

## ABSTRACT

### TEMPORAL EFFECTS OF HORMONES ON NUCLEIC ACID SYNTHESIS IN MAMMARY ORGAN CULTURES

By

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Mammary tissues from pregnant mice undergo specific changes in structure and function when cultivated in vitro on synthetic media containing insulin + corticosterone + prolactin. Previous studies have shown that the biochemical effects of the three hormones involved are vitally linked to cell proliferation and protein synthesis. This study was undertaken to determine the sequence of action of the three hormones individually and in various combinations on mammary DNA and RNA synthesis in vitro.

The first part of this study was devoted to modifying DNA and RNA procedures for application to mammary explants. An in vivo survey was made of the changes in mammary tissue nucleic acid levels in the course of normal secretory development. Growth continues throughout pregnancy and lactation reaching a maximum on the 6th day post-partum. RNA increases markedly with the onset of lactation and attains maximum levels on the 6th day. The decline in lactational function that follows results from both a decline in cell number and in functional activity.

The second part of this study consisted of cultivating pre-lactating mammary explants with the eight possible combinations of the three hormones involved in mammary differentiation in vitro.

Hormonal effects on DNA and RNA were studied as a function of time. The results obtained show that explant survival as well as secretory activity falls with time in culture except for explants cultured with insulin + corticosterone + prolactin. Stimulation of DNA and RNA synthesis and survival of explants are dependent on the presence of insulin, irrespective of other hormonal supplements. A principal effect of insulin appears to be the stimulation of DNA synthesis. Its stimulation of RNA appears to be a secondary effect resulting from the increase in cell number and is thus unrelated to the induction of functional activity. Corticosterone, or prolactin alone, or corticosterone + prolactin have little or no effect in maintaining DNA levels. Corticosterone in combination with insulin increases DNA content on the second day, but the triple hormone system was the most effective combination increasing DNA on the second, 3rd, and 5th days. Thus, corticosterone enhances the effect of insulin on DNA while having no effect by itself. The effect of prolactin on DNA synthesis is dependent on prior stimulation by insulin and corticosterone as well as the continual presence of the latter two hormones. Corticosterone or corticosterone + prolactin have an overall inhibitory effect on RNA synthesis, but this inhibition is completely abolished when insulin is also present in the medium. In addition, corticosterone significantly increases the stimulatory effect of insulin on RNA. Prolactin stimulates RNA synthesis, since prolactin-containing combinations stimulate explant secretion and RNA content. Insulin + corticosterone + prolactin was most effective combination inducing RNA synthesis, maintaining its stimulated content, and

initiating maximum secretion after the second day of culture. Thus, in addition to the information obtained with the hormones individually, the need for synergism between the three hormones for long-term effects was also demonstrated in this study.

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## I. INTRODUCTION

The development of the mammary gland involves changes in its glandular epithelium, namely, proliferation and differentiation, the latter characterized by the secretion of a cell-specific product, "milk". Until recently, investigators have relied largely upon morphological methods to study mammary gland development in the mouse. However, the need for precise and rapid measurements of mammary growth and function has resulted in the application of biochemical approaches. These include DNA determination for measuring mammary growth and RNA determination for measuring the functional state of the cells. Studies on nucleic acids levels during various stages of the growth and development of the mouse mammary gland and under various experimental conditions in vivo and in vitro will be reviewed below.

### Nucleic Acid Content as a Measure of Mammary Development

#### A. Mammary Growth.

The use of DNA content as a measure of cell number was first suggested by Davidson and Leslie (1950). It requires that the amount of DNA in the cell nucleus remain constant for the somatic tissue of a given species. Although the mammary gland undergoes periods of cellular multiplication and secretory activity, Lewin (1957) indicated that the ratio of DNA content to the number of nuclei was relatively constant from the 7th day of pregnancy to the 12th day of lactation in

the mouse. The results of the studies on the mouse mammary gland by Lewin (1957), Brookreson and Turner (1959), and Munford (1963) indicate that cellular multiplication and secretory activity are not antagonistic phenomena as was thought earlier from histological studies.

Brookreson and Turner (1959) and Wada and Turner (1959) examined the mammary DNA content of virgin, pregnant, and lactating Swiss mice. They found that the total DNA per 7 glands of each mouse was 1.757 mg in virgin mice. The amount increased gradually during pregnancy and lactation, reaching a maximum of 10.211 mg at the 14th day post-partum. The percentage growth was 30.6% of the total growth during the first 12 days of pregnancy, 45.7% in the latter half of pregnancy, and 21.9% from parturition to the 14th day of lactation.

It should be mentioned that the amount of DNA/mg defatted-dehydrated mammary extract (DNA concentration) decreased during the course of lactation. This result led Munford (1964) to suggest that concentration be regarded as a measure of cell size rather than cell number, since concentration is affected by extracellular milk and colostrum, which can confound differences between different stages of development. The total increase in cell number reached a maximum around the 4th to 6th day of lactation (Lewin, 1957), although Munford (1963) found that the maximum was reached on the 7th day of lactation.

During weaning of 20-day lactating mice, DNA levels decrease rapidly to a level of 1.93 mg on the 15th day of involution (Anderson and Turner, 1963b). This amount is comparable to the virgin content of 1.76 mg DNA. On the other hand, Wada and Turner (1959) reported

that after three month's involution, mouse mammary glands contain DNA equivalent in amount to that in 6-day pregnant mice.

Considering mated mice lacking fetuses as pseudopregnant, Wada and Turner (1959) and Brookreson and Turner (1959) found that maximal mammary growth was reached by the 6th day of pseudopregnancy. The amount of DNA was comparable to that found in 6-day pregnant mice. Apparently, some factor associated with pregnancy and which is not present in pseudopregnancy, contributes to the stimulation of mammary growth.

The effect of recurring pregnancies on the extent of mammary gland growth was investigated by Wada and Turner (1959). They found an additional 28.70% growth of the mammary gland at the 18th day of the second pregnancy with a further increase on the 18th day of the third pregnancy. Quadripara mice showed 9.68% decline in total DNA in comparison to tripara mice. From these observations, the above workers suggested that the decline in mammary gland growth reflects a decline in the secretion of hormones essential for growth.

In all the preceding studies, DNA determinations were made on intact mammary glands which include parenchyma cells, adipose stroma, and lymph nodes. Nicoll and Tucker (1965) investigated the contribution of stromal and lymph node DNA to the total DNA of intact mouse mammary glands. They found that mammary gland-free pads showed no change in total DNA between virgin and midlactating states. Although lymph node DNA constitutes a substantial fraction of total DNA, mammary parenchymal cells are primarily responsible for the difference in the total DNA content of the mammary gland between virgin and lactating states.

## B. Mammary Secretion.

Because of the well-established relationship between RNA and protein synthesis, RNA and the ratio RNA/DNA are widely used to estimate the state of functional activity of various tissues. In the mammary gland, RNA measurements have been used as indices of the functional state of the mammary cells.

In lactating mice, the total RNA content of the right inguinal glands rises steadily from parturition to a maximum of 589.7  $\mu\text{g}$  RNA-P from about the 14th to 16th day of lactation, then falls steadily from the 16th day to the end of lactation, i.e. 322.8  $\mu\text{g}$  RNA-P/right inguinal glands on day 19 and 98.9  $\mu\text{g}$  on day 26 (Mizuno, 1961). Similar results were also obtained by Chikamune and Mizuno (1958), and Chikamune (1963) (cited in Denamur, 1964). RNA/DNA ratios (Mizuno, 1961) reach a maximum on the 14th day. The decline in lactational function thus occurs not only because of the decrease in mammary cell number, as shown by the fall in total DNA, but also because of the slowing down of cellular synthetic activity, as shown by the decrease in total RNA and RNA/DNA ratios.

No differences were found in total DNA, RNA, or RNA/DNA ratios between the right and left inguinal glands of the same animal (Mizuno, 1961), which indicates that mammary gland growth and function are equal on both sides.

### Experimental Control of Mammary

#### Nucleic Acid Levels in Vivo

Nucleic acid measurements in experimental studies in the mouse mammary gland have been little studied. Damm and Turner (1957)

stimulated lobuloalveolar development in male mice by injecting various levels of estradiol benzoate and progesterone (constant ratio 1:1000, daily for 10 days). Total DNA was found to increase with increase of hormones up to 0.75  $\mu$ g estradiol benzoate and 0.75 mg progesterone. Mammary growth as calculated by DNA concentration reached a peak with lower doses, i.e. 0.125  $\mu$ g of estradiol benzoate and 0.125 mg of progesterone.

Optimal growth was stimulated by estradiol benzoate and progesterone administered for 19 days in either estrogen-primed male (to develop duct system) or ovariectomized female mice. The total DNA values obtained in both cases was nearly half that obtained in 18-day pregnant controls (Anderson, Brookreson, and Turner, 1961; Anderson and Turner, 1963a). From these results, it appears that even the most efficient estrogen and progesterone treatment results in mammary development that is consistently less than that found at the end of gestation.

Castration of mice at the beginning of lactation does not interfere with normal mammary growth, indicating that ovarian hormones are not required during lactation (Brookreson and Turner, 1959).

Damm and Turner (1961) tested a number of crude and purified pituitary extracts and lactogen in combination with estradiol benzoate. Although all combinations produced lobuloalveolar development, the level of DNA did not approach that found at the end of pregnancy. Crude and purified FSH and growth hormone together with estradiol benzoate exhibited very little or no mammogenic effect. Estradiol benzoate and progesterone with different combinations of thyroxine,

hydrocortisone, prednisone, lactogen, and growth hormone were also ineffective in stimulating mammary growth equal to that observed at the end of pregnancy (Anderson and Turner, 1963a). The highest level of DNA was obtained in ovariectomized-adrenalectomized animals given prednisone and different combinations of estradiol benzoate and progesterone.

The effect of concurrent pregnancy on nucleic acid content was studied by Mizuno (1960). He found that the DNA and RNA content of pregnant-lactating mice at 19 days of lactation was significantly higher than that of nonpregnant-lactating mice. However, there was no significant difference in RNA/DNA ratios between the two groups. This observation indicated that the synthetic activity of the gland was not changed or inhibited, but that mammary gland proliferation was actively stimulated by pregnancy and that the hormonal conditions suitable for mammary growth can coexist with that for lactation.

In unilaterally-ligated glands, the unligated side is always more developed and more active than the ligated side (as indicated by higher nucleic acid content) (Mizuno, 1961). This difference was not due to a lower DNA content in the ligated side but rather to an increase in DNA in the unligated one. The latter could be due to compensatory cell proliferation by increased suckling intensity. Administration of progesterone or prolactin or concurrent pregnancy increased DNA, RNA, and RNA/DNA ratios of the ligated glands more than the unligated ones. Concurrent pregnancy had a greater effect on the above parameters than did hormone treatment.

## Hormonal Control of Mammary

### Development in Vitro

The hormonal regulation of mammary gland differentiation has been studied mainly in vivo. In a detailed morphological study, Nandi (1959) demonstrated that a combination of ovarian and adrenocortical hormones, prolactin, and growth hormone promoted mammary differentiation in hypophysectomized-ovariectomized-adrenalectomized and hypophysectomized-ovariectomized mice.

Although the hormones influencing mammary growth and differentiation in the mouse have been established in in vivo studies, precise knowledge of the action of hormones is still limited. With the development of the organ culture technique, several workers have used it to study the direct effects of hormones on mammary growth and differentiation. The minimal hormonal requirements affecting maintenance and development of secretory activity of mid-pregnant mouse mammary explants in a chemically-defined medium have also been established at the morphological level.

Elias (1957) and Elias and Rivera (1959) indicated that explants of alveolar lobules are dependent on cortisol and prolactin for secretory responses in vitro. Tissues cultured without hormones undergo extensive degeneration. The addition of ovarian hormones, as well as somatotropin, alone or in combination, did not affect the previous in vitro responses (Elias and Rivera, 1959). Insulin is required to maintain organ cultures of mammary tissue in a synthetic medium especially when cortisol is also added (Elias, 1959; Rivera and Bern, 1961).



Cultures from pregnant, early prelactating, and late pre-lactating mice were maintained in medium containing insulin and cortisol, with loss of secretory activity in late prelactating tissue (Bern and Rivera, 1960; Rivera and Bern, 1961). In these studies using C3H mice, the minimal hormonal combination capable of initiating secretion in vitro was either somatotropin or prolactin in addition to insulin and cortisol.

Rivera (1964a) indicated that an adrenal corticoid and insulin are the minimal requirements for survival of prelactating explants. Prolactin or somatotropin or both prolactin and somatotropin are additionally required to induce secretion. Multiparous tissue is more sensitive to pituitary hormones than is nulliparous tissue. Minimal effective concentrations of pituitary hormones also differ in different strains of mice. Strain A (known to be refractory to somatotropin) was found to require at least 4 times more prolactin and 7.5 times more somatotropin (in combination with aldosterone and insulin) than tissues from C3H mice (highly sensitive to somatotropin) for comparable secretory activity. Similar work has been done on the hormonal sensitivity of several other strains of mice (Rivera, 1966).

Rivera, Forsyth, and Folley (1967) studied the lactogenic activity of growth hormone from several mammalian species both in C3H and A strains of mice. Ovine, bovine, and porcine growth hormone were 20-50% as active as ovine prolactin, while simian and human preparations showed activity equal to or greater than prolactin. Various adrenocortical hormones were also studied for their secretory effect on mammary explants. Cortisol, as well as the naturally-occurring

glucocorticoid in the mouse, corticosterone, or the mineralocorticoid, aldosterone, were equally capable of initiating mammary secretion (Rivera, 1964b).

Induction of lobuloalveolar differentiation in glands from immature mice have been reported (Prop, 1960; Rivera, 1964c). Some lobuloalveolar differentiation occurred in partly-defined medium supplemented with aldosterone, insulin, estrogen, progesterone, growth hormone, and prolactin. Loculoalveolar differentiation comparable to that found in pregnancy could be obtained in glands cultured in media containing insulin, progesterone, aldosterone, prolactin, and growth hormone in vitro provided the donor animals were pretreated in vivo with lobuloalveolar mammogenic hormones (estrogen, progesterone, prolactin, and growth hormone) (Ichinose and Nandi, 1964).

As regards the biochemical aspects of this system, it was found that DNA synthesis in 48-hour mammary cultures (as measured by incorporation of  $H^3$ -thymidine) was stimulated to the same extent in media containing insulin and prolactin or insulin, hydrocortisone, and prolactin and was double the DNA synthesized by insulin alone (Juergens et al., 1965). Further studies by Stockdale and Topper (1966) demonstrated that insulin alone is necessary and sufficient to initiate maximum DNA synthesis after 24 hours culture of mid-pregnant explants and after 48-72 hours of virgin mice explants. Immature explants can undergo DNA synthesis and mitosis in the absence of insulin, although this does not lead to functional differentiation (Voytovich and Topper, 1967). Autoradiographic studies by Stockdale, Juergens, and Topper (1966) also indicate that the three-

hormone combination has no unique effect on mitosis.

Turkington (1968a) tested further the hormonal requirements for stimulation of DNA synthesis. He found that only insulin or growth hormone increased DNA synthesis, both in midpregnant and virgin mammary explants, but they did not alter the rate of DNA replication. Addition of puromycin and cyclohexamide prevented the increase in DNA synthesis, indicating that protein synthesis is required for this process. Insulin + growth hormone, as well as thyroid hormones, alone or in combination with other hormones, and human placental lactogen failed to alter DNA synthesis. Human placental lactogen in combination with insulin and hydrocortisone was found to act as a potent "pro-lactin" in mouse mammary gland in vitro (Turkington and Topper, 1966).

With regard to RNA, insulin was shown to increase RNA synthesis more than 60% in explants of pregnant mice when compared with explants cultured without insulin (Mayne, Barry, and Rivera, 1966). In these experiments RNA synthesis was measured by the increase in C<sup>14</sup>-adenine incorporation into RNA during the first 3 hours of culture. Extending the culture period to 12 hours maintained the high incorporation rate in the presence of insulin while RNA content remained essentially constant (Mayne and Barry, 1967). This indicates that an increased rate of RNA synthesis is required to maintain RNA levels in the tissue.

Stockdale, Juergens, and Topper (1966) found that the initial rate of RNA synthesis was maintained for 48 hours, but doubled after 24 hours in the presence of either insulin, insulin + hydrocortisone, insulin + prolactin, or insulin + hydrocortisone + prolactin. After 48 hours, it remained constant with insulin and insulin + hydrocortisone, increased slightly with insulin + prolactin and

significantly in the presence of the three hormones. On the other hand, Mayne, Forsyth, and Barry (1968) found that in cultures with insulin and corticosterone, RNA synthesis and content did not change much by 24 hours and had fallen considerably by 48 hours. In the presence of insulin + corticosterone + prolactin, RNA increased after 24 and 48 hours. This augmentation could be detected as early as six hours after culture.

Studies on functional differentiation showed that production of a casein-like phosphoprotein is hormonally-controlled and requires the same three hormones (insulin, hydrocortisone, and prolactin) required for maximum histological responses (Juergens et al., 1965). This group of milk proteins was therefore used as a means of studying biochemical differentiation of the mammary gland. The three hormones were found to stimulate maximal casein synthesis after 48 hours of culture. All other hormonal combinations elicited minimal or no stimulation. The effect was selective for casein phosphoproteins, which was increased four times the initial value by 48 hours. The casein synthesized consists of four major electrophoretic components having the same patterns of mobility as casein from normal mouse milk (Turkington, Juergens, and Topper, 1965). The observation that the three-hormone combination had no unique effect on DNA synthesis led to the conclusion that the effect on casein synthesis was due to an increase in specialized cell function rather than increased cell number. The augmentation in casein synthesis is partially suppressed (20-45%) by actinomycin D, indicating that casein synthesis is dependent on continuous synthesis of DNA-dependent RNA.

The synthesis of whey proteins ( $\alpha$ -lactalbumin and  $\beta$ -lactoglobulin) is also stimulated by the three hormones to the same extent as casein synthesis. The ratio of whey proteins to casein synthesized by mid-pregnant mammary explants corresponded well with the ratio found in mouse milk. Thus, in each case, stimulation represents increased synthesis per cell (Turkington, Lockwood, and Topper, 1966; Lockwood, Turkington, and Topper, 1966). Maximum non-milk protein synthesis is achieved with insulin as the only hormonal supplement, which according to DNA studies, reflects cell proliferation rather than differentiation.

Experiments by Mayen, Barry, and Rivera (1966) showed that although protein synthesis increased in mammary explants of rats, the blocking of RNA synthesis did not affect total protein synthesis in these explants. They concluded that it is unlikely that insulin plays a primary role in inducing differentiation of the mammary gland.

In explants of midpregnant or virgin mice, casein synthesis occurs about 30 hours after the peak of DNA synthesis, suggesting that DNA synthesis and/or mitosis may be necessary for augmented casein synthesis (Stockdale and Topper, 1966). This notion was supported by the observation that when new cell formation was blocked by colchicine, augmentation of casein synthesis was inhibited (Stockdale and Topper, 1966; Turkington, 1968b).

The above data led Stockdale and Topper (1966) to hypothesize that mammary gland epithelium consists of two cell populations: a non-dividing group, which produces casein at a rate unaffected by the three hormones and responsible for base-line casein synthesis and a second group, which does not produce casein unless cell division

first occurs. This latter group undergoes mitosis under insulin stimulation and synthesizes casein only when hydrocortisone and prolactin are also present. The second group of cells is thought to have a limited functional life.

Further studies by Turkington and Topper (1967) indicate that androgens inhibit mammary DNA synthesis and prevent the augmentation of casein synthesis in vitro, but do not inhibit base-line casein synthesis. The rate of casein synthesis in the presence of varying concentrations of androgens was directly proportional to DNA synthesis, indicating that DNA synthesis is a prerequisite for hormone-dependent mammary differentiation.

The sequence of action of the three hormones in relation to cell cycle was studied by Lockwood, Stockdale, and Topper (1967a; 1967b). Since DNA synthesis and mitosis occur during the first 48 hours of culture but cease after 72 hours, these investigators postulate that insulin is required for the initiation of DNA synthesis and is also necessary during the  $G_1$  phase post-mitotically, that prolactin elicits differentiative responses after mitosis, while hydrocortisone action precedes that of prolactin, i.e. it is not able to act post-mitotically.

To study further the effect of insulin on initiation and maintenance of cell proliferation, Lockwood, Voytovich, Stockdale, and Topper (1967) assayed for DNA polymerase in explants of virgin mice. Their results suggested that insulin-dependent DNA synthesis is elicited at least partially by insulin-dependent de novo appearance of DNA polymerase activity. The finding that DNA polymerase activity

is maintained on the 4th and 5th days of culture when DNA synthesis is diminishing indicates that there are factors other than polymerase lacking which inhibit DNA synthesis after 5 days.

The mode of action of prolactin and human placental lactogen was further investigated by Turkington (1968b), who found that casein synthesis can be induced in explants cultured 96 hours in insulin and hydrocortisone only by addition of either prolactin or human placental lactogen. This post-mitotic induction of casein depends upon the continuous presence of insulin. RNA synthesis appears to precede milk protein synthesis. Addition of colchicine with prolactin after 96 hours of culture in the presence of insulin and hydrocortisone does not interfere with the action of prolactin.

From the results discussed above, it is apparent that the biochemical effects of the three hormones involved in mammary development in vitro (insulin, adrenal steroid, and prolactin) are vitally-linked to cell proliferation and protein synthesis. Most of the work has been focussed on functional differentiation, with emphasis on the triple hormonal requirement for maximum casein and RNA synthesis after two days culture and on the need for prolactin during this period. The important role of insulin in stimulating cell proliferation has also been emphasized. However, much less attention has been paid to the synergistic activities of the three hormones, their temporal sequence of activities, and the duration of their effects. Knowledge of the temporal sequence of hormone action in terms of the developmental events affected is of utmost importance in the elucidation of hormonal mechanisms.

In the present investigation, the activities of the eight possible combinations of the three hormones on mammary DNA and RNA were studied as functions of time. Accordingly, the purpose of this study was to analyze the sequence of action of insulin, corticosterone, and prolactin, individually and in various combinations, on mammary DNA and RNA synthesis in vitro. Concomitantly, an in vivo survey of mammary nucleic acid levels was made to obtain information on the patterns of change occurring during normal physiological development of the mouse strain used in this study. Preliminary studies were devoted to modifying DNA and RNA procedures for application to mammary explants.



## II. MATERIALS AND METHODS

### A. Preparation of Defatted-Dehydrated Mammary Extracts for Nucleic Acid Determination.

Animals (Swiss-albino mice) were mated at estrous. Pregnancy was dated from the following morning when a vaginal plug was noted. Mammary tissues were taken from 12, 15, and 19 day pregnant and 1, 3, 6, and 10 day lactating mice. The litter size of lactating mice was adjusted to 10 pups per animal to insure the suckling of all glands. The animals were killed by cervical dislocation, and the inguinal-abdominal mammary glands were removed and rapidly transferred to ice-cold 10% trichloroacetic acid (TCA) and weighed. All subsequent steps were carried out in the cold to minimize enzymic degradation. Tissues were minced finely with a scalpel and then homogenized in 10% TCA, washed three times with 10% TCA and twice with 1M sodium acetate in methanol. Lipids were then removed by washing the extract twice with methanol:chloroform (2:1), twice with 100% ethanol and once with ether. The ether was evaporated in a hood and the powder obtained was then placed into a dessicator under vacuum for two days. The dry weights of the defatted-dehydrated extracts (DDE) were recorded.

### B. Methods Used for Determination of Nucleic Acids.

#### 1. Development of the Method.

DNA and RNA were extracted and separated from the DDE by a modification of the Schmidt and Thannhauser method (1945) following recommendations by Hutchison and Munro (1961). Basically, the method

is as follows: the finely-minced tissue is extracted with acid and lipid solvents, the residue is then incubated in 1N potassium hydroxide at 37°C for at least 15 hours. DNA is precipitated by 6N hydrochloric acid and 5% TCA, while RNA and phosphoproteins remain in the filtrate. Phosphorus determination is used to estimate the amount of nucleic acids in each fraction. The effects of the modifications recommended by Hutchison and Munro (1961) on nucleic acid content of the mammary gland were tested and the results are described below.

a. Time of Alkaline Hydrolysis.

Hutchison and Munro (1961) recommended hydrolysis of tissue extract for 1 hour with 0.3 N potassium hydroxide at 37°C instead of 15 hours in 1N potassium hydroxide as originally described (Schmidt and Thannhauser, 1945). However, Denamur (1964) suggested a hydrolysis time of 18-24 hours for mammary tissues. Accordingly, the effects of 1, 3, 4, and 18 hours of alkaline hydrolysis on RNA and DNA content of mammary tissue were tested.

10 day lactating animals were used in this survey. RNA was estimated by U.V. absorption at 260 mμ and by the orcinol reaction (Mejbaum, 1939) and DNA was determined by U.V. absorption at 260 mμ and by the diphenylamine reaction of Dische (1930) as modified by Burton (1956). The results are summarized in Table I.

Excepting for the diphenylamine reaction at 18 hours of alkaline hydrolysis, no considerable differences were noted in DNA content by varying the time of alkaline hydrolysis. A hydrolysis time of 3 hours at 37°C was chosen, since at this time the RNA and DNA contents, as measured by U.V. and colorimetric reactions, agreed closely.

b. Time of Hot Acid Extraction of DNA.

Increasing the time of hot acid extraction from 30 minutes to 1 hour had no effect on the final estimation of DNA. In 10-11 day lactating mice, mammary DNA content was 20.7  $\mu\text{g}/\text{mg}$  DDE after 30 minutes as compared to 20.6  $\mu\text{g}/\text{mg}$  DDE after 1 hour of hot acid extraction as estimated by U.V. reading at 260  $\text{m}\mu$  and 15.8  $\mu\text{g}/\text{mg}$  DDE after 30 minutes as compared to 16.4  $\mu\text{g}/\text{mg}$  DDE after 1 hour as estimated by the diphenylamine reaction. Thus, the optimum temperature-time condition selected was 30 minutes at 70°C.

c. Recovery of DNA and RNA.

The RNA in the DNA fraction of 10 day lactating mice accounted for 8% of the total RNA as measured by the orcinol test. The contamination of the RNA fraction with DNA accounted for 26% of total DNA using the diphenylamine reaction (Table II). According to Tucker and Reece (1962), the material reacting with diphenylamine is not DNA. In a similar experiment, they indicated that the mammary RNA curve showed a bathochromic shift from the normal spectra of standard and mammary DNA. In the present study, mammary RNA gave an absorbance maximum at 670  $\text{m}\mu$  as compared to a maximum at 600  $\text{m}\mu$  in case of DNA curves. Increasing the time of alkaline hydrolysis to 15 hours decreased the amount of RNA contamination to 5% but DNA contamination was increased to more than 50%.

To check further the loss of DNA during the separation of RNA and DNA, 100  $\mu\text{g}$  of purified calf thymus DNA was added to the DDE of pregnant and lactating glands before separation of the nucleic acids. An average of 94% of the added DNA was recovered (Table III). With

tubes containing only 100  $\mu$ g purified DNA, there was 91.7% recovery of DNA even after subjection to the entire procedure used for separation and estimation of nucleic acids.

From the above results, it appears that less than 10% of DNA or RNA is lost during the procedure.

## 2. Method Used.

Duplicate 3 mg samples of the DDE were hydrolyzed in 0.6 ml of 0.3 N KOH for 3 hours at 37°C. Tubes were then transferred directly to an ice bath, and all the subsequent steps were carried out in the cold. The alkaline digest was neutralized with 0.51 ml of 0.3 N HCl, then acidified with 0.2 ml of 10% perchloric acid (PCA). The pH was checked at each step. Tubes were kept for 1 hour in the ice bath to insure complete precipitation of the DNA fraction. The supernatant was removed by centrifugation in a refrigerated centrifuge for 10 minutes at 4500 rpm. The precipitate was washed twice with 5% PCA, and the combined supernatants were adjusted to a final volume of 10 ml. Duplicate 3 ml samples were analyzed for RNA by the colorimetric orcinol reaction for ribose (Mejbaum, 1939). DNA was extracted from the precipitate with 2 ml 5% PCA at 70°C for 30 minutes. The precipitate was washed twice by centrifugation with 2 ml 5% PCA, and the combined supernatants were adjusted to a final volume of 6 ml. Duplicate 2 ml samples were analyzed for deoxyribose by the diphenylamine reaction of Dische (1930) as modified by Burton (1956).

RNA (purified yeast RNA) and DNA (calf thymus DNA) standards were run with each experiment. The standard curve obtained was used

to calculate mammary RNA and DNA content. 1 O.D. unit was found to be equivalent to 234.3  $\mu\text{g}$  RNA in the orcinol reaction and 298  $\mu\text{g}$  DNA in the diphenylamine reaction.

### C. Culture Technique.

#### 1. Mammary Tissues:

Tissues were taken from 12-day pregnant, nulliparous, Swiss-albino mice. Explants were dissected with fine scissors under sterile conditions from gland edges. They were roughly equal in size, each explant weighing about 0.5 mg. Explants were placed into a small petri-dish containing sterile hormone-free medium until transferred to culture plates.

#### 2. Culture Medium and Hormones:

The basal culture medium was chemically-defined hormone-free medium 199 (DIFCO Laboratories) to which 50 i.u./ml penicillin G was added. Bovine insulin (I) (Calbiochemical, Lot number 64693) was dissolved in a minimal amount of 0.005 N HCl and added to an appropriate volume of medium 199 to yield a stock solution of 100  $\mu\text{g}/\text{ml}$ . Ovine prolactin (PL) (NIH-P-S-4, NIH-P-S-7) was dissolved in a small volume of 0.0001 N NaOH to which medium 199 was added to yield a stock solution of 100  $\mu\text{g}/\text{ml}$ . Corticosterone (B) (Upjohn, Lot number VAN 1879) was dissolved in absolute alcohol to yield a stock solution of 100  $\mu\text{g}/\text{ml}$ . Protein hormone solutions were sterilized by passage through millipore filters with an average porosity of 0.45  $\mu$ .

The 8 different hormonal combinations used are as follows:  
medium 199 without hormone supplementation, insulin, corticosterone,

prolactin, insulin + corticosterone, insulin + prolactin, corticosterone + prolactin, and insulin + corticosterone + prolactin. The appropriate hormones were added to basal medium 199 to a final concentration of 5  $\mu$ g/ml (insulin and prolactin) and 1  $\mu$ g/ml (corticosterone).

### 3. Culture Method:

The watch-glass organ culture procedure (Elias and Rivera, 1959) was used with slight modifications to allow more explants to be cultured (Mayne, Barry, and Rivera, 1966). Each culture assembly consisted of a large Falcon petri-dish into which 3 circles of Whatman filter papers were placed and saturated with 5 ml sterile distilled water. 3 halves of smaller petri-dishes (35 X 10 mm) were placed on top of the filter papers. 3 ml of medium was pipetted into each of the small dishes.

75 randomly-selected explants, dissected from each of 2 animals, were added to each large plate. 25 explants were floated on the medium in each of the small petri-dishes. Cultures were placed in a plastic box and incubated at 37°C. A mixture of 95% O<sub>2</sub> and 5% CO<sub>2</sub> was bubbled through distilled water into the box. The pH of the media was maintained at 7.4 by adjusting the rate of gas flow.

Cultures were terminated after 1, 2, 3, and 5 days. In the case of 5-day cultures, the medium was changed on the third day. Duplicate cultures were usually prepared for every hormonal combination.

Tissue samples from each experiment were taken for histology to determine explant survival and secretory responses. They were fixed in Carnoy's solution, sectioned at 7  $\mu$ , and stained with

hematoxylin and eosin.

D. Isotope Incorporation Studies.

Explants were incubated with  $H^3$ -thymidine, 0.5  $\mu$ c/ml (Schwartz, Lot number 6706, specific activity 6 c/m mole) and either 0.5  $\mu$ c/ml  $H^3$ -uridine (Lot number 8600, specific activity 8 c/m mole) or 0.25  $\mu$ c/ml  $C^{14}$ -uridine (New England Nuclear, Lot number 373-166-1, specific activity 20-30 mc/m mole) 4 hours before termination of the cultures. After the 4-hour pulse period, tissues were blotted on filter paper and weighed. DDE was prepared as described in part A. RNA and DNA fractions were then separated and their contents determined. The soluble extracts were neutralized with 3 N NaOH. Triplicate 0.1 ml portions were pipetted onto small (23 mm) Whatman filter papers, left at room temperature overnight to dry, placed into scintillation vials, and counted in 10 ml of toluene-PPO-POPOP (1000 ml - 4 g - 0.5 g) solution in a liquid scintillation counter.

In vivo controls to determine the initial rate of RNA and DNA synthesis in pregnant mammary explants were also prepared. 12-day pregnant mice were injected intraperitoneally 30 minutes before killing with either  $H^3$ -thymidine (2.3  $\mu$ c/g body weight) or  $H^3$ -uridine (2  $\mu$ c/g body weight). DNA and RNA assays were performed as described above.

### III. RESULTS

The results obtained in this study are presented in three sections. The first is concerned with mammary nucleic acid content during pregnancy and lactation in vivo, the second, the effects of hormones on nucleic acid content in mammary tissues in vitro, and the third, the effects of hormones on precursor incorporation into mammary nucleic acids in vitro.

#### Nucleic Acid Content During Pregnancy and Lactation

Nucleic acid content of whole abdominal-inguinal mammary glands from 12, 15, and 19-day pregnant and 1, 3, 6, and 10-day lactating mice were determined according to the method outlined above.

The results obtained on DNA content are summarized in Table IV. Total mammary DNA per abdominal-inguinal glands from either side of the animal increased gradually during the course of secretory development. Total DNA content of 12-day pregnant glands averaged 0.77 mg and increased to a maximum of 3.40 mg on the 6th day of lactation. The amount on the 10th day of lactation remained fairly high, 3.15 mgs. On the other hand, DNA concentration ( $\mu\text{g DNA/mg DDE}$ ) increased from 43.28  $\mu\text{g}$  on the 12th day of pregnancy to 45.38  $\mu\text{g}$  on the 15th day of pregnancy, after which it decreased to 31.34  $\mu\text{g}$  on the 19th day. During lactation it decreased from 37.08  $\mu\text{g}$  on the first day to 19.90  $\mu\text{g}$  on the 6th day and reached 22.38  $\mu\text{g}$  on the 10th day of lactation.



No marked differences were noted in the amount of DNA/mg wet weight during pregnancy and lactation. The lowest value was 3.07  $\mu$ g DNA on the third day of lactation, and the highest was 3.60  $\mu$ g on the first day of lactation.

Results obtained on RNA content and RNA/DNA ratios are shown in Table V. Total RNA per abdominal-inguinal glands increased from 0.73 mg at 12 days of pregnancy to a maximum of 18.34 and 16.32 mgs on the 6th and 10th days of lactation. The amount of RNA/mg wet weight and the amount of RNA/mg DDE also showed a maximum on the 10th day of lactation.

The RNA/DNA ratio rose from 0.95 on the 12th day of pregnancy to 5.58 and 5.27 on the 6th and 10th day of lactation, respectively.

It should be noted that some variation occurred in nucleic acid content and RNA/DNA ratios between animals. Such variations might be due to variations in body weights, wet weight of the glands, or percentage of secretory epithelium among different animals.

#### Effect of Hormones on Nucleic Acid Content in Mammary Cultures

##### A. DNA Content.

The influence of various hormone combinations on DNA content is presented as a time study in Table VI. Initial control values were obtained in tissues prepared in the same manner as experimental explants, except that they were not cultured. In these in vitro experiments, the results are expressed as  $\mu$ g DNA/mg wet weight to minimize the effects of variations in size and weight of the

explants. Figure 1 indicates the percentage change in DNA content from initial control values.

1. 1-day cultures.

Explants cultured in medium without hormones and in medium containing corticosterone showed the lowest DNA contents of 1.72 and 1.76  $\mu$ g, respectively; these figures represent 20% and 18% decrease from initial controls. On the other hand, explants cultivated with insulin alone showed 41% increase in DNA over initial controls. The combination of corticosterone and insulin increased DNA content by only 20%, and prolactin alone and prolactin + corticosterone by 16% and 20%, respectively, of the initial controls. Insulin and prolactin together effected maximum increase in DNA content (54%) while the three hormones combined gave only 14.4% increase over initial controls.

2. 2-day cultures.

Explants cultured without hormones or with corticosterone alone showed little change from the initial controls. The DNA content of explants cultured with insulin alone was lower than in 1-day cultures but was still 15.7% greater than that present initially. Insulin + corticosterone or prolactin alone increased DNA by 31% and 26%, respectively. Insulin + prolactin and corticosterone + prolactin maintained the increase in DNA obtained on the first day, namely 54% and 23%, respectively. The 3-hormone combination further increased DNA content by 22%. As noted on the first day of culture, the greatest stimulation of DNA occurred with insulin + prolactin.

### 3. 3-day cultures.

DNA content decreased below initial control values in medium 199 alone and in media containing corticosterone or prolactin alone or corticosterone + prolactin. Explants cultured with insulin, insulin + corticosterone, and insulin + prolactin showed some increase over initial controls, but the percent increase was much less than that obtained on either the first or second day of culture. Maximal stimulation of DNA (48%) occurred in explants cultured with the 3-hormone combination.

### 4. 5-day cultures.

DNA content decreased below initial control levels in most of the hormone combinations, the lowest levels occurring in explants cultured in media without hormones or containing only corticosterone. Prolactin stimulated 23.6% increase over initial controls, an increase comparable to that obtained after 2 days in culture. As was apparent on the third day, the 3-hormone combination stimulated DNA by 41%, and this increase was greater than that stimulated by any other combination on the fifth day.

In summary, it appears that DNA content fell in hormone-free media or media containing corticosterone alone. The decrease in DNA was progressive and apparent from the first day of culture and reached its lowest levels on the fifth day. Media containing insulin, either alone or in combination with corticosterone or prolactin, increased DNA content up to the third day of culture. By the fifth day, the 3-hormone combination was the most effective in maintaining increased DNA levels.

## B. RNA Content.

The influence of various hormones on RNA content is summarized in Table VII and Figure 2. Figure 2 shows the percent change in RNA content from the initial control value of 2.4  $\mu$ g DNA/mg wet weight.

### 1. 1-day cultures.

The hormone-free system showed a decrease in RNA of almost 31% from that of the initial controls. Corticosterone alone showed an even greater inhibitory effect, 47% that of the initial controls. On the other hand, RNA content was increased by insulin alone, prolactin alone, insulin + corticosterone, insulin + prolactin, and insulin + corticosterone + prolactin. The 3-hormone combination stimulated RNA to a lesser extent than did the other combinations.

### 2. 2-day cultures.

As on the first day, hormone-free media and media containing only corticosterone caused a decrease in RNA content relative to the initial controls. Only media containing insulin + prolactin or insulin + prolactin + corticosterone increased RNA content above the levels obtained in 1-day cultures. All other hormone combinations had a lesser effect on RNA than on the first day of culture.

### 3. 3-day cultures.

RNA content decreased further in hormone-free media and media containing only corticosterone. RNA also fell below initial control levels in tissues cultured with prolactin, insulin + corticosterone, and corticosterone + prolactin. Small increases in RNA content were obtained with insulin and insulin + prolactin, but the maximum

increase at 3 days (32%) was obtained in the presence of the three hormones.

#### 4. 5-day cultures.

RNA content was below initial control levels with all hormone combinations except for insulin + corticosterone and insulin + corticosterone + prolactin. Even with the 3-hormone combination the maximum increase in RNA content was only 13%.

In summary, RNA content decreased below initial control levels in media without hormones and media containing only corticosterone, the latter showing a greater inhibitory effect than the former throughout the 5-day culture period. Insulin alone increased RNA content during the first three days of culture, after which its effect was decreased by 10% from initial controls. Prolactin alone stimulated RNA synthesis on the first day of culture, but required synergism with insulin for maximal stimulation on the second day. The 3-hormone combination also stimulated RNA content on the second day, and although the level decreased on the 3rd and 5th days, it remained above initial controls and all other hormonal combinations at these later culture periods.

### Effect of Hormones on Rates of

### Nucleic Acid Synthesis in Mammary Cultures

#### A. DNA Synthesis.

Tables VIII and IX summarize the effects of various hormone combinations on the incorporation of  $H^3$ -thymidine by mammary explants. In Table VIII the results are expressed as specific radioactivity of

DNA (DPM/ $\mu$ g DNA) and in Table IX, as DPM/unit wet wt. of tissue. The patterns of incorporation did not differ according to the way the data were expressed. The rate of DNA synthesis in the in vivo control was 60 DPM/ $\mu$ g DNA or 189 DPM/mg wet wt.

The highest incorporation was obtained on the first day in culture, but fell in all explants with time. In general, higher rates of incorporation were obtained in explants cultured with insulin-containing media, i.e. insulin alone, insulin + corticosterone, insulin + prolactin, and insulin + corticosterone + prolactin.

#### B. RNA Synthesis.

The effects of the various hormone combinations on RNA synthesis are summarized in Tables X and XI. In Table X the results are expressed as specific radioactivity of RNA (DPM/ $\mu$ g RNA) and in Table XI, as DPM/mg wet wt. The rates of RNA synthesis in the in vivo control were 6 DPM/ $\mu$ g RNA or 20 DPM/mg wet wt.

The rate of incorporation of labelled uridine into RNA, measured as DPM/mg wet wt., was highest in 1-day cultures. In the absence of hormones the rate remained nearly constant on the 2nd, 3rd, and 5th days of culture. With corticosterone, incorporation was always lower than with the hormone-free system, while with prolactin alone, it remained roughly constant during the first 3 days of culture. Combinations containing insulin generally stimulated higher rates of incorporation than did insulin-free medium. Except for the first day, the highest rate of incorporation occurred in explants cultured with the three-hormone combination. As regards the specific radioactivity

measurements, no specific pattern was apparent. The initial rate of RNA synthesis in vivo was extremely low, and all hormone combinations, including the hormone-free system, augmented RNA synthesis relative to the initial control throughout the entire culture period.

### C. Statistical Analysis

The in vitro data were subjected to the four factor analysis of variance test to determine whether there were significant differences in nucleic acid content or synthesis in time and/or in response to the different hormone combinations. The results are presented in Tables XII, XIII, XIV, and XV. Insulin-containing, corticosterone-containing, and prolactin-containing combinations were analyzed, i.e. individual combinations were not considered as separate factors. However, individual hormones or combinations thereof, although not statistically analyzed, will be discussed below.

As Table XII indicates, the average DNA content of explants cultured in all insulin-containing combinations was significantly greater ( $P < 0.01$ ) than in explants cultured in its absence. The presence of prolactin also increased the content of DNA significantly ( $P < 0.05$ ). There were no significant differences ( $P > 0.05$ ) in DNA content after different times in culture.

On the other hand, Table XIII shows that the rate of  $H^3$ -thymidine incorporation decreased with time ( $P < 0.01$ ). It also shows that insulin increased the rate of DNA synthesis ( $P < 0.01$ ) whereas corticosterone and prolactin had no significant effect ( $P > 0.05$ ). The effect of time on explants cultured in the presence of insulin differed significantly ( $P < 0.01$ ) from its effect on

explants cultured in other media, indicating that although rates of synthesis decreased with time in all systems, the rate or trend of decrease differed according to the presence or absence of insulin.

Statistical analysis of the hormonal effects on RNA content and synthesis are presented in Tables XIV and XV, respectively. In both cases, the effects of hormones decreased significantly ( $P < 0.01$ ) with time. Insulin was the only hormone to increase significantly ( $P < 0.01$ ) both RNA content and synthesis. The effect of insulin on RNA content was increased significantly ( $P < 0.05$ ) in the presence of corticosterone, while prolactin-containing combinations increased RNA content ( $P < 0.05$ ). The lack of significant differences ( $P > 0.05$ ) between different hormone combinations with time indicates that the temporal decrease in RNA synthesis is independent of the hormonal environment.

### Histology

Tissue samples were examined from each culture and the responses of alveoli were graded according to the following criteria for maintenance of alveolar structure and level of secretory activity of surviving alveoli.

#### a. survival of alveoli

- most alveoli degenerate
- + some alveoli maintained
- ++ most alveoli maintained

#### b. secretory activity of maintained alveoli

- most alveoli small; little or no stainable secretion in lumina; few cells with vacuoles



- + alveoli moderately distended; small amounts of stainable secretion in lumina; moderate number of cells with vacuoles
- ++ alveoli greatly distended; large amounts of stainable secretion in lumina; most cells with vacuoles

The results are presented in Table XVI. Explants in all systems were completely or partially-maintained at the end of the first day of culture. By the second day, extensive breakdown of alveolar structure occurred in cultures without hormones. Alveoli were maintained by all media containing insulin, but only partly-maintained by all other hormonal media. On the 3rd and 5th days, alveoli were maintained only by media containing insulin + corticosterone or the three-hormone combination and partially-maintained in media containing insulin and insulin + prolactin.

No secretory activity was noted in explants cultured in media without hormones or in the presence of insulin, corticosterone, or prolactin alone or insulin + corticosterone during the entire culture period. Explants cultured with insulin + prolactin showed, after 1 day of culture, an increase in the size of alveolar lumina, containing small amounts of stainable material. This response was increased to some extent on the second day and persisted throughout the 5-day culture period. Explants cultured with corticosterone + prolactin contained surviving cells that were vacuolated and contained small amounts of secretion in the alveolar lumina. However, no secretion was noted in these cultures on the 3rd or 5th days. In contrast to the other systems, the three-hormone combination effected a

progressive increase in the amount of stainable material in the alveolar lumina. The amount of secretion was small and the alveoli were only moderately distended on the first day, but these responses increased considerably by the second day and were maintained through the 3rd and 5th days of culture.

TABLE I: Influence of Time of Alkaline Hydrolysis on RNA and DNA  
Content<sup>\*\*</sup> of the Mammary Gland

Time of alkaline hydrolysis <sup>*</sup>	RNA ( $\mu$ g/mg DDE)		DNA ( $\mu$ g/mg DDE)	
	U.V. at 260 m $\mu$	orcinol	U.V. at 260 m $\mu$	diphenyl- amine
1 hour	111.7	110.2	19.7	14.7
3 hours	117.5	117.9	20.7	16.1
4 hours	116.1	110.1	22.5	19.5
18 hours	122.9	115.9	20.5	6.6

\* Temperature of incubation was 37°C.

\*\* DDE from 10-day lactating mice.

TABLE II: DNA and RNA Recovery<sup>\*\*</sup>

Time of alkaline hydrolysis <sup>*</sup>	RNA ( $\mu\text{g}/\text{mg}$ DDE)			DNA ( $\mu\text{g}/\text{mg}$ DDE)		
	in RNA fraction	in DNA fraction	% contamination	in DNA fraction	in RNA fraction	% contamination
3 hours	115.4	9.6	8.2	25.7	6.7	26.0
15 hours	112.5	5.6	4.9	26.1	13.8	52.8

\* Temperature of incubation was 37°C.

\*\* DDE from 10-day lactating mice.

TABLE III: Percent Recovery of Known Amounts of Purified DNA Added to 3 mgs DDE

Stage of Development	DNA content/3 mg DDE without addition of DNA ( $\mu\text{g}$ )	DNA content/3 mg DDE with added 100 $\mu\text{g}$ pure DNA ( $\mu\text{g}$ )	% recovery of added DNA
15 day pregnant	124.7	221.9	97.2
1 day lactating	119.6	205.8	86.2
1 day lactating	136.8	240.0	103.2
3 day lactating	80.9	170.1	89.2

TABLE IV: DNA Content of Mammary Glands of Pregnant and Lactating Mice

Stage of Development	No. of mice	Total DNA (mgs)*	DNA ( $\mu$ g/mg DDE)*	DNA ( $\mu$ g/mg wet wt.)*
12 days pregnant	8	0.77 $\pm$ 0.05	43.28 $\pm$ 2.10	3.31 $\pm$ 0.24
15 days pregnant	8	1.06 $\pm$ 0.11	45.38 $\pm$ 3.07	3.43 $\pm$ 0.57
19 days pregnant	4	2.12 $\pm$ 0.33	31.33 $\pm$ 2.76	3.11 $\pm$ 0.31
1 day lactating	8	2.23 $\pm$ 0.22	37.07 $\pm$ 2.19	3.59 $\pm$ 0.53
3 days lactating	6	2.43 $\pm$ 0.33	30.03 $\pm$ 2.76	3.06 $\pm$ 0.32
6 days lactating	5	3.40 $\pm$ 0.47	19.89 $\pm$ 2.54	3.47 $\pm$ 0.56
10 days lactating	6	3.14 $\pm$ 0.61	22.38 $\pm$ 0.83	3.21 $\pm$ 0.40

\* Means and standard errors

TABLE V: RNA Content and RNA/DNA Ratios in Mammary Glands of Pregnant and Lactating Mice

Stage of development	No. of mice	Total RNA (mgs)*	RNA ( $\mu$ g/mg DDE)*	RNA ( $\mu$ g/mg wet wt.)*	RNA/DNA*
12 days pregnant	8	0.73 $\pm$ 0.08	40.52 $\pm$ 3.18	3.12 $\pm$ 0.31	0.96 $\pm$ 0.07
15 days pregnant	8	1.34 $\pm$ 0.27	54.63 $\pm$ 5.67	4.28 $\pm$ 0.91	1.22 $\pm$ 0.12
19 days pregnant	4	4.65 $\pm$ 1.00	67.03 $\pm$ 5.49	6.66 $\pm$ 0.84	2.21 $\pm$ 0.26
1 day lactating	8	5.98 $\pm$ 0.82	94.70 $\pm$ 5.38	9.70 $\pm$ 1.68	2.61 $\pm$ 0.24
3 days lactating	6	8.83 $\pm$ 1.59	101.50 $\pm$ 6.25	10.98 $\pm$ 1.82	3.53 $\pm$ 0.27
6 days lactating	5	18.34 $\pm$ 2.18	105.06 $\pm$ 6.53	18.33 $\pm$ 2.30	5.58 $\pm$ 0.68
10 days lactating	6	16.32 $\pm$ 2.98	117.15 $\pm$ 4.15	16.83 $\pm$ 2.24	5.27 $\pm$ 0.24

\* Means and standard errors

TABLE VI: Influence of Hormones on Mammary DNA in Vitro.

Hormone Addition	DNA content ( $\mu\text{g}/\text{mg}$ wet wt.) * at time intervals indicated				
	0	1 day	2 days	3 days	5 days
No hormones		1.72 $\pm$ 0.04	2.20 $\pm$ 0.12	1.43 $\pm$ 0.34	1.44 $\pm$ 0.43
I		3.05 $\pm$ 1.16	2.50 $\pm$ 0.15	2.40 $\pm$ 0.36***	1.98 $\pm$ 0.34
B		1.76 $\pm$ 0.67	2.16 $\pm$ 0.11	1.93 $\pm$ 0.17	1.46 $\pm$ 0.14
PL		2.51 $\pm$ 1.03	2.72 $\pm$ 0.85	2.06 $\pm$ 0.47	2.67 $\pm$ 0.73
I + B		2.61 $\pm$ 0.86	2.82 $\pm$ 0.20	2.38 $\pm$ 0.15***	2.00 $\pm$ 0.01
I + PL		3.33 $\pm$ 1.32	3.32 $\pm$ 1.12	2.23 $\pm$ 0.83	2.14 $\pm$ 0.12
B + PL		2.61 $\pm$ 1.00	2.67**	1.76 $\pm$ 0.45	2.24 $\pm$ 0.10
I + B + PL		2.47 $\pm$ 0.57	2.63 $\pm$ 0.01	3.19 $\pm$ 0.29	3.04 $\pm$ 0.72
Initial control	2.16 $\pm$ 0.43				

\* Means and standard errors of duplicate experiments

\*\* Result of single experiment

\*\*\* Mean of four experiments

TABLE VII: Influence of Hormones on Mammary RNA in Vitro.

Hormone Addition	RNA content ( $\mu\text{g}/\text{mg}$ wet wt.) * at time intervals indicated				
	0	1 day	2 days	3 days	5 days
No hormones		1.65 $\pm$ 0.10	1.98 $\pm$ 0.12	1.47 $\pm$ 0.47	1.82 $\pm$ 0.15
I		3.03 $\pm$ 0.70	2.64 $\pm$ 0.20	2.54 $\pm$ 0.30***	2.15 $\pm$ 0.16
B		1.27 $\pm$ 0.43	1.67 $\pm$ 0.04	1.28 $\pm$ 0.05	1.04 $\pm$ 0.10
PL		3.47 $\pm$ 0.82	2.74 $\pm$ 0.14	2.04 $\pm$ 0.38	2.40 $\pm$ 0.35
I + B		3.28 $\pm$ 0.10	2.63 $\pm$ 0.24	2.30 $\pm$ 0.19***	2.48 $\pm$ 0.87
I + PL		3.33 $\pm$ 1.14	3.68 $\pm$ 0.35	2.65 $\pm$ 1.14	1.93 $\pm$ 0.03
B + PL		1.89 $\pm$ 0.50	2.49**	1.73 $\pm$ 0.61	0.61 $\pm$ 0.15
I + B + PL		2.77 $\pm$ 0.27	3.46 $\pm$ 0.39	3.16 $\pm$ 0.05	2.71 $\pm$ 0.58
Initial control	2.40 $\pm$ 0.67				

\* Mean and standard errors of duplicate experiments

\*\* Result of single experiment

\*\*\* Mean of four experiments



TABLE VIII: Hormonal Effects on Mammary DNA Synthesis in Vitro.

Hormone Addition	Specific radioactivity of DNA (DPM/ $\mu$ g DNA) <sup>*</sup> at time intervals indicated				
	0	1 day	2 days	3 days	5 days
No hormones		148 $\pm$ 22	29 $\pm$ 12**	43 $\pm$ 23	19 $\pm$ 8
I		966 $\pm$ 281	162 $\pm$ 44**	68 $\pm$ 35**	102 $\pm$ 67
B		212 $\pm$ 24	26 $\pm$ 10**	13 $\pm$ 12	-
PL		125 $\pm$ 74	22 $\pm$ 17	5 $\pm$ 3	19 $\pm$ 8
I + B		1058 $\pm$ 117	111 $\pm$ 47**	102 $\pm$ 57**	56 $\pm$ 32
I + PL		874 $\pm$ 396	193 $\pm$ 89	34 $\pm$ 23	122 $\pm$ 38**
B + PL		141 $\pm$ 102	61 $\pm$ 58	-	-
I + B + PL		793 $\pm$ 445	400 $\pm$ 254	80 $\pm$ 11	74 $\pm$ 17**
Initial control	60 $\pm$ 0.18				

\* Means and standard errors of duplicate experiments

\*\* Mean of triplicate experiments

TABLE IX: Hormonal Effects on Mammary DNA Synthesis in Vitro.

Hormone Addition	$H^3$ -thymidine incorporated (DPM/mg wet wt.)* at time intervals indicated				
	0	1 day	2 days	3 days	5 days
No hormones		256 $\pm$ 44	71 $\pm$ 32**	54 $\pm$ 19	50 $\pm$ 30
I		3269 $\pm$ 1980	363 $\pm$ 98**	157 $\pm$ 49**	179 $\pm$ 98
B		390 $\pm$ 184	56 $\pm$ 22**	22 $\pm$ 22	1 $\pm$ 1
PL		235 $\pm$ 55	45 $\pm$ 27	9 $\pm$ 5	58 $\pm$ 37
I + B		2864 $\pm$ 1214	198 $\pm$ 135	233 $\pm$ 136**	102 $\pm$ 55
I + PL		2381 $\pm$ 159	540 $\pm$ 78	56 $\pm$ 23	229 $\pm$ 136
B + PL		264 $\pm$ 124	166 $\pm$ 154	1 $\pm$ 0.5	-
I + B + PL		1683 $\pm$ 624	1047 $\pm$ 662	252 $\pm$ 12	176 $\pm$ 26
Initial control	189 $\pm$ 19				

\* Means and standard errors of duplicate experiments

\*\* Mean of triplicate experiments

TABLE X: Hormonal Effects on Mammary RNA Synthesis in Vitro.

Hormone Addition	Specific radioactivity of RNA (DPM/ $\mu$ g RNA)* at time intervals indicated				
	0	1 day	2 days	3 days	5 days
No hormones		374 $\pm$ 184	110 $\pm$ 38	264 $\pm$ 41	165 $\pm$ 43
I		554 $\pm$ 333	243 $\pm$ 136	253 $\pm$ 27	202 $\pm$ 114
B		271 $\pm$ 137	161 $\pm$ 145	250 $\pm$ 14	150 $\pm$ 70
PL		95 $\pm$ 22	132 $\pm$ 77	160 $\pm$ 78	180 $\pm$ 175
I + B		370 $\pm$ 182	219 $\pm$ 166	374 $\pm$ 19	194 $\pm$ 4
I + PL		641 $\pm$ 437	229 $\pm$ 83	186 $\pm$ 159	259 $\pm$ 158
B + PL		243 $\pm$ 183	223 $\pm$ 207	93 $\pm$ 84	358**
I + B + PL		576 $\pm$ 337	299 $\pm$ 130	314 $\pm$ 161	240 $\pm$ 126
Initial control	6 $\pm$ 0.67				

\* Means and standard errors of duplicate experiments

\*\* Result of single experiment

TABLE XI: Hormonal Effects on Mammary RNA Synthesis in Vitro.

Hormone Addition	$H^3$ or $C^{14}$ -Uridine incorporated (DPM/mg wet wt.)* at time intervals indicated				
	0	1 day	2 days	3 days	5 days
No hormones		635 $\pm$ 343	362 $\pm$ 229	368 $\pm$ 64	378 $\pm$ 137
I		1441 $\pm$ 616	587 $\pm$ 282	658 $\pm$ 98	451 $\pm$ 277
B		286 $\pm$ 59	258 $\pm$ 230	319 $\pm$ 6	80 $\pm$ 4
PL		344 $\pm$ 152	369 $\pm$ 228	334 $\pm$ 60	493 $\pm$ 487
I + B		1195 $\pm$ 558	536 $\pm$ 384	744 $\pm$ 49	320 $\pm$ 3
I + PL		1635 $\pm$ 725	812 $\pm$ 222	306 $\pm$ 203	494 $\pm$ 296
B + PL		367 $\pm$ 225	579 $\pm$ 488	108 $\pm$ 87	165**
I + B + PL		1503 $\pm$ 778	1085 $\pm$ 567	980 $\pm$ 489	721 $\pm$ 478
Initial control	20 $\pm$ 6				

\* Means and standard errors of duplicate experiments

\*\* Result of single experiment

TABLE XII: Effect of Hormones on DNA Content in Vitro.

## Analysis of Variance

Source	Sum of Squares	Degrees of Freedom	Mean Square	F values
Total	41.71	63	0.66	1.38
Replication	9.46	1	9.46	19.71 P < 0.01
Time (T)	2.67	3	0.89	1.85
I	5.22	1	5.22	10.88 P < 0.01
B	0.00	1	0.00	-
PL	3.49	1	3.49	7.27 P < 0.05
I+B	0.01	1	0.01	0.02
I+PL	0.54	1	0.54	1.13
B+PL	0.03	1	0.03	0.06
I+B+PL	0.20	1	0.20	0.42
T×Hormone	-	21	-	-
T×I	0.70	3	0.23	0.48
T×B	0.64	3	0.21	0.44
T×PL	0.75	3	0.25	0.52
T×I+B	1.01	3	0.34	0.71
T×I+PL	0.25	3	0.08	0.17
T×B+PL	0.33	3	0.11	0.23
T×I+B+PL	1.67	3	0.56	1.17
Error	14.74	31	0.48	-

Note: Insulin-containing, corticosterone-containing, and prolactin-containing combinations were analyzed, i.e. individual combinations were not considered as separate factors.

TABLE XIII: Effect of Hormones on DNA Synthesis in Vitro.

## Analysis of Variance

Source	Sum of Squares	Degrees of Freedom	Mean Square	F values
Total	6,542,550	63	103,850	3.04
Replication	81,725	1	81,725	2.39
Time (T)	2,668,378	3	889,459	25.99 P < 0.01
I	1,191,645	1	1,191,645	34.82 P < 0.01
B	2,957	1	2,957	0.08
PL	3,555	1	3,555	0.10
I+B	878	1	878	0.03
I+PL	71	1	71	-
B+PL	777	1	777	0.02
I+B+PL	445	1	445	.01
T×H	-	21	-	-
T×I	1,330,490	3	443,497	12.96 P < 0.01
T×B	13,242	3	4,414	0.13
T×PL	89,716	3	29,905	0.87
T×I+B	7,930	3	2,643	0.08
T×I+PL	39,485	3	13,162	0.38
T×B+PL	34,057	3	11,352	0.33
T×I+B+PL	16,480	3	5,493	0.16
Error	1,060,719	31	34,217	-

Note: Insulin-containing, corticosterone-containing, and prolactin-containing combinations were analyzed, i.e. individual combinations were not considered as separate factors.

TABLE XIV: Effect of Hormones on RNA Content in Vitro.

## Analysis of Variance

Source	Sum of Squares	Degrees of Freedom	Mean Square	F values
Total	50.72	63	0.81	1.84
Replication	0.90	1	0.90	2.05
Time (T)	6.31	3	2.10	4.77 P < 0.01
I	14.81	1	14.81	33.65 P < 0.01
B	1.41	1	1.41	3.20
PL	3.11	1	3.11	7.06 P < 0.05
I+B	2.50	1	2.50	5.68 P < 0.05
I+PL	0.93	1	0.93	2.11
B+PL	0.63	1	0.63	1.43
I+B+PL	0.04	1	0.04	0.09
T×H	-	21	-	-
T×I	0.17	3	0.06	0.14
T×B	0.56	3	0.19	0.43
T×PL	1.81	3	0.60	1.36
T×I+B	1.70	3	0.57	1.30
T×I+PL	0.88	3	0.29	0.66
T×B+PL	0.58	3	0.19	0.43
T×I+B+PL	0.76	3	0.25	0.57
Error	13.62	31	0.44	-

Note: Insulin-containing, corticosterone-containing, and prolactin-containing combinations were analyzed, i.e. individual combinations were not considered as separate factors.

TABLE XV: Effect of Hormones on RNA Synthesis in Vitro.

## Analysis of Variance

Source	Sum of Squares	Degrees of Freedom	Mean Square	F values
Total	2,750,801	63	43,664	2.04
Replication	999,000	1	999,000	46.57 P < 0.01
Time (T)	361,933	3	120,644	5.63 P < 0.01
I	231,121	1	231,121	10.77 P < 0.01
B	5,293	1	5,293	0.25
PL	324	1	324	0.02
I+B	3,843	1	3,843	0.18
I+PL	22,275	1	22,275	1.04
B+PL	25,520	1	25,520	1.19
I+B+PL	3,081	1	3,081	0.14
T×H	-	21	-	-
T×I	170,463	3	56,821	2.65
T×B	25,687	3	8,562	0.40
T×PL	69,674	3	23,225	1.08
T×I+B	56,387	3	18,796	0.88
T×I+PL	75,887	3	25,296	1.18
T×B+PL	22,028	3	7,343	0.34
T×I+B+PL	13,307	3	4,436	0.21
Error	664,978	31	21,451	-

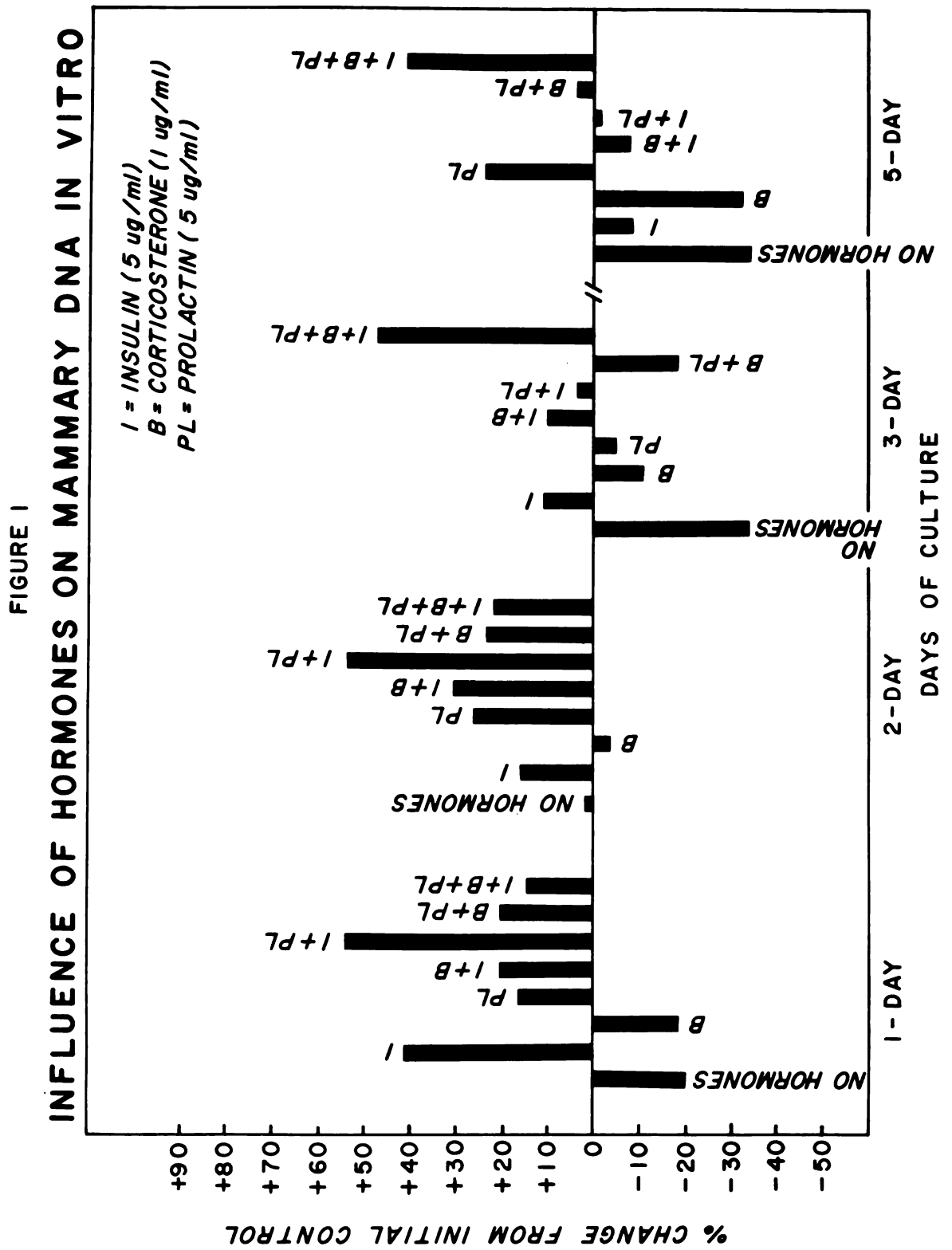
Note: Insulin-containing, corticosterone-containing, and prolactin-containing combinations were analyzed, i.e. individual combinations were not considered as separate factors.

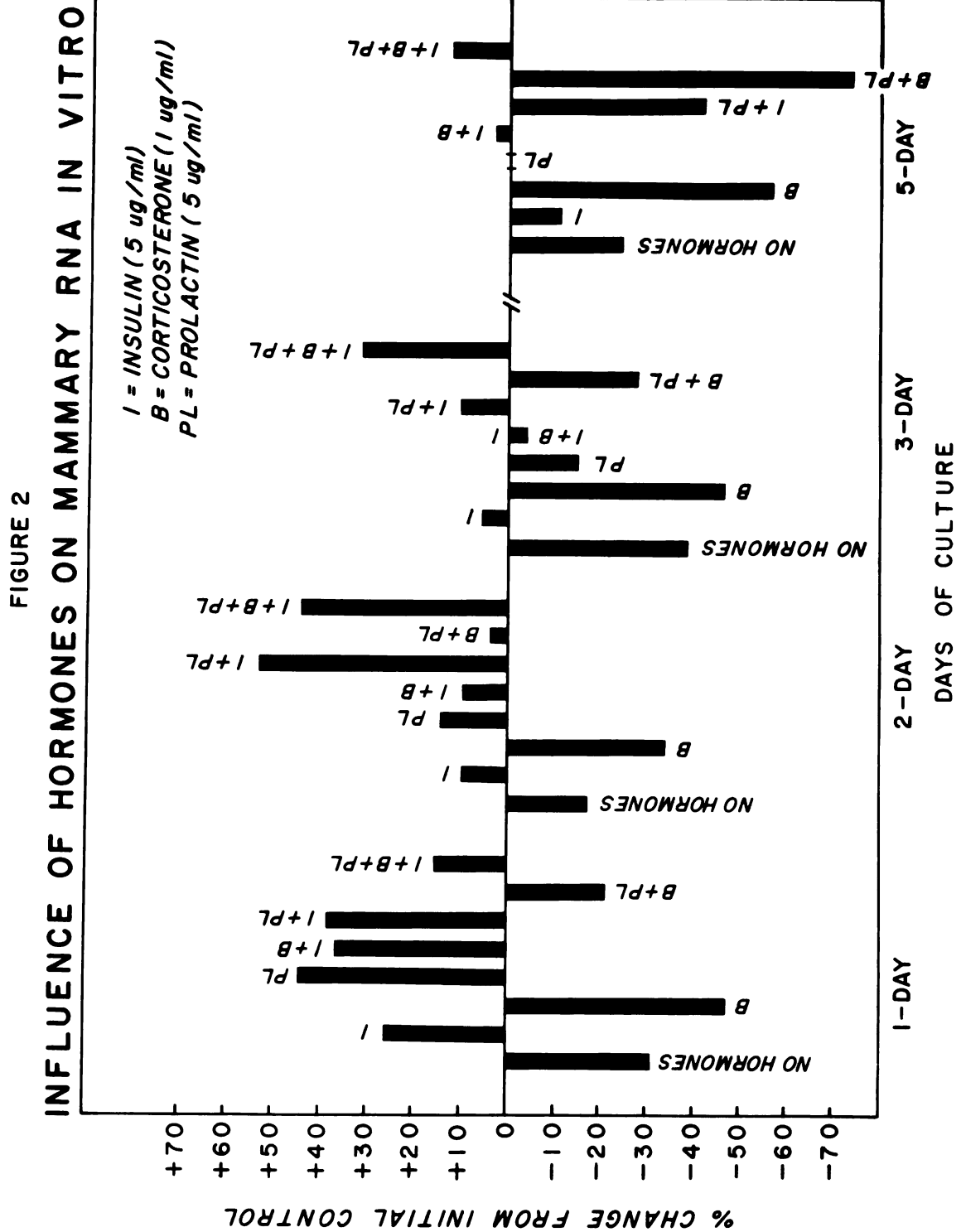


TABLE XVI: Histological Responses of Mammary Explants to Hormones in Organ Culture

Hormone Addition	Responses* of mammary explants at time intervals indicated							
	1 day		2 days		3 days		5 days	
	maintenance of alveoli	secretory activity	maintenance of alveoli	secretory activity	maintenance of alveoli	secretory activity	maintenance of alveoli	secretory activity
No hormones	+	-	-	-	-	-	-	-
I	++	-	++	-	++	-	+	-
B	++	-	+	-	+	-	-	-
PL	+	-	+	-	-	-	-	-
I+B	++	-	++	-	++	-	++	-
I+PL	++	+	++	+	-	+	+	+
B+PL	++	-	+	+	-	-	-	-
I+B+PL	++	+	++	++	++	++	++	++

\* see text for explanation of symbols





#### IV. DISCUSSION

The aim of this study, as stated in the introduction, was to analyze the sequence of action of the three hormones individually and in various combinations on DNA and RNA synthesis in vitro. Before discussing the temporal effects of these hormones in vitro, a brief account will be given of the changes occurring during the normal development of intact mammary glands of Swiss-albino mice and of the histological appearance of the cultured tissues.

##### A. Nucleic Acid Content During Pregnancy and Lactation.

The in vivo study of mammary nucleic acid levels in this strain of mouse was done on intact abdominal-inguinal glands. Total DNA increased steadily from the 12th day of pregnancy reaching a maximum on the 6th day of lactation, at which time total DNA was 4.3 times greater than on the 12th day of pregnancy. These results confirm previous reports that growth continues throughout lactation (Lewin, 1957; Brookreson and Turner, 1959; Munford, 1963) and that maximum growth occurs on the 6th day (Lewin, 1957) and not on the 14th day post-partum as reported by Brookreson and Turner (1959) and Wada and Turner (1959).

Total RNA increased markedly with the onset of lactation. Both RNA content and RNA/DNA ratios reached maximum values on the 6th day of lactation. According to Mizuno (1961) and Munford (1964) the decline in lactational function results from the decrease in number and

functional activity of the mammary cells.

DNA concentration expressed on a wet weight basis showed no change during the period analyzed, whereas if expressed on a dry weight basis, a decrease occurred during the course of lactation, reaching its lowest value ( $19.8 \mu\text{g}/\text{mg DDE}$ ) on the 6th day post-partum. If concentration of DNA is regarded as a measure of cell size (Munford, 1964), the decrease in concentration may be due largely to increased milk content of the gland. RNA concentration expressed on a wet weight basis showed the same pattern of change as did total RNA. Since DNA content/ $\mu\text{g}$  wet wt. is approximately constant, this method of expressing RNA can be taken to assess the functional state of the cells.

#### B. Effects of Hormones on Morphology of Explants.

The histological observations are in agreement with and extend previous reports (Rivera and Bern, 1961; Stockdale, Juergens, and Topper, 1966; Mayne, Forsyth, and Barry, 1968). Explants regardless of the medium (hormone-free and hormone-containing) are completely or partially-maintained at the end of the first day of culture. It was not until the end of the second day that the insulin requirement for tissue survival became apparent. Degeneration of alveoli occurred in explants cultivated with prolactin alone, supporting earlier experiments (Elias, 1957). Prolactin in combination with insulin or insulin + corticosterone stimulated secretory vacuoles in the alveolar cells as well as secretion in the alveolar lumina throughout the five days of culture. Corticosterone + prolactin also stimulated cellular

vacuolation in maintained alveoli on the second day, but not on the 3rd or 5th days of culture, suggesting that the need for insulin for secretion might be only for maintenance of alveolar structure.

C. Effects of Hormones on Mammary DNA in Vitro.

Considerable variation was found between results per given experiment as shown by the high standard errors. But since each experiment was done only in duplicate, and since the pattern of hormonal effect between experiments did not change, variations in the absolute values may only reflect inherent variations in hormonal responsiveness between tissues from different animals.

The initial rate of DNA synthesis in vivo was estimated by injecting  $2.3 \mu\text{c H}^3\text{-thymidine/g}$  body weight. The result obtained (60 DPM/ $\mu\text{g}$  DNA or 189 DPM/mg wet wt.) was much lower than those obtained in all explants cultured for one day. Higher quantities of radioisotope might have given a higher initial in vivo rate. However, if the rate of incorporation of DNA precursor is independent of the amount of precursor injected, then one can only conclude that in vitro conditions permit mammary cell proliferation to occur at a rate greater than in vivo, at least during the first day of culture.

Analysis of variance revealed significant differences ( $P < 0.01$ ) between the amount of DNA synthesized at different times of culture. The rate of synthesis was highest during the first day of culture. Incorporation rates fell considerably by the 2nd day and even more on the 3rd and 5th days. In accordance with the data of Stockdale and Topper (1966), insulin was the only hormone required for

initiation of DNA synthesis on the first day. All media containing insulin increased DNA synthesis significantly more than did media deficient in insulin, irrespective of the hormone supplement. On the first day, explants cultured with insulin alone showed the highest rate of synthesis. On the second day, the rate fell considerably in tissues cultured with insulin or insulin + corticosterone, but fell to a much lesser extent in tissues cultured with insulin + prolactin, and even less in the presence of all three hormones. By the third day, DNA synthesis fell considerably in all of the above cultures, the insulin + prolactin medium stimulating the lowest rate of synthesis. In the above cultures, the biochemical results corresponded well with the histological findings. These results suggest that the increased rate of DNA synthesis on the first and second days is required for the maintenance and growth of mammary explants.

As regards DNA content, all combinations containing insulin stimulated DNA to an extent significantly greater than did media without insulin. The high rate of precursor incorporation on the first day is accompanied by an increase in DNA content relative to the initial controls. These data indicate that the increased incorporation does, in fact, reflect increased synthesis and not increased uptake of precursor.

Tucker, Paape, and Sinha (1967) in an in vivo study showed that ACTH was not of major importance in promoting mammary development resulting from increasing the stimulus of suckling. Cortisol in lower doses had no influence on DNA content of pregnant rabbits after five days of administration (Denamur, 1964). Our results show that

corticosterone alone has no effect on DNA synthesis and DNA content, which were nearly equivalent to values obtained with hormone-free control and below initial control levels. However, corticosterone in combination with insulin increased DNA content to a maximum on the second day, which suggests that the corticosterone effect is detectable only subsequent to a stimulatory effect of insulin on DNA.

Prolactin alone or in combination with corticosterone had no effect on DNA synthesis, and these cultures showed complete degeneration of the alveoli by the third day of culture. Thus, the DNA content of tissues cultivated with prolactin alone may only represent that of the adipose tissue, whose survival may be independent of hormones. Statistical analysis showed that combinations containing prolactin significantly ( $P < 0.05$ ) increased DNA content over those lacking it. This increase is mainly due to the combinations containing insulin + prolactin and insulin + corticosterone + prolactin.

The triple hormone system showed a high incorporation rate on the second day, which was followed by an increase in DNA content of more than 40% from the initial control and over 30% the DNA content of explants cultured with insulin + corticosterone on the 3rd and 5th days of culture. This indicates that prolactin synergizes with insulin and corticosterone to stimulate maximal synthesis of DNA after the first day of culture. Thus, prolactin's effect on DNA is dependent upon prior stimulation by insulin and corticosterone and upon synergism with the latter two hormones for the manifestation of its effect.

In general, it appears that insulin alone is capable of initiating DNA synthesis in mammary organ cultures. The site of



action of insulin on this process is as yet unknown, although de novo synthesis of protein appears to be involved (Turkington, 1968a). Corticosterone both enhances the effect of insulin and exerts its action after insulin initiates DNA synthesis. The three-hormone combination, in contrast to all other combinations, stimulated considerable increase in DNA content after 5 days in culture, indicating that maximum growth requires the full complement of the three hormones.

D. Effects of Hormones on Mammary RNA in Vitro.

According to Stockdale, Juergens, and Topper (1966), RNA synthesis was doubled (relative to the initial rate obtained after exposure to tritiated-uridine for four hours in vitro) in tissues cultured with insulin-containing media by 24 hours. The rate of synthesis was maintained by media containing insulin or insulin + corticosterone for 48 hours, increased by media containing insulin + prolactin, and further increased when all three hormones were present. The results of this study are in accordance with the above findings on the first day. Contrary to their results, the rate of incorporation decreased after 48 hours by the above hormone combinations. Tissues cultured with the three-hormone combination showed the highest rate of incorporation during the second, third, and fifth days of culture. RNA content paralleled RNA incorporation.

The observation that insulin increases both RNA and DNA indicates that the increase in RNA by insulin is due to its increase of cell number. There was no change in the RNA/DNA ratio during the five days when compared to initial controls. This is confirmed by

histological findings; although there was a high rate of incorporation of  $H^3$ -uridine in explants cultured with insulin or insulin + corticosterone, neither secretion nor cell vacuolation was stimulated by these combinations during the entire culture period. This suggests that the increase in RNA was not accompanied by stimulation of functional activity. Only combinations containing at least insulin and prolactin induced secretion.

The effects of corticosterone on RNA synthesis were interesting. This steroid decreased RNA synthesis and content to a greater extent than did hormone-free media. However, the inhibitory effect on RNA did not immediately affect the appearance of the tissues, since they were at least partially-maintained until the third day of culture. Furthermore, the inhibitory effect of corticosterone on RNA was not apparent in the presence of insulin, whose effect was significantly increased ( $P < 0.05$ ) by the presence of corticosterone.

Prolactin's effect on RNA synthesis was nearly constant throughout the entire culture period and similar to that of the hormone-free control. This consistent rate of synthesis may reflect the activity of the adipose tissue, since by the third day of culture most of the epithelial cells had degenerated in both cases. The increase in the RNA content (Table VII) of explants cultured with prolactin alone may be due to increased synthesis during the first few hours of culture. A study of the effect of prolactin on RNA synthesis and content during the first few hours should be done to elucidate prolactin's effect on the initiation of RNA synthesis. Histologically, secretion was not induced by prolactin alone but by

corticosterone + prolactin only during the second day of culture. This morphological effect was accompanied by an increase in both RNA content and incorporation. The preceding observations indicate that corticosterone + prolactin induces secretion, whereas insulin permits alveolar maintenance. In support of this view, the experiments of Turkington (1968b) show a rise in RNA and casein synthesis 24 hours after the addition of prolactin (or human placental lactogen) to media containing only insulin + hydrocortisone for 96 hours.

From these data it may be postulated that prolactin initiates RNA synthesis, although maximum synthesis requires the presence of corticosterone and insulin in addition to prolactin. The increase in RNA synthesis due to insulin may be due to an increase in cell number. Corticosterone has an overall inhibitory effect on RNA synthesis, which is completely abolished in the presence of insulin but not in presence of prolactin alone.

In summary, the following conclusions may be made.

1. Insulin appears to be the only hormone required for initiating DNA synthesis. Its effect on RNA synthesis does not appear to be related to induction of functional activity.
2. Prolactin initiates RNA synthesis, but the maintenance of this effect requires the presence of corticosterone and insulin.
3. The incomplete hormone system which contains insulin + corticosterone shows a small rise on DNA content on the second day, suggesting that corticosterone affects DNA synthesis either subsequent to insulin's initial action or by secondarily enhancing the effect of insulin.

4. The fact that only the three-hormone combination can bring about maximum DNA synthesis throughout the 5 days of culture suggests that the effects of prolactin on DNA is dependent on prior stimulation by insulin and corticosterone and synergism with the latter two for the manifestation of its effect.
5. The unique effect shown by the three-hormone system on both RNA and DNA throughout the 5 days of culture indicates that the system requires synergism between the 3 hormones for its function.

## SUMMARY

Normal in vivo mammary proliferation in Swiss-albino mice reaches a maximum on the 6th day of lactation. RNA increases markedly with the onset of lactation and attains maximum levels on the 6th day. The decline in lactational function that follows results from both a decline in cell number and in functional activity.

The results obtained in vitro show that explant survival as well as secretory activity falls with time in culture except for explants cultured with insulin + corticosterone + prolactin. Stimulation of DNA and RNA synthesis and survival of explants are dependent on the presence of insulin, irrespective of other hormonal supplements. A principal effect of insulin appears to be the stimulation of DNA synthesis. Its stimulation of RNA appears to be a secondary effect resulting from the increase in cell number and is thus unrelated to the induction of functional activity. Corticosterone, or prolactin alone, or corticosterone + prolactin have little or no effect in maintaining DNA levels. Corticosterone in combination with insulin increases DNA content on the second day, but the triple hormone system was the most effective combination increasing DNA on the second, 3rd, and 5th days. Thus, corticosterone enhances the effect of insulin on DNA while having no effect by itself. The effect of prolactin on DNA synthesis is dependent on prior stimulation by insulin and corticosterone as well as the continued presence of the latter two hormones.

Corticosterone or corticosterone + prolactin have an overall inhibitory effect on RNA synthesis, but this inhibition is completely abolished when insulin is also present in the medium. In addition, corticosterone significantly increases the stimulatory effect of insulin on RNA. Prolactin stimulates RNA synthesis, since prolactin-containing combinations stimulate explant secretion and RNA content. Insulin + corticosterone + prolactin was most effective combination inducing RNA synthesis, maintaining its stimulated content, and initiating maximum secretion after the second day of culture. Thus, in addition to the information obtained with the hormones individually, the need for synergism between the three hormones for long-term effects was also demonstrated in this study.

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