Mean and SE of mean.

[c]Cumulative litter weight gains were recorded between days 8 and 16 for all litters.

LWG responses of the 16- and 20-day treatment groups. Total mammary DNA did not change significantly ($P>0.05$) between day 16 (32.2 mg) and 24 (31.0 mg) but declined ($P<0.01$) to 25.5 mg by day 32 of lactation. Mammary RNA content declined linearly ($P<0.01$) from day 16 to 32 of lactation. Mammary RNA/DNA ratios were 5.3, 4.4 and 3.6, respectively. The changes in ratio paralleled very closely total RNA changes. These results, which confirm the results of Experiment I, indicated that lactational performance declined during the advancing stages of lactation.

The mean resting level of plasma corticosterone for a group of virgin rats killed at metestrus was 10.65 µg per 100 ml of plasma (Table 19). This plasma level of corticosterone was in close agreement with the resting corticosterone levels reported by Guillemin (1958) for female rats. These results substantiated that the management procedures used in this experiment allowed one to measure resting levels of corticosterone in rat plasma.

The total corticosterone content per two adrenals for the groups killed at 16, 24 and 32 days of lactation was 2.01, 1.66 and 1.35 µg, respectively, a significant linear decline ($P<0.01$). The adrenal corticosterone content at day 32 of lactation was not significantly different ($P>0.05$) than the level found in the virgin rats (1.23 µg). When expressed as µg per 100 mg of adrenal

TABLE 19. Adrenal and plasma corticosterone levels in intensely[a] suckled rats during extended lactation.

	Virgin rats[b]	Days of lactation[b]		
		16	24	32
Number of rats	20	20	20	20
µg corticosterone/ 2 adrenals	1.23±0.12	2.01±0.28	1.66±0.14	1.35±0.11
µg corticosterone/ 100 mg of adrenal	2.25±0.14	3.39±0.39	3.05±0.22	2.56±0.19
µg corticosterone/ 100 ml of plasma	10.65±0.90	18.76±3.03	18.32±1.67	15.97±1.64
Adrenal (mg)	49.9 ±2.24	57.2 ±1.99	54.3 ±1.50	53.2 ±1.75
Adrenal mg/100 g of body wt.	25.0 ±1.1	22.2 ±0.7	22.2 ±0.6	21.7 ±1.0
µg corticosterone bound/100 ml of plasma	---	63	58	75

[a]Litters 16 days old replaced with 8-day-old foster litters.

[b]Mean and SE of mean.

tissue the values were 3.39, 3.05 and 2.56 µg, respectively. Again this represented a significant linear decrease ($P<0.05$) with advancing stages of lactation. There was a trend, which was not statistically significant ($P>0.05$) for adrenal weights to decrease with advancing stages of lactation.

Peripheral plasma concentrations of corticosterone for rats killed at day 16, 24 and 32 of lactation (18.76, 18.32 and 15.97 µg per 100 ml of plasma, respectively) were not significantly different ($P>0.05$) from each other. Measurements of CBG activity, at each stage of lactation, were made on pooled plasma samples. The quantities of corticosterone bound to CBG per 100 ml of rat plasma were 63, 58 and 75 µg at day 16, 24 and 32 of lactation, respectively (Table 19).

The results of Holzbauer (1957) indicated a positive correlation between the quantities of cortical hormones present in the adrenal and its secretory activity at any given time. Thus, a high adrenal corticosterone concentration was associated with high secretory activity, a low concentration with low secretory activity. Comparisons of secretion rates and the stores of corticosterone in rat adrenals provided direct evidence for this relationship (Holzbauer, 1957). The correlation between corticosterone stores and secretaion rate in the rat adrenal provides a method in which information can be obtained on the activity of the adrenal cortex immediately before extirpation of the gland. With this relationship in mind, the results of the present study suggest that the secretion

rate of corticosterone decreased from day 16 to day 24 and was at its lowest level at day 32 of lactation. In fact, the adrenal corticosterone content at day 32 was comparable to that found in the virgin non-lactating state. However, there were no significant ($P>0.05$) changes in peripheral plasma levels of corticosterone that reflected this decrease in adrenal secretion rate with advancing stages of lactation. Perhaps the target tissues of the body utilize the increased amount of corticoid hormone that is secreted into the plasma pool at the peak of lactation (day 16). If the target tissues of the body promptly utilized any additional amount of corticosterone available at different stages of lactation, then the peripheral plasma levels may remain at a constant level. The basal corticoid level during lactation appears to be at a level of equilibrium higher than that measured in virgin rats. The mammary gland is a tissue that has an apparent corticoid requirement to maintain lactation in the rat (Experiments III and V). Consequently, the mammary tissue may be utilizing the additional corticoid secreted at the peak of lactation. If such a relationship does exist then the secretion rate or the corticosterone content of adrenal gland may be correlated with some end organ response in the mammary gland.

The within-stage, among-rat correlation coefficients between total adrenal corticosterone content and mammary gland RNA, DNA and LWG are listed in Table 20. The largest

TABLE 20. Correlation coefficients of mammary gland nucleic acid content and litter weight gains with adrenal corticosterone content of intensely[a] suckled rats during extended lactation.

Stage of lactation	Correlation Coefficients		
	RNA	DNA	LWG
16	0.60[b]	0.59[b]	0.44[c]
24	0.26	0.11	-0.02
32	0.43	0.29	0.35
Pooled within stage of lactation	0.45[d]	0.35[d]	0.27

[a]Litters 16 days old replaced with 8-day-old foster litters.

[b]Correlation coefficients significantly different from zero ($P<0.01$).

[c]Correlation coefficients significantly different from zero ($P<0.05$).

[d]Pooled correlation coefficients within stages of lactation significantly different from zero ($P<0.01$).

correlation coefficients were between adrenal corticosterone content and mammary RNA (0.60), DNA (0.59) and LWG (0.44) at day 16 of lactation. At day 16 adrenal corticosterone content was the greatest and the mammary gland was stimulated maximally. Decreased adrenal secretion rates at day 24 and 32 of lactation may cause a general decrease in the responsiveness of the mammary tissue. Such a decrease may account for the absence of a significant correlation coefficient between adrenal corticosterone content and the mammary gland traits measured at these two stages of lactation. The pooled correlation coefficients of adrenal corticosterone content with mammary gland RNA and DNA within stages of lactation were significant ($P<0.01$). I conclude that the declines in adrenal corticosterone content may be related to changes in mammary gland function.

An additional factor for consideration is the possible interaction of peripheral plasma corticosterone and CBG activity. The mass of corticosterone bound per 100 ml of plasma appeared to be higher at day 32 of lactation (75 µg%) than at day 16 (63 µg%) or 24 (58 µg%). Gala and Westphal (1965) reported that CBG activity is depressed during lactation but at four days post-weaning it rises to a level comparable to that found in virgins. The higher CBG activity at day 32 of lactation (75 µg% compared with 63 µg% and 58 µg%) may reflect an increase

in binding activity during advanced lactation that approaches the activity found in the virgin rat. Based on the binding affinity for cortisol, the level of CBG activity reported for the virgin rat is 105 µg% (Seal and Doe, 1965). Since the binding affinity for corticosterone in the rat is comparable to the affinity for cortisol (Seal and Doe, 1965), the 75 µg of corticosterone bound per 100 ml of plasma at day 32 of lactation in the present experiment may be a level that approaches the CBG activity of the virgin rat. If the CBG activity is greater at day 32 of lactation, then the amount of free plasma corticosterone available to the mammary gland may be less, regardless of the constant peripheral blood levels between 16, 24 and 32 days.

SUMMARY AND CONCLUSIONS

Intensive nursing, which was maintained by replacing all 16-day-old litters every 4 days with 12-day-old foster litters and supplying at least one pup per mammary gland, failed to prevent declines in mammary DNA, RNA and litter weight gains (LWG) between days 20 and 36 of lactation. Intense nursing maintained pituitary prolactin during extended lactation but ACTH decreased 68%.

Adrenal corticosterone content and concentration decreased significantly from day 16 to 32 of lactation, indicating that ACTH stimulation of the adrenal gland decreases in rats during extended lactation. Peripheral plasma concentrations of corticosterone in lactating rats were higher than the resting levels measured in the virgin state. However, corticosterone concentrations in the plasma did not change from day 16 to 32 of lactation. Measurements of corticoid binding globulin activity in pooled plasma samples indicated that the binding activity of rats killed at day 32 of lactation was greater than the activity found in rats killed at day 16 and 24. Adrenal corticosterone content of rats at day 16 of lactation was significantly correlated with mammary gland

DNA ($r=0.59$, $P<0.01$), RNA ($r=0.60$, $P<0.01$) and litter weight gain ($r=0.44$, $P<0.05$). The decrease in adrenal corticosterone content from day 16 to 32 of lactation was correlated significantly with the decrease in mammary gland DNA ($r=0.35$, $P<0.01$) and RNA ($r=0.45$, $P<0.01$) content.

Oxytocin injections during advanced lactation caused marginal increases in the DNA and RNA content of the mammary gland but did not significantly increase litter weight gain or the synthetic activity per cell (RNA/DNA). Consequently, milk removal appeared to be adequate for maintenance of milk synthesis during advanced lactation.

Hydrocortisone acetate, prolactin and growth hormone were given singularly and in all possible combinations to determine if these treatments would maintain mammary nucleic acid content and lactational performance during extended lactation. Hydrocortisone acetate maintained mammary DNA and RNA content and reduced the normal decline in litter weight gain. Prolactin given with hydrocortisone acetate reduced the DNA response, but stimulated the synthetic activity of the mammary cells (RNA/DNA). Prolactin and growth hormone did not exert a galactopoietic influence on milk yield.

Chronic secretion of rat prolactin from five isotransplanted pituitaries under the kidney capsule of

lactating rats did not increase litter weight gain although mammary gland DNA and RNA content and RNA/DNA ratio were significantly increased. Prolactin is probably not rate limiting to milk synthesis in the rat because pituitary prolactin content did not decrease during extended lactation and neither ovine prolactin injections nor rat prolactin from isotransplanted pituitaries increased milk yield.

A daily dose of 50 µg of 9-fluoroprednisolone acetate (Predef) from day 16 to 32 of lactation maintained mammary gland DNA and RNA content and increased milk yield. A delay in the onset of injections until day 24, or the injection of 50 µg of Predef daily from day 16 to 23 and 100 µg of Predef daily from 24 to 32 days of lactation maintained LWG and increased the mammary gland nucleic acid content. Results of in vitro incubations indicated that Predef injections for 16 days increased the ability of rat livers to metabolize cortisol into polar metabolites while the formation of non-polar metabolites was inhibited.

I conclude that adrenocortical secretions may be rate limiting to milk synthesis during advanced lactation in the rat because pituitary ACTH and adrenal corticosterone content decrease during this time, corticosteroid binding globulin activity of the plasma increased (although total circulating levels of corticosterone did

not change), and the declines in mammary gland nucleic acid content and litter weight gain can be prevented with hydrocortisone acetate or Predef supplementation.

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APPENDICES

APPENDIX I

COMPOSITION OF RAT FEED

The animal ration was composed of the following ingredients:

Ground shelled corn (1/8 inch screen)	607.0 lb
Soybean oil meal, 50% protein	280.0 lb
Alfalfa meal, 17% protein	20.0 lb
Fishmeal, 65% protein	25.0 lb
Dried whey	25.0 lb
Pro-strep 20, 0.54% penicillin and 2.72% streptomycin	4.0 oz
Pro-Gen, 20% arsanilic acid	0.5 lb
Vitamin A, 10,000 units/g	364.0 g
Irradiated yeast, 9.000 units/g	38.0 g
Choline chloride	318.0 g
D. Ca Pantothenate	2.5 g
Riboflavin	1.5 g
Nicotinic acid (Niacin)	15.0 g
Vitamin B-12 (0.1% mannitol trituration)	3.0 g
DL alpha tocopherol acetate, 250 IU vitamin E/g	8.8 g
Menadione (vitamin K)	1.0 g
DL methionine	227.0 g
Limestone	16.0 lb
Dicalcium phosphate	17.5 lb
Iodized salt	5.0 lb
Manganous sulphate ($MnSo_4 \cdot H_2O$) 32.5% manganous	168.9 g

Ferrous sulfate ($FeSo_4 \cdot 7H_2O$)	
20.9% iron	215.2 g
Calcium carbonate ($CaCO_3$)	
40.4% calcium	83.8 g
Zinc carbonate, basic ($ZnCO_3$)	
56.0% zinc	40.2 g
Cupric sulfate ($CuSO_4 \cdot 5H_2O$)	
25.45% copper	12.9 g
Cobalt chloride ($CoCl_2 \cdot 6H_2O$)	
24.77% cobalt	4.7 g
Potassium iodine (KI)	
76.45% iodine	2.2 g

This ration gave the following analysis:

protein	21.2%
fat	3.9%
crude fiber	2.5%
productive net energy	902 C/lb

APPENDIX II

ORCINOL REAGENT USED IN THE RNA PROCEDURE

1. A stock solution of 1.6% ferric chloride ($FeCl_2 \cdot 6H_20$) was prepared by adding 1.6 g of ferric chloride to 1 liter of concentrated hydrochloric acid.
2. A 1% solution of orcinol in 1.6% ferric chloride solution was prepared 1 hour before use.

APPENDIX III

INCUBATION MEDIA FOR ACTH ASSAY

Stock Solution A	5.75% KCL in distilled water
Stock Solution B	8.08% $CaCl_2.2H_2O$ in distilled water
Stock Solution C	10.55% KH_2PO_4 in distilled water
Stock Solution D	19.10% $MgSO_4.7H_2O$ in distilled water
Stock Solution E	200 ml of .9% NaCl containing 0.520 g of glucose
Stock Solution F	100 ml of 1.3% $NaHCO_3$ (solution gassed for 30 minutes with 95% CO_2+5%O_2)

Preparation of incubation media

1. "Krebs Ringer Salt" solution is prepared by adding together:

   4 ml of solution A

   3 ml of solution B

   1 ml of solution C

   1 ml of solution D

   36 ml of distilled water

   The total volume of 45 ml is designated as a "Krebs Ringer Salt Solution."

2. The incubation media (Krebs-Ringer-Bicarbonate with Glucose) is prepared as follows:

42 ml of solution F

18 ml of "Krebs Ringer Salt" solution

200 ml of solution E

The total contents of 260 ml is gassed with 95% O_2 and 5% CO_2 for 10 minutes prior to use.

APPENDIX IV

0.1M PHOSPHATE-GLYCEROL BUFFER pH7.4 USED IN IN VITRO METABOLISM STUDIES

Solution A: 0.2M sodium phosphate, monobasic

Solution B: 0.2M sodium phosphate, dibasic, heptahydrated

0.1M phosphate buffer with 20% glycerol pH7.4:

19 ml solution A

81 ml solution B

40 ml glycerol

60 ml distilled water

APPENDIX V

0.1M PHOSPHATE BUFFER pH7.4

USED IN IN VITRO METABOLISM STUDIES

Solution A: 0.2M sodium phosphate, monobasic

Solution B: 0.2M sodium phosphate, dibasic heptahydrated

0.1M phosphate buffer, pH7.4:

19 ml solution A

81 ml solution B

100 ml distilled water

APPENDIX VI

SCINTILLATION FLUID #1

1. 4 g of PPO or 2, 5-Diphenyloxazole--scintillation grade (Nuclear Chicago)
   and 50 mg of POPOP or 1, 4-bis[2-(5-Phenyloxayolyl)] benzenescintillation grade (Nuclear Chicago)
   in 1 liter of toluene
2. Radioactivity measured in the following system:
   0.2 ml of aqueous phase
   5.0 ml of ethyl alcohol
   5.0 ml of scintillation fluid

APPENDIX VII

SCINTILLATION FLUID #2

1. 7.5 g PPO

   75 mg POPOP

   120 g Napthalene

   500 ml Xylene

   500 ml Dioxane

APPENDIX VIII

FLUORESCENT REAGENT FOR CORTICOSTERONE DETERMINATION

1. Sulfuric Acid: Ethanol (3:1) mixture
2. This mixture was cooled in a flask under tap H_2O and allowed to stand at least 12 hours before use.
3. Sulfuric Acid: 96.4%, 1.84 sp gr
   J. T. Baker Chemical Co.
   Phillipsburg, N. J.
4. Ethanol - 200 proof - "Gold Shield Alcohol"
   Commercial Solvents Corp., Terre Haute, Indiana

APPENDIX IX

PHOSPHATE BUFFERS USED IN THE CBG PROCEDURE

Solution A: 0.4M monobasic sodium phosphate

Solution B: 0.4M dibasic sodium phosphate

0.2M phosphate buffer, pH7.4

19 ml of solution A

81 ml of solution B

100 ml of distilled water

0.1M phosphate buffer, pH7.4

19 ml of solution A

81 ml of solution B

300 ml of distilled water