EFFECTS OF STEROID HORMONES ON THE NUCLEIC ACID METABOLISM OF THE MOUSE MAMMARY GLAND DURING PREGNANCY AND LACTATION

by

Lynne C. Manaugh

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[redacted]

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LIST OF ABBREVIATIONS

AES.....Automatic External Standardization

BSA.....Bovine Serum Albumin

CCA.....cytidine-cytidine-adenine, normally the 3 terminal nucleotides of a transfer RNA molecule.

CDP.....Cytidine triphosphate

C.P.M.....Counts per minute, referring to radioactivity

DNA.....Deoxyribonucleic acid

D.P.M.....Disintegrations per minute, referring to radioactivity

E. coli.....the bacterium Escherichia coli

EDTA.....Ethylenediaminetetraacetic acid

F.....Hydrocortisone, cortisol

g.....gravity, as in a centrifugal force of 1,000 X g

^{3}H.....tritium, the radioactive isotope of hydrogen of molecular weight of 3

I.....Insulin

L-A.....Lobulo-alveolar, referring to mammary development in pregnancy.

M.....Molar concentration

M_2POPOP.....1,4-bis-[2-(4-methyl-5-phenyl-oxazoyl)]-benzene

MH.....Prolactin, Mammotropic hormone

LIST OF ABBREVIATIONS CONTINUED

ml	milliliter
mm	millimeter
mM	millimolar concentration
mμ	millimicron
mRNA	messenger ribonucleic acid
μc	microcurie
μg	microgram
O.D	optical density
PCA	perchloric acid
pH	reciprocal of the hydrogen ion concentration of a solution; degree of acidity
PPO	2,5-diphenyloxazole
RER	rough endoplasmic reticulum
rRNA	ribosomal ribonucleic acid
sRNA	soluble ribonucleic acid
STH	somatotropic hormone, growth hormone
TCA	trichloroacetic acid
Tris	1,3-propanediol,2-amino (hydroxy-methyl)-
tRNA	transfer ribonucleic acid
^{3}H-6-uridine	uridine with a tritiated hydrogen replacement on the number 6 position

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INTRODUCTION

I. General

The mammary gland has been and continues to be an organ of much interest to biologists of several specialties. The first investigations on the mammary gland developed in the area of cancer research, as mammary tumors spontaneously arise or may be induced experimentally in nearly all common experimental animals. Because the mammary gland is a target tissue of several steroid and polypeptide hormones, it has been the subject of intense study by endocrinologists. Recently, as the fields of chemical embryology and molecular biology have developed, workers in these areas have been studying the mammary gland as an example of a complex differentiating system. Salient features of the physiological, morphological and biochemical differentiation of the mammary gland, and the hormonal regulation or differentiation, will be described below. While details of these parameters may vary among species, the basic aspects seem to be quite similar.

A. General Morphology

In the nonpregnant adult female mouse (and rat) the mammary gland consists of a nipple and a few branching ducts embedded in a fat pad. During pregnancy a period of about 20 days, the gland undergoes a two-phase differentiation

(Nandi, 1959). The first phase is that of lobulo-alveolar (L-A) development, or mammogenesis. This is a mitotic process in which the ducts proliferate and cells at the end of the ducts divide (Bresciani, 1965) to form grape-like clusters of alveoli. By about 14 days of gestation the adipose cells of the mammary fat pad have been largely replaced by ductal and alveolar tissue. The alveolar cells at this stage, visualized by electron microscopy, exhibit great variability (Mills and Topper, 1970), but generally they appear fairly non-specialized, with centrally located nuclei, rudimentary Golgi apparati, a small amount of rough endoplasmic reticulum (RER), and numerous free ribosomes. Milk protein and fat droplets are occasionally seen (Wellings, Grunbaum and DeOme, 1960a). A quiescent period follows L-A development, during which little or no cell division occurs, and the mammary gland does not secrete its characteristic products. This period (sometimes called the presecretory phase) lasts from about 14 days of pregnancy until parturition. By the end of pregnancy the alveolar cells' ultrastructure is more highly organized (Wellings, DeOme and Pitelka, 1960b). The Golgi is larger, though the cisternae are empty, and the RER fills most of the cytoplasm.

B. Lactogenesis

Lactogenesis, or milk formation, is the second stage of mammary gland differentiation. In this phase the alveolar cells elaborate lactose, milk fats and milk proteins

(caseins, α-lactalbumin and β-lactoglobulin). Secretion into the alveolar lumen is of the apocrine type, whereby some of the cytoplasm of the secretory cell is budded off into the lumen when the product is discharged. Ultrastructurally, lactogenesis is characterized by 1) enlargement of the Golgi apparatus, which now contains milk-protein droplets (Wellings and Philp, 1967), 2) an increase in the amount of RER, 3) the appearance of many fat and protein droplets in the alveolar lumen and inside the cells, and 4) increased numbers and lengthening of microvilli bordering the luminal surface (Wellings et al, 1960b).

C. Hormonal Requirements

The hormonal requirements for L-A development and lactation have been thoroughly investigated and are well established for certain strains of mice and for rats (see reviews by Cowie and Folley, 1961; Lyons, 1958; and Reece, 1958). By using the classical endocrinological techniques of endocrine gland ablation and hormone replacement therapy, Nandi (1959) showed that an estrogen, a C21 steroid (progesterone or one of the adrenocortical steroids) and prolactin (MH) are essential for ductal branching. The same hormone combination in higher concentration causes L-A development. In some strains of mice (e.g., C3H), and rats (Lyons, 1958), growth hormone (STH) can replace MH as the mammogenic hormone. The placenta, too, may contribute to mammary development; Turkington and Topper (1966a) report that human placental lactogen induces histological and

functional development comparable to that caused by MH. With the completion of L-A differentiation, the mammary gland is structurally capable of lactating. Initiation and continuance of lactogenesis requires the presence of an adrenal steroid and either prolactin or growth hormone. In triple-operated (adrenalectomized, ovariectomized, and hypophysectomized) C3H and BALB/c mice, the best lactational performance was achieved in animals receiving a glucocorticoid, and both MH and STH (Nandi, 1959; Wellings and Nandi, 1968).

In addition to the absolute requirement for an adrenocorticoid and a mammotropic pituitary hormone, other metabolic hormones may have a mediating effect on lactation. Insulin (I) in high amounts can decrease milk yield in cows (Brown, Petersen and Gortner, 1936, cited in Reece, 1958, p. 227), but it is required for lactation to take place (Cuthbert, Ivy, Isaacs and Gray, 1936). Dessicated thyroid, when administered to thyroidectomized and normal cows in small amounts, causes milk production to rise (Graham, 1934, cited in Reece, 1958; Bailey, Bartlett and Folley, 1949), but in pharmacological amounts it inhibits lactation. In the rat thyroxine and tri-iodothyronine, in moderate doses, stimulate mammary gland growth but depress lactation (Schmidt and Moger, 1967). Singh and Bern (1969) have postulated that the mammary gland response to thyroxine is highly dose-related and synergistic with MH. Parathyroidectomy results in reduced milk production, possibly through its control of

calcium metabolism, and parathyroidectomized animals' milk yield can be improved by parathyroid hormone replacement therapy (Folley, Scott, and Amoroso, 1942; Munson, 1955).

Oxytocin is an octapeptide elaborated by the neurohypophysis. It is thought to be primarily active in causing milk ejection by making the myoepithelial cells of the alveoli contract, thus squeezing milk into the ducts (Ham, 1965).

II. In Vitro Studies

A. Hormonal Requirements

In order to determine whether the hormonal effects described above are due to direct or to systemically mediated actions of the hormones, mammary tissue can be put in organ culture. There the gland is subjected to a smaller number of variables, and the direct effects of hormones, individually and in combination, can be observed. Under proper conditions, the complete range of mammary differentiation can be produced in organ culture (Rivera, 1964b; Rivera and Bern, 1961; Elias and Rivera, 1959; Stockdale, Juergens and Topper, 1966). Histologically and ultrastructurally, mammary differentiation *in vitro* is indistinguishable from that occurring in the intact animal. Mammary tissue from pregnant mice may simply be put into organ culture and appropriate hormones added. If mammary gland from a *nonpregnant* animal is to differentiate completely in culture, the mouse must be treated with estrogen and progesterone for a few days before excising the

mammary gland (Ichinose and Nandi, 1964, 1966). This pre-treatment does not cause L-A development in itself, but evidently estrogen and progesterone effect some "covert" change in the mammary gland which allows subsequent development in culture. If they are added directly to mammary gland cultures, estrogen and progesterone do not augment lobulo-alveolar differentiation (Ichinose and Nandi, 1966). It may be that they must enter the mammary gland via the circulation in order to be effective. Puca and Bresciani (1969) have shown that tritiated estradiol binds to mammary tissue. Thus, estrogen may be exerting an influence on development even though it has not been added directly to the culture medium.

The minimum requirement for histologically normal L-A development in culture is open to some question. It is agreed, however, that I and an adrenal corticoid are necessary. The requirement for MH is not so well understood. According to Ichinose and Nandi (1966), a medium containing insulin and a corticoid will allow differentiation to the stage of small, viable end buds, whereas MH must be present for lobules to form. Wellings, Cooper and Rivera (1966) have observed that in the presence of I and aldosterone, alveoli can form and are maintained. Stockdale et al (1966) confirm this finding. Two explanations for this disparity could be proposed: 1) Ichinose and Nandi used strain BALB/c mice, while Wellings' group and Stockdale's group used C3H mice. It may be that strain differences in hormonal responsiveness (Singh, DeOme

and Bern, 1970, Rivera, 1964a) could account for the difference in amount of L-A development observed in the absence of MH, and 2) Wellings and Stockdale cultured tissue from midpregnant mice, while Ichinose and Nandi used gland from 3-4 week old nonpregnant, pre-treated mice. The pregnant mice may have been exposed to some stimulation from MH in the intact animal, even though none was added to the medium. Nonpregnant mice, however, have very little circulating MH (Nandi, 1959). Whatever the explanation for the conflicting observations, it seems clear that for optimal L-A development, I, a corticoid and MH are needed. Recent electron microscopic studies of cultured mammary tissue (Mills and Topper, 1970) show that treatment with I plus F causes ultrastructural changes in alveolar cells. There is an increase in amount of RER, development of the Golgi apparatus and the appearance of nucleoli. These changes are compatible with a cell which is entering a secretory phase. When the tissue is cultured in medium which also contains MH, the cells undergo these same ultrastructural modifications, but much more quickly.

Some uncertainty also exists about which hormones are necessary to allow lactogenesis in culture. First reports, using prelactating gland, indicated that a minimal lactogenic medium contains I, a corticoid and MH (Rivera and Bern, 1961). Lockwood, Stockdale and Topper (1967a) have performed a series of experiments showing that, for lactation, only I and MH are needed. Their procedure uses a two-stage

incubation, in which mammary tissue is cultured for three days in a mammogenic hormone combination (I and F) and then cultured for an additional day in media containing various possible lactogenic hormone mixtures. By the end of the initial three-day incubation, L-A differentiation is complete, and lactogenesis may be analyzed separately. In the second incubation period, lactation occurs in the presence of I and MH, without a corticoid. These data are in conflict with those of Wellings et al, (1966) and Rivera (1964) and with in vivo observations (Nandi, 1959). The explanation may be that the mammary gland binds enough corticoid during the first incubation to maintain it through the second, 24-hour period. This possibility could be tested using a longer second incubation period. Maximal lactation is obtained using the three hormone combination, I, MH and a corticoid (Lockwood et al, 1967a).

Beginning in the mid-1960's and continuing through the present, there has been an effort to exploit the mammary gland differentiating system to elucidate the mechanisms of action of the various mammotrophic and lactogenic hormones (see review by Topper, 1968). These recent investigations have concentrated on demonstrating hormonal effects on 1) lactose synthesis and activity of enzymes catalyzing its synthetic reactions, 2) milk fat formation, 3) synthesis of specific milk proteins, and 4) nucleic acid metabolism. I will describe in some detail the current information about milk protein synthesis and nucleic acid metabolism.

B. Hormonal Effects on Milk Protein Synthesis

Since MH and I (and possibly a corticoid) are responsible for the ultrastructural changes resulting in secretion in mammary gland cultures, it was expected that they might also specifically stimulate the formation of casein, α-lactalbumin and β-lactoglobulin. (Turkington, Juergens and Topper [1965] have shown that the phosphoproteins synthesized by mammary cells in culture are electrophoretically indistinguishable from mouse casein fractions). Juergens et al, (1965), have conclusively demonstrated that this is so. Post-mitotic cells during the second incubation in culture will synthesize casein and whey proteins when stimulated with added MH (Turkington, Lockwood and Topper, 1967b; Lockwood et al, 1966). It has recently been shown by immunofluorescent staining that each hormonally stimulated mammary epithelial cell synthesizes casein and whey proteins simultaneously (Turkington, 1969a). I is also required in the second incubation (Lockwood et al, 1967).

Some interesting details about the respective roles of I and MH in stimulating casein synthesis have recently been reported. Casein is a phosphoprotein, with the phosphate moeity bound to hydroxyl groups of serine by ester linkages (Burnett and Kennedy, 1954). Turkington and Topper (1966b) have shown by the use of puromycin that the phosphorylation of casein occurs after the synthesis of the polypeptide chain. Treatment of mammary fragments with MH in the absence of I will allow formation of the non-phosphorylated polypeptide,

but I is required for phosphorylation (Voytovich, Owens and Topper, 1969). The glucocorticoids, the lactogenic hormone class about which the least is known, may also have an effect on phosphorylation of casein. Turkington, Juergens and Topper (1967a) classified adrenocortical steroids as inactive, moderately active, or highly active in stimulating histological development and in augmenting casein synthesis. There was a direct correlation between activity in alveolar differentiation and suppression of dephosphorylation of casein. It is possible, therefore, that one action of F could be to stabilize casein by preventing or inhibiting its dephosphorylation.

Beitz, Mohrenweiser, Thomas and Wood (1969) have succeeded in synthesizing milk protein in a cell-free system from lactating bovine mammary gland. They used a radio-immunodiffusion assay to identify three casein fractions, α-lactalbumin and β-lactoglobulin. Their system was inhibited by RNAse and puromycin and in general the study showed that the synthesis of milk protein conforms to the mechanism of protein synthesis found in other biological systems. None of the mammogenic or lactogenic hormones had any effect on the relative amounts or rate of synthesis of any of the protein fractions. This suggests that the point of influence of these hormones may not be at the translational stage, but at some earlier point, such as DNA replication or transcription.

C. DNA Synthesis and Mitosis

One area of research which has proven fruitful began

with the observation that midpregnant mammary gland in culture begins synthesizing casein only after a lag period of 8 to 10 hours, even in the presence of optimal lactogenic hormone combinations (Juergens, Stockdale, Topper and Elias, 1965). During the lag period alveoli are proliferating, i.e., L-A development is occurring. A comparison of mature virgin gland to midpregnant gland clarified the relationship between L-A development and casein synthesis (Stockdale and Topper, 1966). The mitotic rate of midpregnant gland reaches a peak at 24 hours of culture and casein synthesis peaks at 48 hours. In virgin glands the mitotic peak takes place at about 48 hours, and casein synthesis is greatest at 72 hours. Therefore, in both cases casein synthesis lags behind mitosis by 24 hours. The question arises whether cell division is <u>obligatory</u> for casein synthesis, and, if so, why? Stockdale and Topper (1966) tried to answer this question by culturing cells in the three-hormone combination with colchicine, a mitotic inhibitor. In this experiment, no augmentation of casein synthesis took place. (If colchicine is added <u>after mitosis is finished</u>, it has no inhibitory effect on casein synthesis). Likewise, androgens in the culture medium inhibit casein synthesis in direct proportion to the degree to which they inhibit DNA synthesis (Turkington and Topper, 1967). These data are strongly suggestive that there is a requirement for mitosis before lactogenesis can occur.

The hormone essential for mammary DNA replication and

alveolar mitosis, in the mouse at least, appears to be insulin. If I alone is present in the culture medium, maximal cell division takes place, though histologically the gland is abnormal (Prop and Hendrix, 1965; Stockdale and Topper, 1966). The means by which I acts in the mammary gland system is not understood. Its effect on cell division could be either direct or permissive--I is known to have a great many metabolic actions, and Lasfargues in his early work (1962) believed it to enhance the effects of mammogenic and lactogenic hormones. The best-known effect, that of increasing glucose transport across the cell membrane, is apparently not causally related to mitosis in the mammary gland, as mitosis occurs when D-fructose is substituted for glucose in the medium (Lockwood, Turkington and Topper, 1966). Another I action more-or-less causally related to DNA synthesis has been observed: there is an elevation of DNA-dependent DNA polymerase concommitant with DNA synthesis in cultures exposed to I (Lockwood, Voytovich, Stockdale and Topper, 1967b). The stimulation of DNA polymerase by I is apparently not simply due to the ability of I to permit survival of the mammary cells: when mammary cells were first cultured 48 hours in the absence of any hormones, followed by 48 hours in I medium, the DNA polymerase activity was enhanced as much as when the cells were initially cultured in I-medium. After two days in culture, DNA synthesis and mitosis essentially stops, while DNA polymerase levels remain high. This indicates that DNA polymerase is not the

only regulatory agent in mammary cell division. Puromycin or Actinomycin D added to the I medium will prevent the rise in DNA polymerase activity. The authors (Turkington, 1968; Lockwood et al, 1967b) suggest that this means that the I effect is related to de novo synthesis of the enzyme rather than activation of preexisting polymerase. At the high inhibitor doses they used (10^{-4} M puromycin; 5µg/ml Actinomycin D for 24 hr) inhibition could have been caused by general toxicity from the inhibitor.

Histones are basic nuclear proteins which are complexed with DNA (Mirsky and Ris, 1951; Wilkins, 1956; Huberman and Attardi, 1966). In the mammary gland, as in several other tissues, histone synthesis is correlated temporally with DNA synthesis, (Marzluff, McCarty and Turkington, 1969). I alone is sufficient to maximally stimulate histone formation. Histones have been studied as possible regulators of gene activity since Huang and Bonner (1962) showed that in pea-seedlings, histone-associated DNA (chromatin) is a relatively inert primer for RNA synthesis compared to free DNA. Thus far, it has not been demonstrated that there is enough specificty in histones alone to account for the complexity of gene activation and inactivation. The mammary gland differentiating system is a case in point. There does not appear to be a selective alteration in the rate of synthesis of any specific fraction; I stimulation results rather in a general stimulation of all histones.

While there has been no clear-cut evidence that any

histone fraction binds to a specific area on the DNA strand, there is the possibility that enzymic modification (e.g., acetylation and phosphorylation) of histones may confer specificity on them, (Gershey, Vidali and Allfrey, 1968), perhaps by altering the tightness of binding to DNA. Turkington and Riddle (1969) have investigated the phosphorylation of histones and other nuclear proteins in cultured mammary tissue. While they did not observe any alteration in relative rates of synthesis of histone fractions, there were striking differences in degree of phosphorylation of various electrophoretically separated fractions. I stimulates phosphorylation of histones and other nuclear proteins several hours before synthesis of the histone polypeptide occurs. MH also stimulates phosphorylation of histones, but only in mammary fragments previously incubated in I and F. The requirement for glucocorticoid is not established, and during the first incubation period it seems to depress phosphorylation about 15 per cent. It is interesting that during this period F causes a decreased incorporation of RNA precursor of about the same magnitude (Green and Topper, 1970). According to Turkington and Riddle (1969), the specific pattern of phosphorylation suggests the possibility that nuclear protein phosphorylation may be involved in the hormonal regulation of RNA synthesis in mammary epithelial cells. Langan (1968) has shown that phosphoprotein complexed to histone decreases the histones' inhibitory effect on the RNA polymerase reaction. If the

phosphorylation of nuclear proteins constitutes a mechanism of gene activation, one would indeed expect to observe increased phosphorylation of nuclear proteins at times of increased mitotic and/or synthetic activity such as occurs during mammary development. In human lymphocytes, stimulated to divide by phytohemaglutinin, phosphorylation of nuclear proteins is stimulated prior to the increased RNA synthesis (Kleinsmith, Allfrey and Mirsky, 1966). These data, however, like those from the mammary gland system, do not show that there is a causal relationship between phosphorylation of histones and other nuclear proteins and RNA synthesis.

D. RNA Synthesis

1. Total RNA

Even before the elevation of DNA polymerase activity and DNA synthesis in I medium, there is increased activity in the transcription and translation apparatus of the mammary epithelial cell. Palmiter (1969) measured incorporation of radioactively labeled uridine, amino acids and thymidine into trichloroacetic acid-insoluble material (i.e., RNA, protein and DNA) and found that there is a rise in RNA and protein synthesis within 1 hour after I is added to the medium. This early synthesis is not enhanced by F and/or MH. Thymidine incorporation into DNA rises several hours after the rise in RNA and protein. These data confirm earlier work of Stockdale, Juergens and Topper (1966) and

Mayne, Barry and Rivera (1966). The latter investigators found that, while actinomycin D inhibited early I-stimulated RNA synthesis by 97 per cent, leucine incorporation into protein was only inhibited by about 25 per cent. Thus, during the early period, at least, protein synthesis may be only partially dependent on new synthesis of RNA.

Correlated with the rise in RNA and protein synthesis in hormonally stimulated mammary fragments in vitro, an elevation of DNA-dependent RNA polymerase can be demonstrated in the nuclei of the mammary cells (Turkington and Ward, 1969). This increased activity occurs very early and is almost complete by 8 hours if I alone is in the medium.

In contrast to the findings that I by itself is sufficient to stimulate early RNA, protein, and RNA polymerase activity, it is likely that MH is also necessary later in the incubation period. Mayne, Forsyth and Barry (1967) report that by 48 hours in culture the RNA content of the mammary explants, cultured in medium containing only I, or I plus F, had dropped by about 20 per cent, whereas if the medium contained all three hormones, RNA content was increased by 37 per cent. These findings are paralled by those of Turkington and Ward (1969), who measured RNA polymerase activity. These investigators state that MH can only act to stimulate RNA polymerase activity in post-mitotic, differentiated mammary cells, i.e., cells which have been pre-incubated in the I plus F medium. They demonstrate this by using the two-stage incubation technique.

When the first incubation medium contains only I, there is no MH-stimulated enhancement of RNA polymerase activity during the second incubation. It might have been informative to compare cells incubated initially in I, F, and MH versus those in I and F. This could demonstrate any additive effects of MH and I in stimulating RNA polymerase. In the system described above, corticoids do not seem to have a stimulatory effect on RNA polymerase, although they do have a permissive effect on MH stimulation of the enzyme activity.

Using the organ culture technique, Green and Topper (1970) measured the effects of the I, F and MH on total mammary RNA and specific activity of the mammary RNA. In this series of experiments the sequential incubation was used: the first incubation period was 4 days, the second was 1 day. MH, added to the second incubation mixture, increased the ^{3}H-uridine uptake by from 16 per cent to 38 per cent. This could be expected, since MH activates RNA polymerase. The surprising finding of this report is that F seems to inhibit uridine incorporation into mammary RNA by about 25 per cent. It appears, however, that the effect of this corticoid is mainly on the fat cells of the midpregnant mammary gland, which still has a high percentage of adipose tissue. Intact mammary fragments did not show any greater inhibition of uridine incorporation than did de-epithelialized gland, which is composed almost entirely of adipose tissue. In these long-term cultures the prolactin effect does not seem to be completely dependent on the "preparation" of the mammary

cells by a glucocorticoid; when explants are incubated in I alone, followed by I + MH, there is a 30 per cent increase in uridine uptake. However, the prior incubation in I + F medium resulted in generally greater stimulation in the presence of MH.

Since total RNA synthesis in mammary tissue in vitro is responsive to hormones, an effort has been made by several workers to discover whether the various types of RNA respond differentially to hormonal stimulation.

2. Hormonal Effects on transfer RNA (tRNA)

Turkington (1969) reports that there is a disproportionate increase in tRNA synthesis in organ cultures incubated in the three-hormone combination. This increased tRNA synthesis can be correlated in time with elevation of transfer RNA (tRNA) methylating enzymes. Prolactin and I exhibit an additive stimulatory effect on tRNA formation and on methylase activity. For maximal stimulation, all three hormones are needed. A specific effect of F seems to be to depress the activity of adenosine 1-methylase to near zero. Adding MH reverses this effect. Contrary to its effect on RNA polymerase, MH is equally effective in methylase stimulation whether or not the mammary cells are pre-incubated in F-containing medium. Therefore it appears that the major regulator of tRNA methylases is MH rather than F. Turkington speculates that tRNAs lacking methylated bases might act in some regulatory way, perhaps to prevent or inhibit the

synthesis of milk proteins during pregnancy. There is some evidence from methionine-starved E. coli mutants that methylation does alter the amino acid acceptor activity and coding properties of some tRNAs (Peterkofsky, Jesensky and Capra, 1966; Littauer, Revel and Stern, 1966).

An intriguing possibility for a control mechanism of protein synthesis at the tRNA level is described by Herrington and Hawtrey (1970). They report that tRNA from nonlactating bovine mammary gland is deficient in amino acid acceptor activity, and that the reason for this deficiency seems to be the lack of the terminal adenine nucleotide at the amino acid acceptor end of the molecule. Unfortunately, the authors do not show data comparing tRNA from nonlactating with tRNA from lactating bovine mammary tissue. Nor do they indicate whether all tRNAs lack the terminal adenine, or whether it is absent on tRNA coding for specific amino acids.

3. Hormonal effects on ribosomal RNA (rRNA)

To this writer's knowledge, there is only one published study describing the synthesis or hormonal regulation of ribosomal RNA in mammary gland differentiation. Turkington and Riddle (1970) have studied relative concentrations of polysomes and monosomes in differentiating gland in culture. They found that an early effect of I is to cause increased rRNA synthesis, stimulation of monosome formation and stimulation of monosome incorporation into polysomes. In post-mitotic mammary cells (exposed first to I and F), MH acted

synergistically with I to cause these same changes. These investigators made the further observation that casein synthesis began almost simultaneously with the rise in polysome concentration, about 4-6 hours after MH was added to the culture. This result is interpreted by the authors as the initiation of translation, or, more specifically, the binding of ribosomes together with messenger RNA (mRNA) to make polypeptide-synthesizing units.

III. In Vivo Studies

The volume of information about molecular events of mammogenesis and lactogenesis in vitro has grown tremendously in the last five years. Some very specific actions of the hormones are known, although much is yet to be discovered about their mechanisms of action. In contrast to the extensive knowledge of the molecular biology of lactogenesis derived from organ culture techniques, there are relatively few published studies dealing with macromolecular syntheses in the intact animal. It is the aim of this dissertation research to begin a systematic investigation of nucleic acid metabolism of the intact mammary gland and the influence of hormones on it. It is obvious that some of the data on mammary gland development and function could only have been obtained by use of some in vitro system. The two-stage incubation technique, for example, is a powerful tool for differentiating the effects of several hormones on L-A development and lactogenesis. Experiments with hormone

treatment of mammary gland in culture better insure that hormonal effects are direct on the target tissue, not systemically mediated. It is easier to control experimental variables in organ culture, too; a single gland from one animal can furnish tissue for several experimental and control groups. Eventually, though, if *in vitro* data are to be meaningful, they should be related to events occuring in the living animal. It is important to show whether the crucial features, at least, of an *in vitro* system hold true *in vivo*. To date this has been done only in a fragmentary way for the mammary gland. Before describing the investigations I have done, I will review current concepts of protein and nucleic acid metabolism of the mammary gland of intact animals of several species.

A. DNA Synthesis and Mitosis

As has been pointed out before, DNA synthesis occurs during early pregnancy in the mammary gland (Folley, 1952; Nandi, 1959). In rats and mice this process requires an estrogen, progesterone, MH, a glucocorticoid and probably I (Kumaresan and Turner, 1967). Cytological studies have revealed little evidence of cell division in the last third of pregnancy (Reece and Warbritton, 1953; Maeder, 1923; Weatherford, 1929). However, in some species there is evidence for a second mitotic period, occurring late in pregnancy and continuing through the first few days of lactation. Greenbaum and Slater (1957) state that between

the 20th day of pregnancy and the third day of lactation, the DNA per gram of mammary tissue increases by about 40 per cent. These data were confirmed by Griffith and Turner (1959), who also measured a DNA increase of that same magnitude over the last day of pregnancy to the fifth day of lactation. In rats the total number of nuclei per mammary gland also increases (Lavrin and Cole, 1964; Baldwin and Milligan, 1966).

Treating early- or late-pregnant rats with 2 mg/day of F, the most potent mammogenic and lactogenic steroid, does not increase the DNA content of mammary glands over that of control pregnant rats (Ferreri and Griffith, 1969). This does not prove that F is not essential for DNA synthesis and mammary growth; the endogenous steroids might already have exerted their maximal stimulatory effect.

Bresciani (1964) reports that E-17β accelerates the rate of DNA synthesis as measured by autoradiography. The majority of DNA labeling was in the end bud cells.

Pseudopregnancy can be produced in the rat by vibrating the cervix of a female on the day of estrous. In pseudopregnancy the mammary gland achieves a degree of development comparable to that of 11-12 days of pregnancy (Sinha and Schmidt, 1969). Sinha and Schmidt compared pituitary MH depletion with mammary nucleic acid content and found a high negative correlation between the two quantities, i.e., the larger the amount of MH released by the pituitary, the greater the amount of mammary DNA per gram of body weight.

This study does not prove, though, that MH is causing stimulation of DNA synthesis. Baldwin and Martin (1968) hypophysectomized rats on the day of parturition and gave various hormone treatments for 2 1/2 days thereafter. Corticosteroid treatment exerted no effect on ^{32}P uptake into DNA, while MH stimulated uptake to 67 per cent of that of sham-operated controls. This finding is in contrast to the in vitro data of Lockwood et al (1967b), who found that MH is not essential for proliferation of mammary alveoli. A possible explanation for the discrepancy, considered by the authors of the former paper, is that the general depression of metabolism resulting from hypophysectomy causes lowered I secretion and/or activity. To test this hypothesis, they treated animals with a long-acting I preparation in addition to the other hormones. Insulin had no effect on ^{32}P incorporation in either the presence or absence of MH, so the contradiction apparently cannot be explained by predicating I as the primary mitotic agent. One possibility could be that there are different hormonal requirements for that mitosis which occurs in early through mid-pregnancy (the stage investigated by Lockwood et al (1967b) and that which occurs at parturition. Furthermore, it may be that the "burst" of DNA synthesis observed at parturition in rats does not even take place in the mouse mammary gland.

It is obvious from the preceeding data that our knowledge about DNA synthesis during pregnancy and lactation, and the hormonal requirements for it, is far from complete. It is

clear that DNA synthesis takes place during early pregnancy in all species investigated. The hormones necessary for this DNA replication and cell proliferation appear to be estrogen, progesterone (or an adrenal steroid), STH (or MH) and probably I. Although added adrenal steroids do not stimulate DNA replication, they are necessary for L-A development and may be related indirectly to mitosis and DNA synthesis. MH and STH seem to be interchangeable in rats in ability to stimulate DNA replication during L-A differentiation, in contrast to *in vitro* findings that I alone is sufficient to stimulate DNA synthesis. Little information is available as to the hormonal regulation of the "burst" of DNA synthesis occurring at parturition, but Baldwin and Martin (1968) indicate that MH, at least, is required.

Closely associated with DNA replication after parturition is a rapid rise in nuclear protein synthesis, especially histone protein (Stellwagen and Cole, 1969). Like DNA synthesis, histone synthesis reaches a peak one day after birth and drops precipitously through the rest of the period studied (day 6 of lactation). Different histone fractions apparently are made at different rates: arginine-rich histones seem to be synthesized three times faster than the lysine-rich ones. These data follow closely the findings of Marzluff *et al* (1969) that in I-dependent mammary mitosis *in vitro*, histone synthesis occurs practically simultaneously with DNA replication.

B. RNA Synthesis

It might be expected that associated with the active proliferative and secretory states of the developing and lactating mammary gland there would be a high rate of RNA synthesis. Several investigators have reported that mammary gland RNA content is higher in lactating than in pregnant rats (Greenbaum and Slater, 1957b; Tucker and Reece, 1963; Sinha and Schmidt, 1969; Lavrin and Cole, 1964; Baldwin and Milligan, 1966). Baldwin and Milligan (1966) show that the sharpest rise in RNA content of the gland takes place on the last day of pregnancy, continues to rise through the lactational period and drops during the weaning stage. All of the above reports deal with rats, but recently Banerjee and Banerjee (1971) stated that in mice, too, RNA levels increase with the onset of lactation.

Few details are known about mammary RNA synthesis in any intact animal. For example, there are no published data showing whether the major fractions of RNA are all synthesized at the same rate throughout development of the gland. However, in the past few years several workers have tried to detect the presence of mRNA in mammary gland tissue.

There is much looseness in the operational definition of mRNA of mammalian cells. The most stringent test of an RNA's messenger capability is to ascertain its ability to code for specific proteins in a cell-free protein synthesizing system. For example, in the mammary gland system,

one would assay an RNA sample for its ability to code for casein, one of the whey proteins, or a lactose synthetase subunit, using E. coli ribosomes in the cell-free system. In very few mammalian systems has it been possible to show that a specific RNA has this capability. There are, however, other, less stringent criteria which may be applied: polydispersity, DNA-like base composition, high hybridizability to DNA, rapid labeling, and association with polysomes. Mammary gland RNA in pregnancy versus lactation has been examined with respect to these characteristics, but no hormonal manipulations have been attempted in order to stimulate mRNA production. Sirakov and Rychlik (1969) state that a 15s RNA, present in higher concentration in lactating than in pregnant mouse mammary gland, has several of the properties of mRNA. It is rapidly labeled, has a molecular weight consistent with a messenger for casein or lactoglobulin, and stimulates protein synthesis in a Nirenberg-Matthiae (1961) system. The authors did not show synthesis of a specific milk protein, however, so these data must be considered only suggestive.

Turkington (1970) measured changes in concentration of hybridizable nuclear RNA of pregnant versus lactating and virgin versus lactating mice. In this technique labeled extracted RNA from pregnant and lactating glands were mixed in liquid, DNA was added and subsequently denatured by heating and rapid cooling. RNA whose base sequence is complementary to the DNA's base sequence will bind to the

single-stranded (denatured) DNA. In Turkington's system, mammary RNA from pregnant mice "competes" with RNA from lactating glands for binding sites on the DNA strands. The RNA which is the more complementary to the DNA base sequence will bind to the greater degree. Nuclear RNA from mammary glands of virgin mice competed incompletely with lactational RNA for DNA binding sites, i.e., the virgin RNA is less complementary to DNA than the lactational RNA. The authors claim that there were also differences in hybridizability of RNA from pregnant versus lactating glands, but the competition curve does not illustrate this convincingly. The study shows that there are some rapidly labeled nuclear RNA species associated with functional differentiation of the mammary gland. It is possible that this is mRNA coding for milk proteins.

C. Hormonal Effects on RNA Synthesis in Intact Mammary Gland

It will be recalled that in culture mammary gland RNA polymerase activity and RNA synthesis are stimulated by I and MH, while the effect of glucocorticoids may be inhibitory, at least on RNA synthesis of the adipose cells. In vivo there is evidence to suggest that both MH and adrenal steroids positively influence RNA synthesis. RNA levels in hypophysectomized lactating rats could be restored to nearly normal by replacement therapy of MH and F (Baldwin and Martin, 1968). Intraductal administration of MH into pseudopregnant rabbits induces two peaks of ^{3}H-uridine

incorporation, one at about 12 hours after injection, the other at between 30 and 36 hours after injection. During prolonged lactation in rats, MH and F, or F alone, prevents the lowering of the RNA:DNA ratio normally seen after the third week of lactation (Thatcher and Tucker, 1970a), but MH alone does not prevent its decline. It was suggested by the authors that during prolonged lactation, at least, adrenal steroids may be rate limiting for RNA synthesis. Ferreri and Griffith (1969) noted that in pregnant rats F did not influence mammary gland RNA content when administered in midpregnancy, but caused about a 20 per cent increase over placebo-treated controls when given at the end of pregnancy. Using the same dose as in the previous experiment, Davis and Liu (1969) did not observe such an increase in rat mammary RNA in late pregnancy. They did, however, show that prior adrenalectomy diminished the RNA levels which could be attained in rats which were ovariectomized 24 hours after adrenalectomy (ovariectomy of pregnant rats is a procedure which induces premature lactation).

IV. Statement of Thesis Problem

It seems clear that the little information we have about nucleic acid metabolism of the mammary gland in vivo during pregnancy and lactation is quite ambiguous and in some cases contradictory. Likewise the role of the several lactogenic and mammogenic hormones is unclear, both in vivo and in

vitro. It is the purpose of the research to be described here to undertake the systematic investigation of this subject, using as experimental model the BALB/c mouse. Hopefully, by using the mouse, some meaningful comparisons can be made with the results obtained by those workers employing organ culture methods for studying mammogenesis and lactogenesis.

The first part of the thesis research entails measuring normal levels of mammary gland RNA and DNA during pregnancy, lactation, and weaning. Following the description of normal metabolism, the results of hormonal manipulations which were done to elucidate the role of certain steroid hormones in lactogenesis and lactation will be reported. Of particular interest is the effect of the glucocorticoid cortisol whose influence *in vivo* seems different from that *in vitro*. This hormone has been difficult to study, too, because of its radically different effects on various cell types. Hopefully, the information about cortisol gained from this study may contribute to the fund of knowledge about its general physiology and mechanism of action.

MATERIALS AND METHODS

I. Animals

A. Strain of Mouse

In all of these experiments 3-4 month old, primiparous BALB/c mice were used and were obtained from Jackson Laboratories, Bar Harbor, Maine. This strain was chosen because it does not carry the mouse Mammary Tumor Virus, which would constitute an additional variable in the experiments.

B. Care of Animals

The mice were routinely housed five-to-a-cage in the animal care quarters. They were given free access to Purina Lab Chow and water, except as noted in section II-E, below.

C. Staging of Pregnancy

When it was necessary to know the stage of pregnancy within one day, mice were bred by placing a male overnight in a cage with three or four females. By the tenth day after conception it could usually be grossly determined whether a mouse was pregnant. The stage of pregnancy could also be confirmed by examining the size and degree of development of the fetuses (Gruneberg, 1943).

D. Removal of the Mammary Glands

The mice were killed by neck fracture. A ventral midline incision through the skin was made and the skin was

peeled away from the peritoneum. The mammary glands were dissected free of lymph nodes and muscle and removed.

II. Operative Procedure

A. Anesthesia

In beginning studies animals were anesthetized with ether, and anesthesia was maintained with an ether nose cone. In this investigator's hands, however, it was difficult to prevent respiratory failure. Later, a much more successful procedure was developed by Weaver (1969), and it was used in all experiments described in this paper.

Anesthesia was induced by placing the mice in an ether jar. They were removed from the jar immediately after they passed from the agitated into the unconscious state. Anesthesia was maintained by delivering vaporized Penthrane (Abbott Laboratories methoxyflurane) through a nose cone (Figure 1). Penthrane proved to be an ideal anesthetic for mice; it could be administered for long periods of time with no apparent harmful effects. In over 100 mice there were no mortalities attributable to Penthrane, in contrast to a mortality rate of 25 per cent with ether.

B. Adrenalectomy

After the mice were anesthetized, a mid-dorsal incision approximately 1.5 to 2.0 cm long was made through the skin. Next, a tear was made about 0.5 cm lateral to the main incision, through the peritoneum and muscle layers, at the level of the kidney, which was visible through the peri-

toneum. The adrenal, located at the anterior end of the kidney, was held with forceps and snipped out with fine scissors. One must be sure not to lacerate the gland, since small fragments of adrenal tissue may regenerate (Manaugh, 1970, unpublished observations). After both adrenals were removed, the peritoneal wounds were gently drawn closed, but not sutured. Two or three wound clips were used to close the skin incision.

C. Criteria of Successful Adrenalectomy

As was mentioned in paragraph B, above, complete removal of the adrenal gland was necessary to achieve the proper results. Since the mouse adrenal gland is very friable and easily broken, it was necessary to carefully observe the mice to be sure that adrenalectomy was complete. The criteria of successful adrenalectomy adopted for these experiments were:

1. The absence of any visible adrenal tissue at autopsy (the site was examined with a dissecting microscope)
2. The presence of symptoms of adrenal insufficiency (particularly evident in mice are weight loss, nervousness, irritability and enlarged lymph nodes)
3. Lack of milk in mammary glands at autopsy
4. Failure of nursing pups to gain weight

D. Sham Operations

Sham operations were performed in the same way as adrenalectomies, up to the point of removal of the gland. The sham-operated animals were kept anesthetized for the same length of time as the adrenalectomized animals.

E. Postoperative Treatment

After the operation, mice were placed one-to-a-cage in a draft-free area. Control and adrenalectomized mice were given access to normal saline and tap water. In order to minimize effects due to differences in the amount of food consumed, the food was measured and the same amount fed to all the mice.

In the experiments in which mice were lactating, the babies were divided equally among the experimental and control mothers. If one or more babies died or were killed by the mother, they were replaced so that the litter size was always the same. In a few cases adrenalectomized mothers killed every baby in the cage, and because of the impossibility of maintaining sufficient suckling stimulus, these mice were not included in the experiment.

F. Ovariectomy

The skin and peritoneal incisions for ovariectomy were made in the same way as for adrenalectomy; in fact, a single incision was made when performing both operations on an animal. The ovary, located posterior to the kidneys, is embedded in a mass of fat. The fat was lifted out, the

fallopian tubes pinched off with fine forceps to control bleeding, and the ovary cut away. The uterus was returned to the peritoneal cavity. The incisions were closed as for adrenalectomy.

G. Criteria of Successful Ovariectomy

Ovariectomy was practiced on several (24-30) animals and successful operation was verified by taking a vaginal smear, as described by Zarrow, Yochim and McCarthy (1964, p. 37). The vaginal smear was obtained by placing a drop of water in the vagina of the mouse and aspirating the drop again. The drop was placed on a slide, dried and stained with Giemsa or Wright's stain. The absence of estrous cornified cells on the smear by 7 days postoperatively indicated that the operation was successful. After the first five trials, 100 per cent of the practice ovariectomies were successful.

H. Postoperative Treatment

The postoperative treatment of ovariectomized was the same as for the adrenalectomized mice, except that tap water was given instead of saline.

I. Hormone Replacement Therapy

1. Hydrocortisone acetate (Sigma Chemical Company) was made up in sterile saline to a concentration of 2 mg per ml. The dose found effective in reversing the symptoms of adrenal insufficiency

(see section II D, above) was 50 µg of the saline suspension of hydrocortisone acetate administered every 12 hours. The hydrocortisone was made up fresh every other day, as it seemed to lose potency after a few days. This finding is confirmed by Monder (1968), who has observed the conversion of hydrocortisone to 21-dehydrocortisol at a rate of up to 2.8 per cent per hour under conditions where the suspension is not kept adequately refrigerated.

2. 17β-estradiol benzoate was given in aqueous suspension (Progynon, Schering Corporation, Bloomfield, New Jersey). The dose was either 1µg or 2µg per day, subcutaneously.

III. Isolation of Nucleic Acids

A. General Introduction

Two methods were used to isolate nucleic acids:

1. A modification of the Schmidt-Thannhauser (1945) procedure. This method was used when it was necessary to know total quantities of RNA and DNA in a tissue homogenate.
2. A hot phenol extraction, from which is prepared "intact" RNA.

B. Schmidt-Thannhauser Hydrolysis

1. General introduction

The Schmidt-Thannhauser (1945) procedure is a technique for sequentially hydrolyzing RNA and DNA from a tissue homogenate. The separation of the RNA from the DNA is based on the fact that RNA but not DNA can be hydrolyzed in dilute alkali. Although this procedure has become a standard one to obtain nucleic acids for quantitation, Hutchison and Munro (1961) emphasize the large number of variables to which the homogenate is subjected (concentration of the acid, length of precipitation, temperature of extractions, etc.). This fact, in addition to the knowledge that various tissues respond differently to the hydrolysis conditions, made it desirable to perform a number of controls and to thoroughly test the method on pregnant and lactating mammary tissue. Below is an outline of the procedure used, followed by a discussion of some of its salient features.

2. Outline of the Schmidt-Thannhauser procedure

   a. Treatment of tissue

      i. If the tissue is to be used immediately after excision, it is placed in a few milliliters of a dilute saline-citrate solution (0.015 M sodium chloride, 0.0015 M trisodium citrate).

      ii. If the tissue is <u>not</u> to be analyzed within a few minutes, it is frozen in liquid nitrogen.

b. The fragment of mammary gland is homogenized in an ice bath, using a motorized ground-glass Ten Broeck homogenizer, to a final volume of 10 ml.

c. To 1 ml of the homogenate is added 1 ml of cold 12 per cent perchloric acid (PCA).

d. The acidified homogenate is centrifuged for 10 min at 1,000 X g and the supernatent is discarded.

e. The pellet is washed with 12 per cent PCA and centrifuged two more times.

f. To the washed pellet is added 1 ml of 0.3 N sodium hydroxide.

g. The alkaline pellet is incubated 1 hr at 37°C, with shaking.

h. The alkaline hydrolysate is acidified by adding 1.5 ml of 12 per cent PCA.

i. The acidified mixture is allowed to stand for 1 hr in an ice bath.

j. The acidified mixture is centrifuged for 10 min at 1,000 X g.

k. The supernatent is diluted to 5.0 ml with 12 per cent PCA in a volumetric flask.

l. To the pellet from the above centrifugation is added 2.0 ml of 6 per cent PCA.

m. The acidified pellet is heated for 20 min at 80 to 85°C.

n. After heating, the acidified mixture is centrifuged for 10 min at 1,000 X g.

o. The supernatent is diluted to 5.0 ml with 6 per cent PCA. The pellet is discarded.

3. Discussion of the procedure (letters below refer to steps of the preceding outline)

a. It is important to freeze the tissue quickly, so that the ubiquitous nucleases do not degrade the DNA and RNA, rendering them acid soluble. Mammary gland frozen in liquid nitrogen may be stored up to three or four weeks with no loss of acid-insoluble nucleic acid.

b. Self-explanatory.

c. Twelve per cent PCA causes proteins, DNA and RNA to precipitate. After centrifugation the supernatent contains 1) inorganic and organic phosphorus compounds of low molecular weight, 2) polysaccharides, 3) coenzyme nucleotides, and 4) low molecular weight polypeptides. For the precipitation, PCA was used rather than trichloroacetic acid (TCA), because TCA is strongly ultraviolet-absorbing and interferes with measurement of nucleic acids by absorbance at a wave length of 260 mμ.

It is important that the 12 per cent PCA homogenate remain cold and that it not stand too long; if the mixture is exposed to PCA too long, loss of both RNA and DNA can take place. Hutchison, Downie and Munro (1962) found that RNA is lost to the acid-soluble supernatent if it is kept in PCA

for 2 hr or more. DNA loss apparently occurs by the formation of apurinic acid (the release of purine bases). Apurinic acid is hydrolyzed in alkali to acid-soluble products (Bonar and Duggan, 1955).

d. Self-explanatory.

e. It is important to determine the minimum number of washes which are sufficient to remove interfering contaminants. Washing the homogenate too many times could lead to the formation of apurinic acid and loss of RNA. However, it is especially important to wash a mammary gland homogenate adequately since the lactating gland produces lactose. This disaccharide interferes with the orcinol determination for RNA. Exogenous lactose was added to mammary gland homogenates in amounts up to about three times those found in fully lactating glands (2 mg per gram of tissue, [Greenbaum and Slater, 1957a]). No significant increase in orcinol-reacting material was found in homogenates which had had the precipitate washed twice, so this practice was adopted.

f-g. Hutchison and Munro (1961) recommend use of as short an alkaline incubation as possible, for two reasons:

i. The shorter the alkaline digestion, the less likely that the DNA will be solubilized;

ii. Long periods of digestion in alkali may result in hydrolysis and solubilization of protein, which can interfere with both orcinol and ultraviolet absorbance measures of RNA.

It was found that a 1 hr incubation was sufficient to render more than 95 per cent of the mammary gland RNA soluble. A longer hydrolysis would probably be necessary to reduce the RNA to mononucleotides, but this is not required for either of the methods of RNA quantitation.

h-k. Reacidifying the hydrolysate and allowing it to remain in the cold for one hour causes the DNA, which has not been hydrolyzed, to reprecipitate. Thus, after the mixture is centrifuged, the RNA is in the supernatent and the DNA in the pellet. In order to insure as much precision as possible in measuring the RNA, the supernatent is diluted to a known volume.

l-o. The hot acid incubation constitutes the DNA hydrolysis, which solubilizes DNA, leaving most of the protein as a precipitate.

It may be noted by those familiar with Schmidt and Thannhauser's original (1945) paper that those workers included several washes with organic solvents in the RNA purification. Initially this investigator used ether, ethanol and chloroform washes, but comparison of extractions which included these steps with those

omitting lipid extraction showed no differences in RNA or DNA values. Most of the lipid floated to the air interface and was poured off during the PCA washes. Of course, if one were quantitating nucleic acids by phosphorus determination, thorough removal of phospholipids would be imperative.

C. Hot Phenol Extraction of RNA

1. Phenol extraction of RNA is based on the principle that DNA and protein are precipitated by phenol, while RNA is not (Kirby, 1956). Furthermore, phenol is an inhibitor of ribonuclease, so it prevents the degradation of RNA during the extraction. Below is an outline of the procedure used here (McNulty, personal communication, 1968), which is followed by a discussion.

2. Reagents

a. Phenol, Fisher Chromatography Grade. (The phenol was redistilled before use. After distillation, the buffer was added and the mixture frozen to retard oxidation.)

b. Buffer: 0.01 M sodium acetate
0.14 M lithium chloride
0.50 per cent sodium dodecyl sulfate
(The pH of the buffer was adjusted to 5.1 with acetic acid.)

c. Absolute ethyl alcohol, Gold Shield.

d. 3.3 M sodium chloride.

3. Sterilization

All glassware was first autoclaved, then heated in the drying oven at 170°C for 3 hr. This denatures ribonucleases, which are stable to autoclaving alone (McNulty, personal communication, 1968). The buffer and sodium chloride solutions were autoclaved at 15 lb for 30 min.

4. Procedure

a. Frozen tissue was ground with sterile sand in a sterile mortar and pestle surrounded by dry ice.

b. To frozen sand-tissue mixture was added 5 ml of buffer and an equal volume of buffer-saturated phenol.

c. The phenol mixture was heated with stirring for 10 min at 65°C.

d. The mixture was cooled in an ice bath for 5 to 7 min.

e. The mixture was centrifuged at 1,000 X g for 10 min.

f. The aqueous phase was carefully aspirated with a Pasteur pipette.

g. The interphase and phenol layer were washed with 2 to 3 ml of buffer and reextracted at 65°C for 5 min.

h. The mixture was cooled and centrifuged as in steps d and e.

i. The aqueous phase was aspirated and added to the aqueous from step f.

j. To the combined aqueous phase was added 4 to 5 ml of phenol. The mixture was extracted two more times at 65°C for 5 min each.

k. To the aqueous phase was added 0.1 volume of 3.3 M sodium chloride.

l. 2.2 volumes of absolute ethanol were added to the solution from step k.

m. The RNA was allowed to precipitate overnight in the freezer.

n. The precipitate was centrifuged at 2,000 X g for 15 min.

o. The precipitate was redissolved in buffer and reprecipitated with ethanol.

p. The precipitate was washed three times with ethanol.

q. The RNA precipitate was dissolved in the appropriate buffer.

r. The RNA solution was stored at -70°C in sterile tubes.

5. Discussion (letters below refer to the preceding outline section)

a. The tissue is powdered while still frozen to minimize degradation by RNAse. This is crucial, as a single break in a 28s RNA molecule may cause it to migrate electrophoretically with

the 18s fraction.

b. The rationale for mixing phenol and buffered tissue was explained in the introductory paragraph, above.

c. Experience has shown that for mammary tissue the highest yield of undegraded RNA was achieved when the extraction was done at 65°C. Georgiev (1967) also found that a better yield was obtained using a hot extraction. He stated that at 60°C, in the presence of sodium dodecyl sulfate, virtually all the RNA, including nucleochromosomal RNA, was liberated. In order to present extraction of DNA, the pH must be kept at about 5, and the length of extraction as short as possible (Scherrer and Darnell, 1962).

d. The cooling stage allows the separation and complete precipitation of phenol out of the aqueous phase.

e. Centrifugation of the phenol-buffer-tissue mixture results in the formation of a four-phase system (see Figure 2). The bottom layer is sand, the second layer is phenol in which is dissolved protein and some DNA, the third layer (the interphase) contains membranous fragments, DNA and a small amount of rapidly labeled RNA (Sibatani, Dekloet, Allfrey and Mirsky, 1962), and the top layer has dissolved in it

the majority of the RNA and the polysaccharide.

f. The aspiration of the aqueous phase must be done carefully. It is preferable to leave some of the aqueous phase behind rather than to contaminate it with DNA and protein from the interphase. This is especially important when one realizes that one of the proteins is RNAse.

g. Reextraction of the interphase once should release the majority of the RNA.

h-i. Self-explanatory.

j. The purpose of the repeated extractions is to free the RNA as completely as possible from contaminating protein. One criterion of freedom from protein is the clarity of the aqueous phase and lack of a whitish "scum" at the interphase.

k. Concentrated saline solution facilitates the precipitation of RNA.

l. Ethanol is the RNA precipitant. It also precipitates some polysaccharides, which makes it difficult to quantitate phenol-extracted RNA by the orcinol method (the lactose will give erroneously high readings).

m-q. Self-explanatory.

r. When RNA is immediately frozen and maintained at $-70^{\circ}C$, it is stable for at least 3 mon.

IV. Quantitation of Nucleic Acids

A. RNA Quantitation

Two methods were routinely used to measure RNA:

1. Ultraviolet absorbtion

In this method, the sample to be tested is placed in a spectrophotometer cell and optical density read at 260 mμ (roughly, the ultraviolet wave length of maximum absorption for nucleic acids) against a buffer blank. The ultraviolet absorption of nucleic acids occurs by virtue of the energy-absorbing properties of the conjugated double bond on the nitrogenous bases. By measuring the absorbance of the sample at 280 mμ also, the 260:280 ratio can be calculated and the nucleic acid concentration read directly off a nomograph (prepared by the California Corporation for Biochemical Research, 3625 Medford St., Los Angeles, California, based on Warburg and Christian, 1942). The 260:280 ratio for pure RNA or DNA is 2.0, and, as protein contamination increases, the absorbance at 280 increases, lowering the 260:280 ratio (Warburg and Christian, 1942). Ultraviolet absorbtion measurement, therefore, quantitates not only RNA but protein contamination. It is not, however, the method of choice to use on RNA hydrolysates obtained from Schmidt-Thannhauser hydrolysis. Even when alkaline hydrolysis time is kept to a minimum of

1 hr, protein contamination occurs and gives falsely high readings. Another drawback is that at the low pH of the RNA hydrolysates (about pH 1), the 280 reading is relatively higher than it is at neutral or alkaline pH. The pH of the solutions must, therefore, be laboriously adjusted before readings can be taken. This method was periodically used to cross-validate the data obtained by the orcinol method (described below), but was not routinely used except on phenol-estracted RNA, on which the orcinol method is impractical. Finally, the ultraviolet absorption at 260 mμ does not distinguish at all between DNA and RNA, so if there is cross-contamination of the fractions, it would not be detected.

2. Orcinol determination

   a. General Description

      The orcinol determination of RNA depends on the hydrolysis of RNA to form furfural from ribose (Hutchison and Munro, 1961). The furfural reacts with orcinol to form a green pigment. An outline of the procedure is below (Brown, 1946).

   b. Reagents

      Orcinol, Fisher reagent grade, is recrystallized before use. For the recrystallization instructions, see Appendix 1. The stock solution is made as follows:

4.0 gm orcinol

2.7 gm ferric ammonium sulfate

The above reagents are diluted to 100 ml with distilled water. This solution is stable for 3 to 4 months after which it begins to fade.

The working solution:

10 ml stock orcinol solution

166 ml concentrated hydrochloric acid

24 ml distilled water

c. Standard RNA

The standards are prepared in triplicate using Worthington's yeast RNA. The following concentrations were used: 10, 20, 40, 50, 100 and 200 µg. One ml of standard is placed in each test tube.

d. Samples

RNA hydrolysates were diluted 1:2 with distilled water and 1 ml was placed in each of duplicate or triplicate tubes. Blanks, in triplicate, were made with 6 per cent PCA.

e. Test Protocol

i. To each of the samples, standards and blanks was added 3 ml of the orcinol working solution.

ii. The tubes were rapidly mixed with a vortex mixer (Deluxe Mixer S8220, Scientific Products, Evanston, Illinois).

iii. The tubes were heated for 30 min at 85°C, cooled and read on a Gilford-Beckman spectrophotometer at 660 mμ.

iv. A standard curve was made using the known values of yeast RNA, and sample values were calculated from the standard curve. See Appendix 2 for an example of an orcinol standard curve.

f. Discussion of the orcinol test

The orcinol test generally is quite reproducible, although the standard curve values begin to decrease as the stock orcinol solution ages several months. As can be seen from Appendix 2 the curve is linear over a large concentration range.

One complication of the orcinol reaction is that purine-bound ribose reacts to a much greater extent with the reagent than does pyrimidine-bound ribose (Paege and Schlenk, 1950). If two samples of RNA have greatly different purine-pyrimidine ratios, then, the orcinol values for them will not be reliable. In order to determine whether yeast RNA is similar enough to mammary RNA in base composition to use as a standard in the orcinol procedure, the following test was done:

i. The concentrations of the yeast and the

mammary gland RNAs were adjusted so that the absorption at 260 mμ was the same.

ii. Aliquots of the RNA samples above were assayed in the orcinol test.

This test was repeated using several different lactating gland RNA and prelactating gland RNAs. No significant difference in orcinol reactivity was found, and therefore it was decided that yeast sRNA would be a satisfactory orcinol standard for future work.

While a standard curve was prepared for each set of readings taken, calculations of RNA values for the samples were made on a programmable Olivetti-Underwood calculator-computer. These values were more accurate than values read off the standard curve, and were quicker to obtain.

It has been mentioned in another section that lactose, a sugar produced in high amounts by the lactating gland, interferes with the orcinol reaction, but that three PCA washes remove essentially all of it. Other compounds which have been reported to interfere with the orcinol reaction are proteins (Fleck and Munro, 1962) and DNA (Schneider, 1955). Since it is expected that some contamination from both of these substances occurs in the course of the washing and hydrolysis, it was necessary to determine 1) the

extent of the interference and 2) whether contaminants were normally present in high enough quantities to invalidate the orcinol test. Therefore, the following studies were performed:

iii. Known amounts of RNA and DNA were mixed, the orcinol test was done, and the optical density at 660 mμ was compared to values for known amounts of RNA alone (see Table 1).

iv. Known amounts of RNA and protein were mixed and the orcinol test performed as above (Table 1).

Inspection of Table 1 shows that 50 μg of DNA mixed with 100 μg of RNA does not contribute to the color reaction. The optical density produced by 100 μg DNA alone is 13 per cent that produced by the same amount of RNA.

The next step of this experiment involved testing several samples of RNA hydrolysates to determine the normal range of contamination by DNA. Results are shown in Table 2. It can be seen that the maximum contamination by DNA was only 7 per cent, and thus, DNA interference does not pose a problem in the orcinol test.

The addition of 50 μg of bovine serum albumin caused an increase in $O.D._{660}$ of 16 per cent over the value for 100 μg of RNA alone (Table 1).

Thus, protein contamination could cause falsely high RNA readings in the orcinol test. Table 3 shows examples indicating the range of protein contamination of RNA hydrolysates. In this study protein was quantitated by a modification of the Lowry, Rosebrough, Fair and Randall, (1951) method, to be discussed. Contamination is expressed as micrograms of protein divided by micrograms of RNA per unit volume, converted to percentage. The highest percentage contamination registered is 9 per cent, which should cause less than a 3 per cent elevation of optical density. Thus it seems that protein contamination is not a major problem for the orcinol test, at the level of protein seen in these hydrolysates. Some RNA samples from each experimental and control group were periodically checked by the Lowry test to be sure that protein levels were acceptably low. On occasions when the DNA-protein pellet broke and spilled into the RNA hydrolysate, the hydrolysate was recentrifuged and the two pellets were recombined.

B. DNA Quantitation

1. Ultraviolet absorbtion

The same comments made about RNA quantitation by 260 mμ absorbance apply to DNA; generally ultra-

violet absorption measurements were made as a check on diphenylamine values. Protein contamination was more of a problem with DNA than with RNA hydrolysates, probably because the DNA fraction undergoes hot acid hydrolysis, which may solubilize some proteins.

2. Diphenylamine method

a. General information

The diphenylamine test used in these studies is a modification (Burton, 1968) of the original method of Dische (1930). The blue color which develops is the result of the reaction of deoxyribose with the diphenylamine. As is the case in the orcinol test, purine-bound pentose contributes much more strongly to the color reaction than does pyrimidine-bound pentose. Calf thymus DNA does not differ significantly from mammary gland DNA in diphenylamine reactivity, however.

b. Reagents

i. Stock solution

1.5 gm diphenylamine (Reagent, ACS grade, Matheson, Coleman and Bell, Norwood, Ohio)
100 ml glacial acetic acid
1.5 ml concentrated sulphuric acid
This solution should be made at least every other day.

ii. Working solution

To the stock solution, above, is added 0.5 ml of 1.6 per cent acetaldehyde, reagent grade, for each 100 ml of stock solution. This solution is prepared immediately before use (i.e., not more than one hour before it is needed).

c. Standard DNA

Worthington's calf thymus DNA was dissolved in 5 mM sodium hydroxide to a concentration of 0.3 mg per ml. Working standards are prepared by mixing one volume of the above DNA solution with one volume of 1 N PCA and heating to 70°C for 15 min, which gives a hydrolyzed DNA solution with a concentration of 150 µg/ml.

d. Standard curve

The standard working solution of DNA was diluted to the following concentrations: 15, 30, 45, 60, 75 and 90 µg/ml. Blanks in triplicate consisted of 1.5 ml of 3 per cent PCA.

e. Samples

One ml of DNA hydrolysate was diluted to 1.5 ml with 3 per cent PCA. Samples were prepared in triplicate. A representative diphenylamine standard curve is found in Appendix 3.

f. Test protocol

i. To 1.5 ml of the standard, blank and sample tubes was added 3.0 ml diphenylamine working

solution. The tubes were mixed.

ii. Tubes were incubated at room temperature for 18 hours in the dark.

iii. The optical density of the tubes was read at 600 mμ on a Gilford-Beckman spectrophotometer.

iv. The standard curve was drawn and calculations for sample values made with the Olivetti-Underwood calculator.

g. Discussion

The diphenylamine method proved to be satisfactory for quantitating DNA from hydrolysates obtained with the Schmidt-Thannhauser procedure. This reaction was not as reproducible as the orcinol test and thus the complete range of standard DNA concentrations should be made up each time the test is done. The range of concentrations which can be measured is also more narrow than is the case in the orcinol test. Although this is not shown in Appendix 3, the curve flattens out at DNA concentrations greater than 100 μg.

The major contaminants which can be expected in DNA hydrolysates are RNA and protein. RNA has not been reported to interfere appreciably in the diphenylamine test, and this has been confirmed in the present study.

Table 4 shows results of a diphenylamine test in which the optical density for DNA mixed with RNA was compared to the optical density of DNA alone. There was no significant difference between the two groups. Protein, on the other hand, appeared to cause a pronounced increase in color development in the diphenylamine reaction (Table 5). Mixing equal amounts of DNA and bovine serum albumin produced an optical density increase over DNA alone of 18 per cent and 26 per cent in the two groups reported here.

DNA hydrolysates from pregnant and from lactating mice were randomly selected and analyzed for the amount of protein they contained (by the Lowry method). The results, presented on Table 6, shows that generally the amount of protein in micrograms is less than 10 per cent the amount of DNA, and is therefore negligible as a contaminant. However, one sample was highly contaminated with protein. Because this could appreciably raise the observed diphenylamine values, it was decided to measure the protein of each DNA hydrolysate. Those samples with high protein (equal or greater than 15 per cent) were discarded.

V. Protein Determination

A. Methods

Protein levels in the RNA and DNA fractions were measured by the Oyama and Eagle (1956) modification of the Lowry et al. (1951) colorimetric determination, in which color development is based on the reaction of the phenol group on tyrosine moieties with the Folin-Ciocalteau reagent in basic cupric sulfate solution.

B. Reagents

1. Solution A

   20 gm sodium carbonate

   4 gm sodium hydroxide

   0.2 gm sodium potassium tartrate

   The above chemicals were diluted to one liter with distilled water.

2. Solution B

   5 gm hydrated cupric sulfate, reagent grade, per liter.

3. Solution C

   50 parts solution A to 1 part solution B, mixed fresh on the day of use.

4. Folin-Ciocalteau reagent (Fisher Scientific Company). This reagent was diluted 1:2 with distilled water immediately before use.

C. Standards

Bovine serum albumin (BSA) was obtained from Sigma

Chemical Company. It was diluted with 0.01 N sodium hydroxide to the following concentrations: 10, 25, 50, 75, and 100 µg/ml.

D. Blanks

1 ml of 0.01 N sodium hydroxide was used as the blank.

E. Samples

RNA and DNA samples were generally diluted 1:4 or 1:5 with 0.01 N sodium hydroxide. This was not necessary from the point of view of diluting the protein, but was done to alkalize the solutions, which were acidic from the PCA. The color does not develop in acid solution. If phenol-extracted RNA samples were being tested for protein contamination, the samples had to be thoroughly washed to be certain that no phenol remained; the Folin-Ciocalteau reagent reacts strongly with the phenol group.

F. Procedure

1. After the samples were diluted, 1 ml each (in triplicate) of sample, standards, and blanks were pipetted into test tubes.
2. To the samples, standards and blanks 5 ml of solution C was added, and the solution allowed to stand for 10 min.
3. The Folin-Ciocalteau reagent (0.5 ml) was jetted into the mixture above, using a 1 ml glass tuberculin syringe without a needle. The tubes were quickly

mixed with a vortex mixer. The rapid adding and mixing of the color reagent was necessary or a great deal of variability among the triplicate samples occurred. The color was allowed to develop for 30 min in the dark.

4. Optical density of the samples was read at 625 mμ on a Gilford-Beckman spectrophotometer.

G. Discussion

In Appendix 4 is an example of a standard curve for BSA, using the Oyama-Eagle method. This technique was satisfactory for both phenol-extracted RNA and RNA and DNA from the Schmidt-Thannhauser hydrolysis. Neither RNA nor DNA interfered with the color development.

VI. Radioactive Labeling

A. Labeling Animals

In all labeling experiments animals were injected intraperitoneally with the radioactive precursor using a 25-gauge disposable needle and disposable 1 ml tuberculin syringe. The uridine-6-^{3}H, with activity of 10.4 curies per millimole, was obtained from New England Nuclear, Boston, Mass. The precursor was diluted with sterile normal saline solution. At the end of the labeling period the animals were killed and the RNA and DNA extracted as usual.

B. Removal of Soluble Precursors

Successive washes from homogenates of ^{3}H-uridine-labeled

mammary gland homogenates were saved, solubilized, and counted as described below. This was done to determine how many washes are necessary to remove the soluble (i.e., unincorporated) label. Table 7 shows the results of such a study. Evidently the customary three washes are enough to decrease unincorporated counts to an acceptable level (to less than 1 per cent of the number of counts in the first wash). Samples of the third PCA wash were routinely counted, to be sure that in all cases the counts were low enough. In the case of phenol-extracted RNA, one reprecipitation and two ethanol washes were sufficient to reduce soluble counts to a level about half that of background. Normally one reprecipitation and three ethanol washes were done, so falsely high counts in phenol-extracted RNA should not be a problem, either.

C. Counting the Samples

1. One-tenth ml of RNA hydrolysate or phenol-extracted RNA was solubilized with Soluene (Packard Instrument Company, Downer's Grove, Illinois). Usually 1 ml of soluene was sufficient to solubilize the sample.
2. Scintillation fluid was composed of the following:

   5 gm PPO (2, 5-diphenyloxazole) from Packard Instrument Company, scintillation grade.

   0.250 gm M_2POPOP (1, 4-bis-[2-(4-Methyl-5-phenyloxazoyl)]-benzene), Packard Instrument Company, scintillation grade.

The above reagents were dissolved in one liter of scintillation grade toluene (Matheson, Coleman and Bell).

3. The samples, to which were added 10 ml of scintillation fluid, were usually counted for 50 min or 100 min in a liquid scintillation counter (Model 3375 Tri-Carb, Packard Instrument Company).

D. Calculation of Disintegrations per Minute and Specific Activity

1. The counts per minute (CPM) were printed out for each sample. The A.E.S. (external standard) ratio was also printed out. By use of a standard curve (see Appendix 5 for this graph), A.E.S. can be converted to per cent efficiency. Disintegrations per minute (DPM) = CPM $\div 10^{-2}$ X per cent efficiency. For example, if the CPM were 550 and per cent counting efficiency were 35, the DPM = 550 $\div$ 0.35, or 1570.

2. The specific activity of an RNA sample can be calculated by dividing the total DPM in that sample by the total RNA. If the radioactivity in a 50 mg quantity of RNA were 5,000 DPM, the specific activity of that RNA would be 100 DPM per mg of RNA.

VII. Polyacrylamide Gel Electrophoresis of RNA

A. General Principles

Separation of RNA species was accomplished by acrylamide gel electrophoresis. The method consists of passing a current through a gel-containing tube which is emersed in buffer. The sample to be fractionated is placed on the upper end of the gel. Separation in acrylamide gel is based on 1) charge on the molecule (This is not a major factor in separating RNA species, as they have a constant charge:mass ratio [Olivera, Baine, and Davidson, 1964]), 2) the shape of the molecule, and 3) the size of the molecule. The rate of movement of RNA molecules through the pores of the polymerized gel is primarily determined by their size and, theoretically, by their shape (e.g., whether they are compact and spherical or linear). The separation by molecular weight is due to the "sieving" effect of the pore size of the gel, which varies inversely with gel concentration. A more complete explanation of the principles and mechanisms of polyacrylamide gel electrophoresis will be incorporated into the detailed description of the specific system used in these studies.

B. Reagents

For a detailed preparation procedure of the reagents, see Appendix 6.

1. Buffer

A Tris, sodium acetate, EDTA buffer was used. The sodium acetate helped to preserve the secondary structure of the RNA and the EDTA, a chelating

agent, prevented RNA from sticking to the surface of the gel (Loening, 1967).

2. Acrylamide-bis-acrylamide stock solution

   Acrylamide: 8.8 per cent

   Bis-acrylamide: 0.44 per cent

   This mixture is diluted to 25 ml with distilled water.

3. Catalyst

   Ammonium persulfate was made up to 135 to 140 mg per cent in distilled water immediately before use, as it is very unstable.

4. Sucrose

   A 70 per cent solution was made in sterile distilled water using ribonuclease-free sucrose obtained from Mann Research Laboratories, New York, N.Y.

5. Working solutions

   The stock acrylamide-bis-acrylamide solution (see above) was diluted with distilled water and catalyst to give a final concentration of:

   a. 2.2 per cent acrylamide
      0.11 per cent bis-acrylamide
      0.035 per cent catalyst, added last

   b. 2.8 per cent acrylamide
      0.11 per cent bis-acrylamide
      0.035 per cent catalyst, added last

   Once the catalyst is added, the solutions must be used immediately, as the polymerization process occurs quickly.

C. Preparation of the Gel Gradients

After the catalyst was added, the solutions were pipetted into the two reservoirs of a gradient mixer (built by Haraguchi, 1969) which was attached to a Harvard Multi-speed Transmission Pump, Model 1201 (Harvard Apparatus Company, Dover, Mass.). The gradient maker was quickly turned on (so that the gels did not polymerize while in the apparatus), and the resultant 2.8 per cent to 2.2 per cent gel gradient was carefully discharged into a 10 cm length of .6 cm diameter quartz Super seal tubing which had been slightly embedded into a block of paraffin (for support and to prevent the gel from running out of the bottom of the tube). When all the gel was in the tube, a small amount of water was gently layered onto the top of the gel to insure that the surface would be flat. The gels were allowed to polymerize for 45 min in the dark.

Several single-concentration gels were used to separate total intact RNA, but these were not as successful as a gel gradient. The large difference in molecular weight (and possible configuration) between sRNA (molecular weight 25,000) and ribosomal RNA (molecular weight about 0.5×10^6 and 2×10^6 for 18s and 28s species, respectively) (Kurland, 1960) made it difficult to fractionate the three major species on a single gel. If a 2.2 per cent gel were used, the 18s and 28s rRNAs could be separated, but the sRNA would tend to run off the end of the gel. Conversely, at a concentration of 2.8 per cent, resolution of the rRNA

fractions was poor. The use of a smooth linear gradient made possible a good fraction of all species on a single gel.

D. Pre-electrophoresis

The gels alone were electrophoresed in buffer for 40 min prior to adding the samples. This served to remove from the gel column any ultraviolet-absorbing impurities.

E. Preparation of the RNA Samples

An appropriate amount of RNA (50-100µg) was mixed to a final volume of 0.1 to 0.2 ml with 70 per cent sucrose. The solution was cautiously pipetted onto the surface of the gel column, which was now under the surface of the buffer. The RNA was mixed with the sucrose to increase its viscosity and insure that it would not be carried away by currents in the buffer.

F. Electrophoresis

The samples were subjected to an electrophoresing current of 5 milliamps per gel for varying lengths of time, as determined below. The power supply was a Canalco Model 200A Constant Current Power source.

G. Scanning the Gels

At 15 to 20 min intervals, the gel-containing tubes were removed from the electrophoresis bath and placed in a Gilford Model 2410 linear transport. The tube was scanned at 260 mµ and, if desired, at 280 mµ on a Gilford-Beckman

spectrophotometer with a Gilford Model 2000 absorbance indicator. The use of the optical grade quartz tubes made it unnecessary to remove the gels from the tube for scanning and thus a single sample could be scanned several times, until the optimum amount of separation is achieved (Roberts, 1969). Repeated brief removals of the tubes for scanning did not seem to result in appreciable diffusion of the RNA bands. Figure 3 is an example of a 260 mμ scan of RNA extracted from prelactating mammary gland.

H. Verification of the RNA Species

In order to determine whether peak I of Figure 3 was the sRNA (4-5s RNA), a standard of Worthington's yeast sRNA (20 μg, altogether) was placed on the gel column. It migrated the same distance per unit time as peak I of mammary gland RNA. Both the yeast RNA and peak I were sensitive to ribonuclease treatment. It was attempted to verify the identities of peaks II and III as 18s and 28s ribosomal RNA by the same means. E. coli marker ribosomal RNA was obtained from Miles Laboratories, Elkhart, Ind., and was electrophoresed. This RNA proved to be both highly degraded and contaminated with DNA; indeed, about 40 per cent of the ultraviolet-absorbing material in each of three samples received was DNA. Therefore it has not been possible to show with absolute certainty that peaks II and III were rRNA. They were both sensitive to ribonuclease. They migrated much more slowly than did the sRNA peak, and

their relative amounts were consistent with 18s and 28s RNAs, however.

I. Quantitating RNA from the Fractionated Peaks

In these experiments the relative sizes of the peaks and the specific activities of the peaks were of primary importance. To quantitate the RNA in each peak, the following facts should be known:

1. The amount of RNA put on the gel. While there may be some loss of RNA to the surrounding buffer, usually it was less than 10 per cent.
2. The background optical density at each point on the scanning curve. This can be adequately determined by scanning duplicate gels to which only sucrose is added.
3. The area under each of the peaks. By subtracting the background absorbance from the total absorbance of the peak, the absorbance of the RNA in the peak can be known. This calculation was repeated for each of the peaks. The amount of ultraviolet-absorbing material not in the major peaks was also calculated. This material probably represented small amounts of degraded RNA and contaminating protein.
4. Since the total absorbance was known (fact 1, above), the quantity of RNA in each peak was calculated by calculating the ratio of absorbance of the peak to

the total absorbance.

J. Scintillation Counting of Acrylamide Gel Samples

All of the experiments to be described here involve using radioactively labeled RNA, and it is necessary to know not only the quantity of RNA in each of the major peaks, but the relative amount of incorporation of radioactive precursor into RNA of those peaks. In order to get this information, one must cut the gel into slices and count the radioactivity in each slice. This was accomplished as follows:

1. The location of absorbance peaks along the length of the gel was recorded on a chart recorder on the Gilford-Beckman spectrophotometer for later comparison to radioactivity peaks.
2. The gel, still in the quartz tubes, was placed on a block of dry ice and the gel allowed to harden, but not freeze solid.
3. The gel was pushed out of the tube with a teflon plunger, care being taken to keep track of the orientation of the gel.
4. With a razor blade the gel was sliced into uniform 2-3 mm slices. The gel must not freeze too hard, or it will chip when sliced.
5. Using forceps, the frozen pieces of gel were put into serially numbered scintillation vials.
6. The gel slices were dissolved by adding 0.1 ml of a

30 per cent solution of hydrogen peroxide to the scintillation vials, which were then incubated overnight in a 35 to 40°C oven. Of several methods tried this was the only method which sufficiently dissolved the gel.

7. One ml of Soluene (Packard Instrument Company tissue solubilizer) was added to the vials and the vials vigorously shaken to be sure that any material sticking to the walls would be dissolved.
8. A 10 ml aliquot of scintillation fluid (see section VI, C 2 for composition of scintillation fluid) was placed in the vials, which were again shaken. Sometimes the liquid in the vials was turbid after addition of the scintillation fluid, indicating that the sample was not completely in solution. The addition of an additional 1 ml of Soluene usually cleared the solution, although this reduced the counting efficiency by about 2 per cent.
9. The samples were placed in a Packard Model 3375 Liquid Scintillation Counter and counted for 50 or 100 min.
10. The disintegrations per minute (DPM) for RNA in each vial was calculated and values for vials containing RNA from a single peak were pooled. The ratio of DPM:RNA-quantity of each peak constituted the specific activity of the peak. Due to inconsistencies in the size of the gel slices these figures

were necessarily rough, but they were able to show differences in specific activity among the three main RNA peaks.

RESULTS

I. Normal Values for Mammary Gland Nucleic Acids

Beginning with day 14 of pregnancy and continuing through the twenty-sixth postpartum day, groups of mice were killed, their mammary glands were removed and RNA and DNA levels were measured. Table 8 is a compilation of the results of those studies. Results are graphically shown on Figure 4.

A. RNA

It can be seen (Figure 4) that RNA levels increase from 2.1 mg per gram of gland weight at 14 1/2 days of pregnancy to 3.5 mg per gram of gland weight at 20 days of pregnancy (approximately the last day of pregnancy in mice). This increase of 75 per cent is significant at the .01 level of confidence (Analysis of Variance, Olivetti Underwood Programma 101 Statistical Analysis manual, program No. 6.10). From parturition through the fourth day of lactation RNA levels rise from 3.5 mg/gram of mammary gland, to 7.7 mg/gram of mammary gland, an increase of 120 per cent. The RNA level at 10 days of lactation, 13.8 mg/gram of mammary tissue, is the highest mean level reached during pregnancy and lactation. This value represents an increase of 590 per cent over midpregnancy, and 515 per cent increase from the first day of lactation. From the tenth to the fourteenth day of lactation RNA drops only slightly (and not significantly)

from 13.8 mg/gram of mammary tissue to 12.8 mg/gram of mammary tissue. From the fourteenth to the twenty-first day of lactation (the normal nursing period is about 21 days) RNA drops from 12.8 mg/gram of gland to 6.5 mg/gram of gland, a decrease of 51 per cent. Average RNA values continue to decline through the first three days of the weaning period. By the third day of weaning, the level of mammary RNA is 3.4 mg/gram of mammary tissue, about the same as it was on the last day of pregnancy. The data show, then, that RNA increases significantly from midpregnancy through parturition. The greatest increase in RNA, however, occurs during the first four days of lactation, and RNA concentration continues to rise until about 10 days of lactation. From 10 to 14 days of lactation the level of RNA remains fairly constant. It begins to decrease steadily from fourteen days of lactation through weaning.

B. DNA

Table 8 and Figure 4 show that the maximum variation in DNA concentration is approximately 0.4 mg/gram of mammary gland, until the 18th day of lactation. During the period of most rapid RNA increase, the first four days of lactation, mean DNA values rise from 1.3 mg/gram of mammary gland to 1.4 mg/gram mammary tissue, a change which is not significant. The variation which does occur appears random. From the eighteenth day of lactation through the third day of weaning (a six day period), mammary gland DNA drops from 1.2 mg/gram

gland to 0.4 mg/gram gland. This is a 67 per cent decrease, significant at $p < .01$, analysis of variance.

C. RNA:DNA Ratio

The ratio of milligrams of mammary RNA to DNA increases from 1.5 at 14 1/2 days of pregnancy to 2.4 at 20 days of pregnancy. The rate increase continues sharply through the first 6 days of lactation and continues to rise, though not so steeply, through the thirteenth day of lactation. The change in RNA:DNA ratio parallels the change in RNA concentration until about the thirteenth day of lactation, when it levels off. From the thirteenth day of lactation to the third day of weaning the decrease in RNA:DNA is statistically not significant.

D. Radioactive Labeling of Normal Mice

Tritiated uridine incorporation into mammary gland RNA was measured in two groups of mice: 1) 18- to 19-day pregnant mice, and 2) 5- to 7-day lactating mice. These two periods were chosen to show maximum differences in incorporation rate; at this time of lactation total RNA is increasing at a high rate (see Figure 4). Both groups received 1 microcurie (μc) per gram of body weight of the labeled RNA precursor, injected intraperitoneally. The labeling period was three hours. Results of this experiment are presented in Table 9.

The mean DPM per gram of mammary gland in lactating mice is about five times greater than the mean DPM for mammary gland of pregnant mice. The specific activity

of RNA of the lactating glands also exceeds that of the prelactating gland, by 177 per cent. The mean total RNA concentration of the lactating mammary gland is greater by 272 per cent than that of glands of pregnant mice.

II. Acrylamide Gel Electrophoresis Of Phenol-Extracted Mammary Gland RNA Of Pregnant And Lactating Mice

In Figures 5A and 5B the relative sizes of radioactivity and optical density peaks of two RNA samples are graphed. Figure 5A is an example scan of mammary RNA extracted from a 17- to 18-day pregnant mouse. The mouse had been labeled for three hours with 2 μc per gram of body weight of ^{3}H-uridine. The other scan is of mammary RNA of a similarly labeled 6- to 7-day lactating mouse. Since the total amount of RNA applied to the columns was nearly the same, the specific activities of the peaks can be compared.

It can be noted that the optical density peaks do not in all cases coincide with the radioactivity peaks. This is probably due to inconsistencies in the thickness of the gel slices, rather than to a difference in migration rate.

At the 2.2 per cent end of the gel is a small peak which is not shown on Figure 3 (Materials and Methods). The optical density peak coincides with a tritium peak, it is DNAse-insensitive and RNAse-sensitive, indicating that it is RNA. This RNA fraction was not always isolated in the phenol extraction.

In comparing the two gel profiles the most obvious

observation which can be made is that the specific activities of all of the peaks of the lactating gland RNA are greater than those of the prelactating gland RNA. For reasons brought out earlier (Section VII-I, Materials and Methods) only rough quantitation is possible, but integration of the areas under the four major peaks of each profile shows that the specific activity of the lactating gland RNA is greater by 49 per cent than the specific activity of the prelactating gland RNA.

The relative distribution of ^{3}H-uridine among the mammary RNA fractions is also quite different in prelactating versus lactating gland. While the percentage distribution of DPM is about in the same ratio as the optical density in prelactating RNA, this is obviously not the case in lactating mammary RNA. A relatively much higher incorporation into the sRNA peak (113 per cent) is observed in the lactating mammary RNA. There is also a shoulder on the heavy end of the sRNA radioactivity peak which is not apparent in the RNA from mammary glands of pregnant mice. This shoulder may be an artifact, although it has been observed in the other five scans of labeled, phenol-extracted RNA of lactating (but not prelactating) glands which were analyzed.

III. Ovariectomy of Pregnant Mice

Since ovariectomy of mice always resulted in abortion (Harris, 1927), it was difficult to assess the effect of this

operation. Mice ovariectomized late in pregnancy often refused to nurse their own prematurely delivered or foster pups. Table 10 compares mammary RNA levels of three-day postovariectomy mice to mammary RNA levels of 1) three-day post sham-operated mice, and 2) intact mice after three days of lactation.

The mean mammary gland weights of ovariectomized, sham-operated and three-day lactating mice are 0.99 grams, 1.03 grams and 1.04 grams, and these are not significantly different. The mean concentration of mammary RNA of ovariectomized mice is 6,101µg per gram of gland, that of sham-operated controls is 4,076µg per gram of gland. This difference is significant at $p<0.05$ level. The mean RNA:DNA ratios (4.9 and 3.3 for ovariectomized and shams, respectively) are also significantly different. The RNA concentration and RNA:DNA ratios of ovariectomized mice are also larger than those values in three-day lactating mice, but the differences are not significant at the $p<0.05$ level.

IV. Ovariectomy of Lactating Mice

Ovariectomy of mice in early (1-2 days) or mid-lactation (7-8 days) has no perceptible effects on any of the parameters measured. Table 11 compares ovariectomized (three-day post-operative) to sham-operated mice at the two stages of lactation. Mice which were ovariectomized postpartum, in contrast to pregnant animals, exhibited no abnormal behavior in caring for their pups.

V. Effect of Adrenalectomy of Pregnant Mice on Mammary Gland Nucleic Acids

In this series of experiments, adrenalectomy of a mid-pregnant or late-pregnant BALB/c mouse always resulted in abortion within a few hours; this has not been reported for mice, but Hoar (1961) states that 93 per cent of his adrenalectomized guinea pigs lose their litters by resorption or abortion. The mother mice do not seem traumatized or "shocky" immediately after the operation, and continue to eat quite normally. Because of this phenomenon, it was not possible to test the effect of adrenalectomy on mammary nucleic acid throughout a three day pregnancy period, as had originally been planned. Adrenalectomized mice, as did ovariectomized ones, often destroyed or refused to nurse their own or foster pups. This abberant behavior occurred in more than half the operated mice. The babies of these animals did not gain weight normally and the mammary glands of the mothers were not milk-filled at autopsy, as were normal lactating galnds.

Table 12 shows the mammary RNA, DNA, RNA:DNA ratios and gland weights for mice which did nurse their litters. The RNA:DNA ratio of the experimental animals (3 days post-operatively) is the same as that of intact 18- to 19-day pregnant mice. It should be kept in mind that this is the time at which the experimental animals were adrenalectomized. Although the RNA:DNA ratio of the two groups presented on Table 12 are the same, the other values differ:

1) Mammary gland weight in the adrenalectomized mice

is only about 40 per cent that of 18- to 19-day pregnant mice.

2) The mammary gland RNA concentration of the adrenalectomized animals (1,934μg/g gland) is 63 per cent that of the pregnant controls, while the mean DNA value is about 62 per cent of the controls.

The nucleic acid concentration of three day postoperative adrenalectomized mice can also be related to three other control groups (Table 10): a) three-day postoperative ovariectomized mice, which also abort their litters within a few hours after the surgical procedure, b) three-day lactating intact mice, which have been nursing the same length of time as the adrenalectomized mice, and c) three-day postoperative sham-operated mice, which would have been nursing litters for about one day. All the measured and calculated values are lower in the adrenalectomized pregnant mice than in mice of any of the control groups.

In summary, it can be said that adrenalectomy of pregnant mice (which by termination must be considered nursing mothers) results in:

1) Loss of total mammary gland mass
2) A decline in mammary gland RNA and DNA concentration relative to mean values of animals at 18 to 19 days of pregnancy. The percentage reduction in RNA and DNA levels is about equal, resulting in no net change in RNA:DNA ratio.

VI. Effect of Adrenalectomy on Mammary Gland Nucleic Acid Levels of Lactating Mice

The consequence of adrenalectomy is even more dramatic in lactating mice than it is in pregnant ones (Table 13). The mammary gland weight is 40 per cent that of the control (sham-operated) animals. RNA concentration, the mean value of which is 1,533μg/g tissue, is only 13 per cent of the mean concentration of the control lactating mice. RNA:DNA ratio is lower by 86 per cent than the RNA:DNA ratio of the controls. The DNA concentration of the glands is the variable least influenced by adrenalectomy; in adrenalectomized animals the DNA/g mammary gland is 89 per cent that of the control animals; this difference is not significant (by Student's t-test, $p<0.10,>0.05$).

VII. Effect of Ovariectomy-Adrenalectomy on Pregnant and Lactating Mice

Table 14 shows results of adrenalectomy and ovariectomy performed at the same time on 1) 18- to 19-day pregnant mice, and 2) 3- to 4-day lactating mice. Comparing the results of adrenalectomy-ovariectomy of the pregnant mice to the results of adrenalectomy alone (Table 12) shows that the double operation produces no additional significant changes in any of the measured or calculated values. The same statement can be made of adrenalectomy-ovariectomy on lactating mice; the mean gland weight, RNA concentration, DNA concentration and RNA:DNA ratios are not statistically different in

double-operated mice from those values in mice which were adrenalectomized only (see Table 13).

VIII. Effect of Hormone-Replacement Therapy on Mammary Gland RNA and DNA Levels of Lactating Mice

In the experiment reported below, mice were adrenalectomized or sham-operated 2-3 days after the birth of the babies and allowed to nurse their litters. Three days after the operation, at which time it was evident (by the signs enumerated in Materials and Methods, Section II, C2 and 4) whether adrenalectomy had been successful, hormone replacement therapy was started. A saline suspension of 50 µg of hydrocortisone acetate was injected subcutaneously every 12 hours. The control animals were given a saline placebo injection subcutaneously every 12 hours. Hormone replacement was carried out for 3 days, at which time the animals were killed and their glands removed.

Table 15 is a compilation of results of hydrocortisone replacement therapy compared to placebo treatment of adrenalectomized mice. Mice had been nursing approximately 8-9 days by termination of the experiment. At this time the original pups of the placebo-treated mice had all died and had been replaced. The RNA and DNA levels as well as the RNA:DNA ratios of the hormone-treated mice were all higher, though not significantly so, than the levels of the sham-operated controls. The mean RNA concentration of the placebo-treated group was lower by 91 per cent than the

hormone-treated group's RNA level. The DNA level was 34 per cent lower than hormone-replacement group's DNA concentration, which represented a significant decrease ($p<.05$, Student's t-test). The RNA:DNA ratio of the placebo-treated mice showed an 86 per cent depression compared to that of the mice which received hydrocortisone. The litters of the sham-operated controls and the hormone-treated mice were in good condition and gaining weight. Some of the hormone-treated mothers' babies had died during the first 3 days following adrenalectomy, but their replacements gained normally after the institution of hydrocortisone therapy.

Hydrocortisone therapy was also given to adrenalectomized-ovariectomized mice, and the results were indistinguishable from those produced by treatment of mice which had been adrenalectomized alone.

Estrogen replacement therapy was administered to the single- and double-operated mice in the form of 17-β estradiol in aqueous suspension. The dose was either 1 µg or 2 µg per day injected subcutaneously. This treatment produced no discernible effect on lactation, on mammary gland weight, RNA concentration, DNA concentration or RNA:DNA ratio at the dose given, and this avenue of investigation was not pursued further.

IX. Early Effects of Hydrocortisone Treatment of Adrenalectomized Lactating Mice on the Specific Activity of Their Mammary Gland RNA

Two- to three-day postpartum mice were adrenalectomized. Two days after the operation the mice were injected with 100 µg of hydrocortisone acetate in saline suspension or with a similar volume of saline solution only. One hour after injection all the mice were intraperitoneally injected with 1.5 µc of ^{3}H-uridine per gram of body weight. After a 3 hour labeling period they were killed, their mammary glands removed, and the RNA and DNA extracted by the Schmidt-Thannhauser procedure. The radioactive samples were counted in the Packard Tri-Carb liquid scintillation counter. Table 16 lists the RNA concentrations, DNA per gram of mammary tissue and specific activities of the mammary RNA of the hydrocortisone-treated versus the placebo-treated mice.

The animals in this investigation were killed 4 hours after being injected with hydrocortisone or placebo. The mammary RNA concentrations of the two groups are not significantly different, while the specific activity of the mammary RNA of the hormone-treated animals is about 4 times greater than that of the saline-injected mice. There is a greater variation of specific activities in the hydrocortisone-treated mice, too, than there is in the controls. The standard deviation of the placebo-injected animals was ±1.19, while that of the hydrocortisone-treated

mice was ±19.8. The mean specific activity of the mammary RNA of the control adrenalectomized mice was about the same as the specific activity of an 18- to 19-day pregnant mouse (see Table 9).

DISCUSSION

I. Normal Values for Mammary Gland Nucleic Acids

A. RNA

The increase in mammary gland RNA concentration observed during pregnancy and lactation was not surprising. The magnitude of the increase, about sixfold from mid-pregnancy through the tenth day of lactation, is comparable to that observed in the rat during the same period (Baldwin and Milligan, 1966). These workers measured an increase of from 2.0 mg/gram of mammary gland at 15 days of pregnancy to 13 mg/gram of mammary gland at 10 days of lactation, or a 550 per cent increase. Thus, the data agree closely in absolute values as well as in relative change. Greenbaum and Slater (1957) reported about a 400 per cent increase in RNA-phosphorus from the last day of pregnancy through the tenth day of lactation (also in rats), as compared to a 515 per cent rise observed by this author. It thus seems that the time sequence of RNA synthesis is similar in the two species. The decrease of RNA concentration seen in mice from the fourteenth through the twenty-first day has not been reported by Baldwin and Milligan (1966) nor by Greenbaum and Slater (1957b) in their studies on rats; the decline in RNA apparently does not occur in this species until weaning actually begins.

At least two explanations could be advanced for both the increase in RNA concentration occurring from

midpregnancy through midlactation and the decrease occurring from midlactation through weaning. The variation in RNA could result from either 1) a change in total concentration of cells per unit weight of mammary tissue, or 2) a change in the average RNA content of the mammary cells. In order to distinguish between these alternatives, the DNA content of the glands was measured.

B. DNA

The amount of DNA per gram of mammary tissue did not decrease significantly until about the seventeenth to eighteenth day of lactation. This data is in conflict with the findings of Griffith and Turner (1959) and Lavrin and Cole (1964), who measured a "burst" of DNA synthesis and mitosis at the beginning of lactation (see Introduction, p. 18). The fact that the data in this thesis does not show a significant change in DNA content does not, however, rule out the possibility that there is mitotic activity at this time in the mouse mammary gland. DNA content was measured per gram of wet weight tissue, and increases in protein concentration of the gland (as could be expected at the onset of lactation) might mask small increases in DNA concentration. The decrease in DNA per gram of mammary tissue seen in late lactation through weaning undoubtedly does represent a loss of mammary cells. Light and electron microscopic observations show that there is autolysis of mammary parenchymal cells during the weaning or involutional

stage of mammary development (Wellings and DeOme, 1963), and this change is reflected by lowered DNA concentration.

C. RNA:DNA Ratio

Because it is not known whether the DNA content and cell number remains perfectly constant through late pregnancy and lactation, it was decided to consider RNA:DNA ratio as the principal measure of RNA concentration. Of course, if this measure is to be valid, it must be assumed that the DNA content per cell remains constant. Simpson and Schmidt (1969) measured the DNA content of rat mammary gland cell nuclei in pregnancy, lactation and involution. They observed no significant differences in DNA content per nucleus from pregnancy through lactation; however, there was a significant 16 per cent reduction in the average DNA content of the mammary gland cell during involution. The authors attributed this decrease in DNA to the lack of metabolic DNA produced once lactation was over. It is also possible that the lowered DNA levels result from leakage of DNA from partly autolyzed cells, cells which still have their nuclear membranes partly intact. Whatever the cause of the difference, it is not known whether such a change occurs in mice. Furthermore, the 16 per cent difference was measured at the seventh day of weaning, while the latest measurements made by this writer were at three days of weaning. Even if there are slight variations on either side of the diploid DNA value, they could not account

for the 67 per cent drop in DNA observed from the eighteenth day of lactation through the third day of weaning. Keeping in mind the above consideration, the plot of RNA:DNA ratio of Figure 4 shows that the ratio increases at about the same rate as RNA per gram of mammary gland through the first six days of lactation. Even if there is a wave of DNA synthesis and mitosis at the beginning of lactation, the average RNA per cell is increasing also. This indicates that either the rate of synthesis of RNA is increasing during this period, or that the rate of degradation of RNA is decreasing; each process would result in a net accumulation of RNA per cell. The RNA:DNA ratio continues to rise until the fourteenth day of lactation, but not so steeply as during the previous 6 days. From the fourteenth to the twenty-fourth day postpartum, there is no significant change in the RNA:DNA ratio, and both RNA and DNA concentrations drop. One explanation for this finding is that the rate of RNA synthesis or degradation is not altered during late lactation and weaning; rather, entire cells are lost. It thus may be that there is not a mechanism for "turning off" RNA synthesis in mammary gland development, but only for "turning it on".

D. Radioactive Labeling of Normal Mice

The results of the labeling studies comparing pregnant to lactating animals confirms the conclusions drawn from the measurements of total mammary RNA concentration. The

approximately 500 per cent greater specific activity of the mammary gland tissue of lactating over prelactating mice (Table 9) is the product of two factors:

1) The 272 per cent greater total concentration of RNA in the lactating glands.
2) The 177 per cent greater specific activity of mammary RNA of lactating mice.

Since the specific activity of the RNA of lactating glands is greater than that of the prelactating glands, it is probable that the increased rate of RNA accumulation in the mammary gland cells is caused by elevated RNA synthesis. Although decreased degradation of RNA may also be involved, the predominant effect shown by the increased precursor incorporation is one of more rapid RNA formation.

As can be seen from Table 9, there is some overlap between values for the two groups of mice in this study. For this reason a greater-than-usual number of animals were used. One reason for the large variation in RNA specific activity could be that the rate of RNA synthesis is quite inconstant. Since the labeling period is only three hours, much of the inconstancy may have been "picked up."

II. Labeling Studies- Acrylamide Profiles

The small peak on the "heavy end" of the acrylamide profiles of both prelactating and lactating mammary RNA may represent the 45s ribosomal RNA precursor (Scherrer, Latham,

and Darnell, 1963; Penman , 1966), but this is quite unlikely. The ribosomal precursor turns over very rapidly and would only be likely to be detected in RNA isolated from cell nuclei (Scherrer and Darnell, 1962). Since this fraction cannot be consistently isolated from mammary gland homogenates, it may represent an artifact, possibly an RNA-protein complex.

Since labeled RNA hydrolysates from lactating mammary glands have higher specific activities than hydrolysates from prelactating glands, it was expected that the tritium-labeled peaks of lactating RNA would be higher than those of prelactating RNA. This is what is observed (compare Figures 5A and 5B). It was of interest to know whether precursor was being incorporated into all fractions at the same rate, and the acrylamide profiles show that in the mammary gland of the pregnant mouse, over a three hour labeling period, this is the case. This is demonstrated by the fact that the relative heights of the labeled peaks are approximately the same as the optical density peaks. Over a shorter labeling period, however, differences in the rate of labeling of the various RNA fractions might be observed.

The sRNA fraction (peak I, Figure 5B) is obviously of higher specific activity than either of the rRNA fractions (peaks II and III, Figure 5B). Since the ratio of total sRNA to rRNA is not elevated, the sRNA is evidently turning over more rapidly than the other fractions. This finding

is in contrast to the work of Turkington (1969b), who reported a disproportionate increase in mammary tRNA during pregnancy, as well as an increase in the amino acid acceptor activity (tRNA is normally the major component of the sRNA fraction). The greatest change in sRNA concentration occurred between the tenth and twentieth days of pregnancy, and 4s RNA experienced the largest percentage increase. Turkington, however, electrophoretically fractionated unlabeled sRNA, while this investigator fractionated labeled RNA. Turkington shows an accumulation of tRNA until about the end of pregnancy; during lactation, tRNA does not increase proportionately over other types of RNA. This writer has noted a higher specific activity of sRNA (relative to total RNA and to rRNA) in lactation, but no elevation of unlabeled (total) sRNA. It may be that during the presecretory phase, when the mammary gland is metabolically fairly inactive (compared to the early pregnancy period of active cell proliferation and to the lactation period of high synthetic activity), sRNA accumulates. Then, when lactation begins, the sRNA begins to be metabolized more rapidly and so no longer accumulates although its rate of synthesis may be great or greater than it was during pregnancy. Quite obviously there is not sufficient information to confirm or deny this hypothesis, but it is a theory which can incorporate data from both this report and from Turkington's (1969) investigations.

The functional significance of an increased turnover rate of sRNA in this system can only be conjectured. It is

not surprising that this fraction of the cellular RNA should be rapidly metabolized, since the lactating mammary gland is actively synthesizing protein, lipid and carbohydrate. Von Ehrenstein (1970) succinctly describes the functions of tRNA in the cell-s protein-synthetic system (page 141):

> Aminoacyl-tRNA complexes, containing the activated and adapted amino acids, function as direct amino acid precursors in protein synthesis. In addition, tRNA and aminoacyl-tRNA complexes also have regulatory functions in the process. For example, they provide the starting signal and may also govern the rate of polypeptide chain assembly (modulation). Furthermore, aminoacyl-tRNA's are more directly involved in repression of biosynthetic pathways than are the free amono acids. They probably also serve as a link in the close coupling between the overall rate of RNA synthesis and the intracellular level of amino acids.

The elucidation of the specific role of sRNA in mammary gland development must await a time when this aspect of RNA metabolism can be studied in detail.

It is possible, too, that only the cytosine-cytosine-adenine (CCA) terminal of the tRNA is turning over. This part of the molecule can be enzymatically excised or added independently of the rest of the molecule (Holt, Joel, and Herbert, 1966). It has been reported that in E. coli (Lieberman, 1956) and in mammalian systems (Lehninger, 1970, p. 572) uridine triphosphate (uridine is the labeled precursor used in these experiments) is converted to cytidine triphosphate. Therefore, it could be that the elevated percentage of label in the sRNA fraction represents CCA turnover and not de novo sRNA synthesis.

III. Ovariectomy of Pregnant Mice

Ovariectomy of late-pregnant mice does not prevent them from lactating; in fact, ovariectomy has been used to induce premature lactation (Liu and Davis, 1967). It induced premature lactation in mice in this study, too. A further finding is that neither RNA, DNA, nor RNA:DNA ratios are depressed three days after ovariectomy of pregnant mice (Table 10). The mean RNA:DNA ratio of the ovariectomized mice (4.9) is 148 per cent greater than the ratio (3.3) of the sham-operated mice. The nucleic acid values are slightly, though not significantly, greater than for three-day lactating mice, too. The reason why the experimental animals' RNA and RNA:DNA ratios are higher than the controls' is probably related to the fact that the ovariectomized mice, having delivered their babies within several hours after the operation, have been lactating almost three days by the termination of the experiment. The sham-operated animals do not abort their litters, so at termination of the experiment they have been lactating only about one day. Thus it appears that the factor determining the RNA concentration of the mammary gland in this experiment is the length of time lactation has been going on, not the presence or absence of ovarian stimulation. Some workers believe that lowered estrogen levels are in fact one of the stimuli for institution of lactation (Meites and Sgouris, 1954), possibly by releasing inhibition of pituitary lactogen.

IV. Ovariectomy of Lactating Mice

As Table 11 shows, ovariectomy causes no effect on mammary nucleic acid content, whether in early- or in mid-lactation. The mammary gland, which is dependent upon estrogen for its lobulo-alveolar development early in pregnancy, is evidently refractory to loss of the hormone late in pregnancy and through lactation. The lack of effect of oavriectomy on mammary nucleic acid is in accord with Lyons' and Flux's (1955) reports that lactation is not inhibited in ovariectomized rats. Nandi (1958), too, finds lactation in mice unaffected by ovariectomy. An explanation for this estrogen-refractory state which occurs by late pregnancy could be that the estrogen receptor sites of the mammary gland are saturated. Although estrogen-binding molecules have not yet been isolated from mammary tissue, as they have from uterus (Korenman and Rao, 1968, Jensen, Suzuki, Kawashima, Stumpf, Jungblut, and DeSombre, 1968), estrogen does specifically bind to mammary gland tissue (Puca and Bresciani, 1969). Alternatively, the mammary cells might stop producing estrogen receptor molecules by late pregnancy, resulting in insensitivity of the gland to estrogens. An estrogen-binding assay similar to that of Noteboom and Gorski (1965) might be used to compare the binding of estrogens to mammary tissue during lobulo-alveolar development to binding late in pregnancy or in lactation. Essentially, Noteboom and Gorski's technique involves

measuring the uptake of radioactively labeled estrogen by target tissues, *in vivo* or *in vitro*.

V. Effect of Adrenalectomy on Mammary Gland Nucleic Acid Levels of Pregnant Mice

It was unfortunate that none of the adrenalectomized pregnant mice were able to carry their litters to term, as it would have been instructive to know the effects of the operation over a several-day period of pregnancy. Furthermore there was not an adequate control for this experiment: the metabolic changes following birth of the babies certainly introduce a number of variables into the system. An attempt was made, however, to relate the data from these animals to groups to which they were similar in other ways.

Since the gland weight, RNA concentration and DNA concentration of the mammary gland in adrenalectomized mice 3 days postoperatively was lower than it was at the time of the operation (18-19 days of gestation, Table 12), it can probably be concluded that there has been some cell loss in the mammary glands. The fact that the RNA:DNA ratio was not significantly altered is reminiscent of the situation occurring in weaning, (see Table 8), where RNA and DNA concentrations drop radically, but the ratio of the two quantities remain unchanged. As was proposed before, this may indicate that the individual viable cells are maintaining their net RNA synthesis undiminished.

In contrast to ovariectomy, adrenalectomy does not

result in premature lactation, although both operations can cause early birth of the litters. This lends weight to the theory that lowered quantities of estrogen and/or progesterone may causally be related to the institution of lactation. Mietes and Nicoll (1966) propose that the lowering of estrogen levels releases the hypothalamic inhibition of MH, which is followed by release of MH and lactation. On the other hand, the absence of adrenal steroids may be directly inhibitory to lactation. Davis and Liu (1969) adrenalectomized mice 24 hours before ovariectomizing them, and found that prior adrenalectomy depressed ovariectomy-induced lactation (gauged by casein synthesis) by 16 per cent.

Though there is little reported work which is comparable to that described here, Banerjee, Rogers and Banerjee (1971) report that 24 hours after adrenalectomy of 15 day pregnant mice, the incorporation rate of ^{3}H-uridine is 20 to 30 per cent lower than the rate of incorporation in controls. Table 12 shows that a decrease in <u>total</u> RNA of approximately 36 per cent occurred by 3 days after adrenalectomy. This was accompanied by a 38 per cent decrease in DNA concentration (Table 12).

VI. Effect of Adrenalectomy on Mammary Gland Nucleic Acids of Lactating Mice

The mammary gland weight loss of animals adrenalectomized during lactation is the same as the weight loss of pregnant, adrenalectomized animals (about 40 per cent).

The percentage drop of RNA and RNA:DNA ratio is much greater for mice adrenalectomized during lactation, however. The 87 per cent drop in RNA concentration of adrenalectomized lactating mice is more than double the drop in the RNA concentration of the adrenalectomized pregnant mice (36 per cent, see Table 12). Probably more significant is the finding that the RNA:DNA ratio is also lower by 86 per cent, while in pregnant animals which have been adrenalectomized this ratio is unchanged. This may indicate that adrenalectomy is exerting different effects on the mammary gland according to the physiological state of the animal (pregnant versus lactating). Since the RNA:DNA ratio is a reflection of RNA concentration per cell, a lowered ratio in lactating adrenalectomized animals probably means that the rate of synthesis or degradation of RNA in the mammary cells is changed.

Comparing the effect of adrenalectomy on prelactating gland to that on lactating gland, it could be proposed that:

1) the predominant result of adrenalectomy on a pregnant animal is loss of cells, while the net synthesis of RNA is unchanged. Banerjee et al (1971) did report a 20 to 30 per cent reduction in ^{3}H-uridine incorporation, which could mean that RNA synthesis is lowered. In their system however, they calculated specific activity in terms of CPM/mg of PCA-insoluble material, while in this paper specific activity is defined as CPM/µg RNA. Thus, if the concentration of cells were reduced in 24-hour postadrenal-

ectomy mammary glands, the CPM/mg of PCA precipitate (which includes the majority of the protein of the homogenate) would be lower. The mechanism whereby the proposed mammary gland cell loss occurs will be discussed later in this section. The adrenalectomy effect could also be considered as an abrogation of a "permissive" influence: lactation and elevated RNA synthesis which would normally occur following parturition is prevented by loss of some adrenal secretion.

2) Adrenalectomy of lactating mice does not appear to cause a diminution in mammary cell concentration, as evidenced by lack of change in µg DNA/g of mammary tissue. Even if there is a small amount of cell loss (the mammary gland weight is diminished following adrenalectomy), the main effect of the operation is to cause a lowering of net RNA synthesis. This striking effect is in accord with the findings of Banerjee et al (1971), who measured an 80 per cent reduction in ^{3}H-uridine incorporation into PCA-insoluble material 24 hours after adrenalectomy in 5-day lactating mice. Accompanying this decline was a virtual cessation of casein-like protein synthesis. These workers found, too, that "total protein" synthesis declined by only 50 per cent, indicating that much of the RNA metabolism of lactation is probably involved in the synthesis of milk protein. Since there have been proposed two consequences of adrenalectomy on mammary tissue, it would be appropriate to consider the possible mechanisms by which the effects are produced.

If, as has been suggested, the main result of adrenalectomy on pregnant mammary gland is cell loss and inhibition of RNA synthesis, it may be that a rather nonspecific phenomenon is occurring. Adrenal steroids may be required simply for maintenance of the normal metabolic processes of the mammary gland. When adrenalectomy takes place, a general slowing of the anabolic processes may cause cells to die. Adrenal steroids (glucocorticoids, specifically) are catabolic for most of the tissues on which they act and adrenalectomy results in retention of protein nitrogen in these tissues (Glenn, Bowman, Bayer, and Meyer, 1961; Kostyo, 1965). The only other organ, besides the mammary gland, in which glucocorticoids stimulate protein and nucleic acid synthesis is the liver (Feigelson, Gross and Feigelson, 1962; Leon, Arrhenius and Hultin, 1969; Breuer and Davis, 1963; Garren, Howell and Tomkins, 1964).

One effect of adrenalectomy is to increase sensitivity to stress (Turner, 1966). Associated with several stress conditions and with inflammation is increased lysosomal rupture (Kovanic, 1968; Janoff and Zweifach, 1964). In consequence of this finding, Berg and Bird (1970) have tested the fragility of muscle and liver lysosomes following adrenalectomy. They measured release of ribonuclease, cathepsin D, β-glucuronidase and aryl sulfatase from lysosomes and found that adrenalectomy increased the release of these lysosomal enzymes from both tissue homogenates and lysosomal fractions. This phenomenon could be acting in

the mammary gland system also. If there were release of proteolytic enzymes, RNAse and DNAse in mammary glands of adrenalectomized mice, it could result in autolysis of the cell, in the most extreme case. If the leakage of hydrolytic enzymes were slow, the effect could be to cause an increased degradative rate of RNA, DNA and protein. In any case, such a process could be reflected in a lowered RNA and DNA concentration, such as is seen after adrenalectomy of pregnant mice. It is difficult to see why this process would not act as well on the mammary glands of lactating mice where the cell loss, if any, is small.

In mammary glands of lactating mice the effect of adrenalectomy is not exclusively on RNA metabolism. As has been mentioned, adrenalectomy has a score of metabolic consequences, among them lowered blood glucose (Turner, 1966) and decreased gluconeogenesis (Winternitz, Dintzis and Long, 1957; Landau, Mahler, Ashmore, Elwyn, Hastings and Zottu, 1962). These changes could decrease the available energy source for the mammary gland, whose glucose utilization increases markedly in lactation (Glock and McLean, 1958). This might partly account for the increased sensitivity of the lactating gland to adrenalectomy; its elevated glucose requirement, which would not then be met, could result in a greater depression of RNA and protein syntheses than in the prelactating gland.

VII. Ovariectomy-Adrenalectomy

The double operation, adrenalectomy-ovariectomy, was performed as an added control. Flux (1955) has reported that if ovaries are removed along with the adrenals, there is a greater depression of lactation than if adrenalectomy alone is performed. In Flux's studies, ovariectomy alone had no inhibitory effects on lactation. He suggested that in adrenalectomized rats the ovaries can "substitute" for adrenal hormones in maintaining lactation. Although lactation *per se* was not monitored in the experiments reported in this dissertation, no additional effect on nucleic acid values was produced by ovariectomy plus adrenalectomy.

VIII. Effects of Hormone Replacement

It has been directly and indirectly shown that the adrenal gland is capable of producing and secreting androgens, estrogens and progestogens, as well as glucocorticoids and mineralcorticoids (Soffer, Dorfman and Gabrilove, 1961, pp. 40-52). In his review on adrenal sex steroids Short (1960) states that over 40 steroids have been isolated from adrenal gland extracts. Quantitatively, the secretion of estrogens is probably not important in the intact animal. Since ovariectomy of lactating mice produces no effect on mammary nucleic acid levels, it could be proposed that the adrenal gland may be stimulated to produce

estrogens, and that this is the reason why ovariectomy is without effect. In order to test this possibility, estrogen replacement therapy was instituted. It produced no effect, and it is therefore concluded that while the adrenal may secrete estrogens, they do not constitute an important influence in lactation in mice.

A complete reversal of the effects of adrenalectomy was produced by treatment of 3-day postadrenalectomized mice with 100µg/day of hydrocortisone acetate.

While cortisol is probably not produced by the adult mouse adrenal (Hofmann, 1956; Southcott, Bandy, Newson and Darrach, 1956), it is the most potent of the glucocorticoids in vivo (Nandi and Bern, 1961) and in vitro (Rivera, 1964b). Moreover, this has been the glucocorticoid used for stimulating lactation by most investigators in the field of mammary gland metabolism. In order to make meaningful comparisons with their work, it was decided to use it here, also.

The dose of hydrocortisone used here, 100µg/day, is higher than a physiological dose, but, once again, it was used in concentrations comparable to those reported by other workers (Ferreri and Griffith, 1969; Davis and Liu, 1969; Nandi, 1959; Baldwin and Martin, 1968). Besides, as Gaunt and Tobin (1936) have shown, the dose of adrenocortical extract necessary to maintain lactation is twice that required to keep the animals alive.

Since several workers have reported that mammary

secretion in adrenalectomized animals can be maintained or reinstituted by giving adrenocortical steroids, (Cowie, 1961; Cowie and Tindal, 1955; Nandi, 1959; Nandi and Bern, 1961) it was not unexpected that hydrocortisone should reverse the inhibitory effect of adrenalectomy on mammary nucleic acid levels. Baldwin and Martin (1968) have found that treatment of hypophysectomized rats with MH plus hydrocortisone, but not with MH alone, will cause mammary gland RNA concentration to remain at normal levels.

The in vitro effects of hydrocortisone on mammary RNA synthesis have been discussed in the introduction to this paper (Introduction, p. 23), but in review, it can be said of them that they are necessary for RNA synthesis, although their effect is described by Green and Topper (1970) as "preparatory", rather than stimulatory. In Green and Topper's experiments, the requirement for hydrocortisone ended once the mitotic stage of development was complete. Their finding is in contrast to those reported here, in which the adrenal steroid requirement extended into the lactation period.

It has been proposed by some workers that one of the stimuli for institution of lactation is elevation of adrenocorticoid levels (Talwalker, Nicoll, and Meites, 1961). It is true that increased levels of circulating corticosterone have been measured during lactation in rats (Thatcher and Tucker, 1970b) but probably lactogenesis instigation involves a more complex process than simply the elevated synthesis of

corticoids.

It is known that progesterone is secreted by the adrenal gland of rats (Feder, Resko, and Goy, 1968) and mice (Uphouse, Wilson and Schlesinger, 1970). In the rat plasma progesterone levels rise significantly at about 2 days following parturition (Grota and Eik-Nes, 1967). Although at high doses progesterone is not found to be stimulatory to lactation (Reece, 1958), it may be stimulatory at the physiological levels which would be found in the intact or in the ovariectomized mouse. Thus, it is possible that one of the effects of adrenalectomy is to reduce progestogen levels; the resultant drop in RNA concentration derives from insufficient progesterone stimulation, rather than lack of glucocorticoids. Some glucocorticoids (e.g., deoxycorticosterone) possess significant progestational activities (Miyake, 1962). Although to this writer's knowledge it is not known whether hydrocortisone acetate has progestational activity, this possibility cannot be ignored.

The results described in this dissertation research show that the effect of hydrocortisone on adrenalectomized mice is manifested soon after hormones administration. Four hours after administration of the hormone, label uptake was elevated by about 400 per cent over that of the placebo-injected mice (see Table 16). Banerjee and Banerjee(1971) have shown an even earlier effect of hydrocortisone: by 1 hour after an injection, there is a 2-fold increase in incorporation of ^{3}H-uridine into the nucleolar area of the

mammary cells of adrenalectomized mice, as measured by autoradiography.

Gaye and Denamur (1969) measured precursor incorporation into protein and RNA under stimulation of MH in intact rabbits, and found that the elevation did not occur until 12 hours after injection. It is therefore possible that hydrocortisone is influencing an earlier step in transcription than is MH.

The mechanism of action of hydrocortisone cannot be determined from theseexperiments, as there are far too many interacting variables. It has been pointed out earlier in this thesis that a great many hormones are involved in lactation. However, it is known from in vitro experiments that RNA synthesis is essential for mammary gland secretion (Turkington, 1970; Mayne and Barry, 1970), and therefore conditions which inhibit the normal metabolism of RNA would probably also interfere with lactation. The effect of steroid replacement on RNA synthesis, even though an early one, may be the secondary or tertiary effect of some other (possibly quite non-specific) process.

One of the effects of glucorticoids which has been touched on earlier is their influence on lysosomal membranes. Many workers have reported that hydrocortisone stabilizes lysosomes of liver cells and cells in tissue culture (deDuve and Wattiaux, 1966; Weismann and Dingle, 1961; Wiener and Marmary, 1969). This might be one of the ways in which hydrocortisone treatment causes increased RNA synthesis in mammary gland cells: a decrease in the release and/or

synthesis of hydrolytic enzymes (possibly resulting from a change in membrane permeability), could allow net RNA synthesis to rise, i.e., the total cellular metabolism could be shifted to the "synthesis side."

In considering the early stimulation by hydrocortisone of ^{3}H-uridine incorporation, the possibility must be intertained that this stimulation is a function of increased precursor pool size. Such an effect, too, might be related to hormone-caused changes in membrane permeability. If the mammary cell membrane were made more permeable to uridine, for example, an increase in specific activity of RNA could be registered without a true elevation of the rate of synthesis of RNA. Greenman, Wicks, and Kenney (1965) have shown that cortisone does produce an increase in specific activity of tissue (liver) phosphate pools. These data were confirmed by Loeb and Tolentine (1970), who reported that the elevation of specific activity of ^{32}P-phosphate-labeled RNA is almost twice as great as that of ^{14}C-orotic acid-labeled RNA, after cortisone treatment. Loeb and Tolentino measured the rate of turnover of ribosomal RNA, however, and found that at least some of the increased label incorporation represents a true increase of RNA synthesis. In view of the magnitude of the increase in label incorporation (about 400 per cent) in hydrocortisone-treated mice, and because of the several-fold elevation of total RNA over a 3-day treatment period, it is likely that in mammary gland, too, hydrocortisone is affecting an increase in net RNA synthesis.

Since the influence of hydrocortisone on the liver is most like that on the mammary gland, a few of its actions on the liver will be considered. It has been demonstrated quite conclusively that hydrocortisone specifically binds to certain tissues, notably liver (DeVenuto, Kelleher, and Westphal, 1962; Beato, Brandle, Biesewig, and Sekeris, 1970; Snart and Agarwal, 1979). The first group of investigators state that the hormone does not bind to muscle tissue, on which it exerta a catabolic effect. Baxter and Tomkins (1970), in their investigations on tyrosine transaminase induction in liver hepatoma tissue culture cells, found a correlation between the amount of cortisol binding with degree of induction of the enzyme. Apparently corticoid binding has not been measured in mammary tissue. It would be interesting to compare the rate and affinity of binding of the hormone in pregnancy to that in lactation. This, too, could explain differences in sensitivity of the gland to hydrocortisone at various stanges of development.

In liver, hydrocortisone or other glucocorticoids have been reported to produce rather specific effects on a number of macromolecular processes which are related to RNA synthesis: 1) They apparently stimulate synthesis of rapidly labeled DNA-like RNA (Niessing and Sekeris, 1970; Kidson and Kirby, 1965; Weber, Srivastava and Singhal, 1965; Murthy, Pradhan and Sreenivasan, 1970). 2) They have been reported to stimulate synthesis of rRNA and sRNA in vivo (Kenney and Kull,

1963; Wicks, Greenman and Kenney, 1965; Mansour and Nass, 1970). 3) Treatment with corticoids results in increased template activity of liver chromatin (Dahmus and Bonner, 1965). 4) Glucocorticoids stimulate DNA-dependent RNA polymerase (Barnabei, Romano and DiBitonto, 1965). 5) Although it has been reported that hydrocortisone binds to histones (Sluyser, 1966), Monder and Walker (1970) believe that a contaminant, 21-dehydrocortisol, is the substance which binds, and that the situation is probably not physiological.

Any of the actions of glucocorticoids listed above could result in stimulation of RNA synthesis if they occurred in the mammary gland. All of them occur within a few hours of treatment with the hormone, and so could account for the stimulation of uptake of isotopically labeled uridine measured at 4 hours after hormone injection. On the other hand, the influence of the hydrocortisone on nucleic acid metabolism might ultimately be related to changes in permeability of the mammary cell membrane to nutrients such as glucose, or to nucleic acid precursors. Munck (1971) has presented data to show that glucocorticoids inhibit glucose uptake in certain tissues, but, in their paradoxical way, these same hormones might exert an opposite effect on mammary cells.

The next steps in the elucidation of the mechanism of action of glucocorticoids on mammary nucleic acid metabolism should entail a detailed investigation of separate aspects of RNA synthesis. For example, the synthesis of the

various RNA fractions in the adrenalectomized, hydrocortisone-treated lactating mice by acrylamide gel electrophoresis (or some other system of fractionation) should be studied. It may be that sRNA synthesis, which is preferentially elevated during lactation , is especially stimulated by the glucocorticoids. Other studies might reveal whether membrane permeability is influencing mammary gland nucleic acid metabolism. It might be possible to distinguish between uptake of labeled precursor and incorporation into RNA in mammary cells in which RNA synthesis is inhibited (perhaps by Actinomycin D). Whatever the experiments undertaken, a major objective must be to distinguish between specific and general effects of the hormone.

SUMMARY AND CONCLUSIONS

The normal RNA, DNA and RNA:DNA ratios of mammary gland from midpregnancy through lactation have been measured and calculated. RNA concentration and RNA:DNA ratios increase at their greatest rate during the first four days of lactation. DNA concentration does not seem to change significantly until late in lactation. By late lactation RNA and DNA levels drop precipitously, but the RNA:DNA ratio does not change.

Ovariectomy performed in late pregnancy or early or midlactation, does not cause mammary RNA and DNA levels, or RNA:DNA ratios to change. Adrenalectomy performed on pregnant mice causes a reduction in RNA and DNA concentrations, but not in RNA:DNA ratios. This may be a reflection of a decrease in cell number with maintenance of the original rate of RNA synthesis. Adrenalectomy of lactating mice results in no apparent decline in DNA concentration, but causes a large drop in RNA concentration and in RNA:DNA ratios. It is suggested that a different mechanism may be causing reduction of RNA levels in lactating glands than in pregnant ones.

Replacement treatment with moderately high doses of hydrocortisone reverses the inhibitory effects of adrenalectomy on nucleic acid levels of lactating mice. The stimulatory effect of this hormone is seen by 4 hours after administration. Several mechanisms are suggested by which hydrocortisone could cause its effects.

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FIGURES

FIGURE 1

DIAGRAM OF ANESTHETIC APPARATUS

This apparatus, designed and built by Weaver (1971), delivers methoxyflurane (Abbott's Penthrane) through a nose cone. The methoxyflurane is vaporized by bubbling air through the liquid anesthetic. The air flow is adjusted by a valve on the wall spigot. Taken from Weaver, 1971.

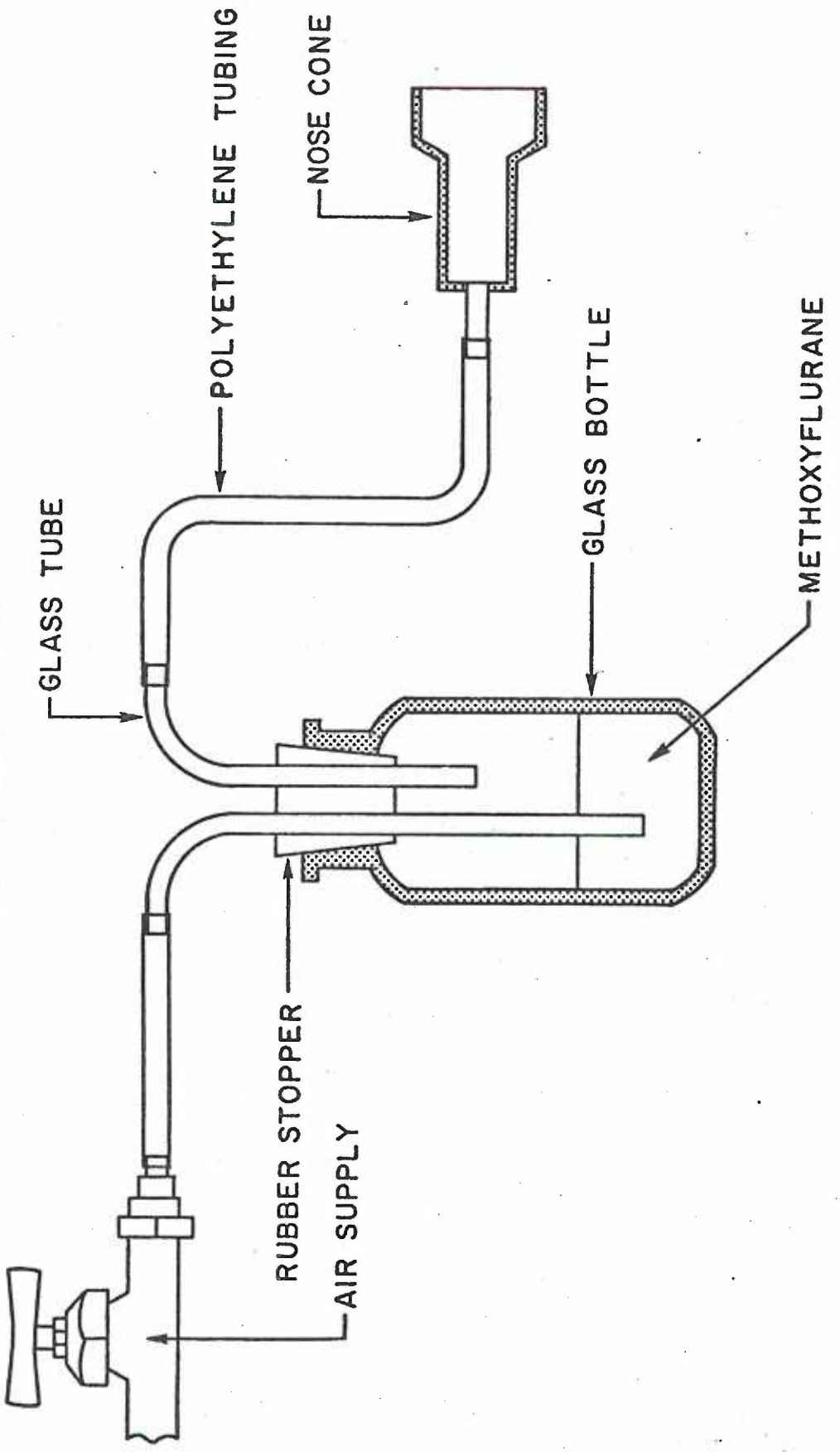


FIGURE 1
ANESTHETIC APPARATUS

FIGURE 2

DIAGRAM OF THE PHASES OF A PHENOL-TREATED TISSUE HOMOGENATE

The diagram illustrates the four phases of the phenol-treated homogenate after the first centrifugation at 1,000 X g for 10 min.

Layer 1 is the sand in which the frozen mammary gland was ground. It is left behind after the first centrifugation.

Layer 2 consists of buffer-saturated phenol in which is dissolved the majority of the protein and DNA, and a small amount of polysaccharide. (Kirby, 1956)

Layer 3, the interphase layer, contains phenol-insoluble protein, membrane fragments and a small percentage of DNA and RNA.

In layer 4, the aqueous layer, is dissolved the majority of the RNA and polysaccharide.

FIGURE 2

PHASES OF A PHENOL-TREATED
TISSUE HOMOGENATE

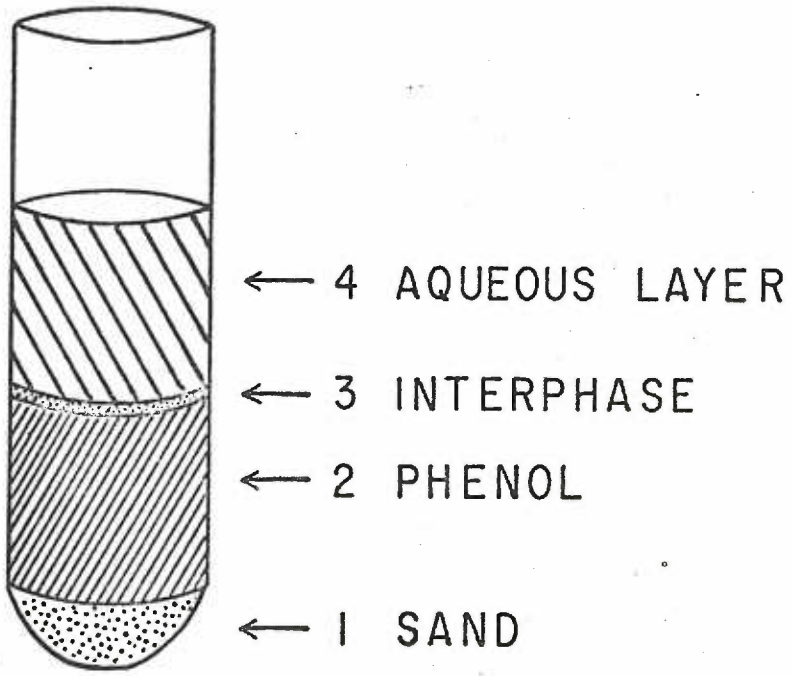


FIGURE 3

ACRYLAMIDE GEL ELECTROPHORESIS PROFILE OF RNA FROM PRELACTATING MAMMARY GLAND

Fifty µg of phenol-extracted RNA from the mammary glands of a 17-18 day pregnant BALB/c mouse was diluted to 50µl with 70 per cent sucrose and applied to a 2.2 per cent to 2.8 per cent polyacrylamide gel gradient column. The gel was electrophoresed at 5 milliamps per gel for 80 min, at which time the gel was scanned at 260 mµ using a Gilford Model 2410 linear transport and Gilford-Beckman spectrophotometer with a Gilford Model 2000 absorbance indicator.

Peak I = sRNA
Peak II = 18s rRNA (see Materials and Methods, section VII-H)
Peak III = 28s rRNA (see Materials and Methods, section VII-H)

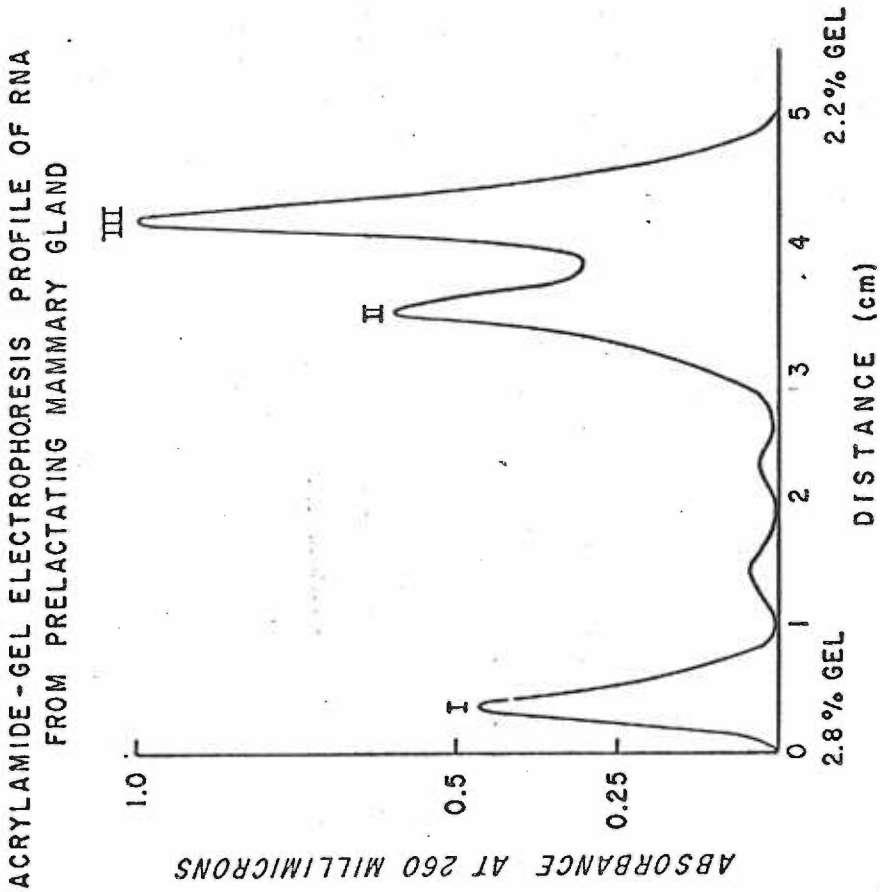

ACRYLAMIDE-GEL ELECTROPHORESIS PROFILE OF RNA
FROM PRELACTATING MAMMARY GLAND
ABSORBANCE AT 260 MILLIMICRONS
1.0
0.5
0.25
I
II
III
0
1
2
3
4
5
2.8% GEL
2.2% GEL
DISTANCE (cm)

FIGURE 3

FIGURE 4

GRAPH OF NORMAL RNA, DNA, AND RNA:DNA RATIOS
OF MOUSE MAMMARY GLAND
DURING PREGNANCY AND LACTATION

The figure is a graphic representation of the mean values of RNA, DNA, and RNA:DNA ratios of intact pregnant and lactating mice. These data are compiled on Table 8.

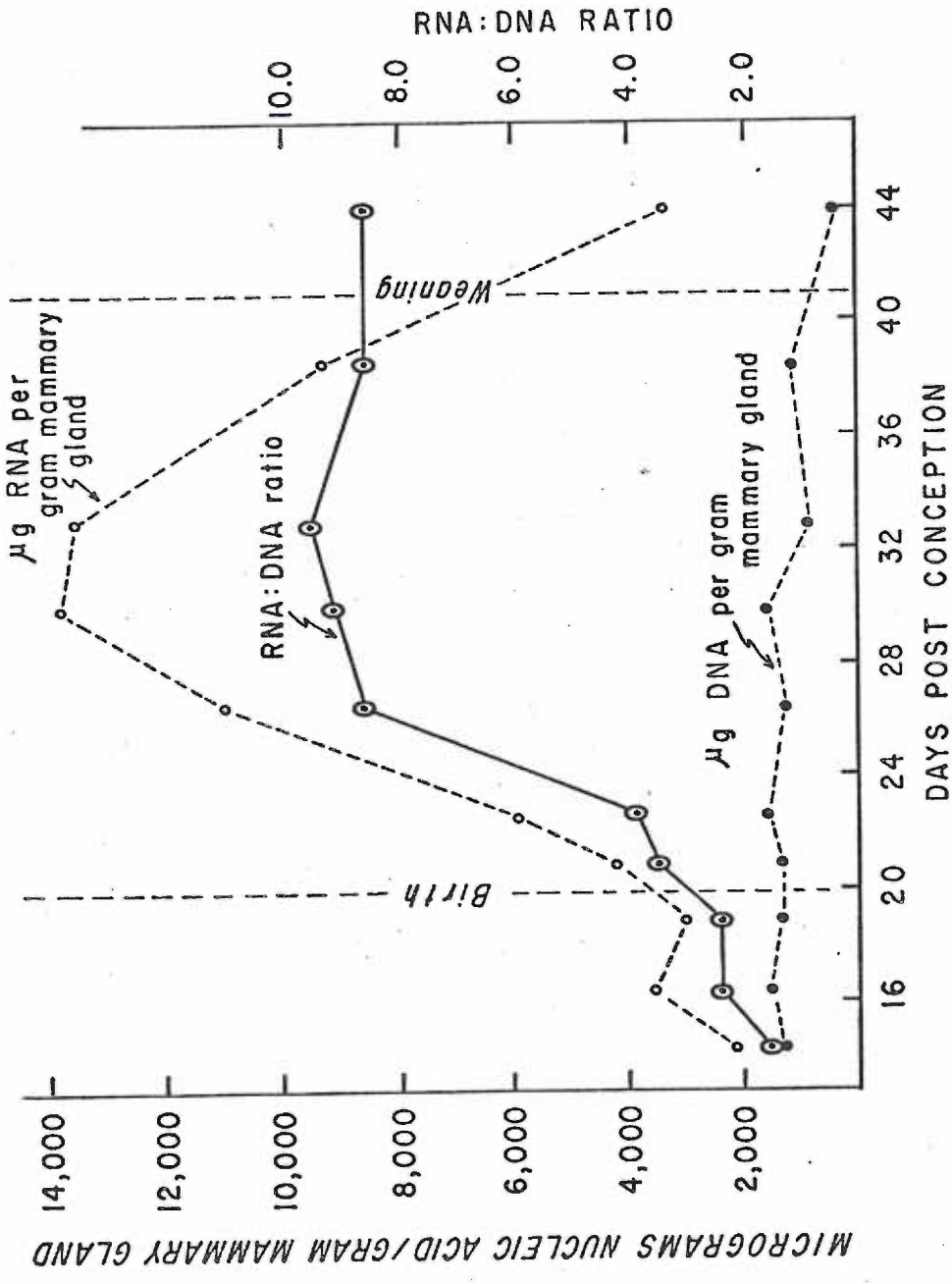

RNA:DNA RATIO
10.0
8.0
6.0
4.0
2.0
Weaning
μg RNA per gram mammary gland
RNA:DNA ratio
μg DNA per gram mammary gland
Birth
16
20
24
28
32
36
40
44
DAYS POST CONCEPTION
14,000
12,000
10,000
8,000
6,000
4,000
2,000
MICROGRAMS NUCLEIC ACID/GRAM MAMMARY GLAND

FIGURE 4

FIGURE 5A

ACRYLAMIDE GEL PROFILE OF TRITIUM-LABELED, PHENOL-EXTRACTED RNA FROM PRELACTATING MAMMARY GLAND

Seventeen- to eighteen-day pregnant BALB/c mice were injected intraperitoneally with 2μc per gram of body weight of ^{3}H-6-uridine. After the 3 hr labeling period the mice were killed and their mammary glands removed. RNA was extracted by the hot phenol method. RNA was diluted to 50μl with 70 per cent sucrose, and 50μg were placed on the gel column. The gels were electrophoresed at 5 milliamps per gel for 95 min. Following the O.D.$_{260}$ scanning, the gels were sliced and the slices prepared for counting (see Materials and Methods section VII-J). The radioactivity peaks were plotted and superimposed on the optical density scan.

Peak I = sRNA (see Figure 3)
Peak II = 18s rRNA (see Figure 3)
Peak III = 28s rRNA (see Figure 3)
Peak IV (see Results, p. 4)

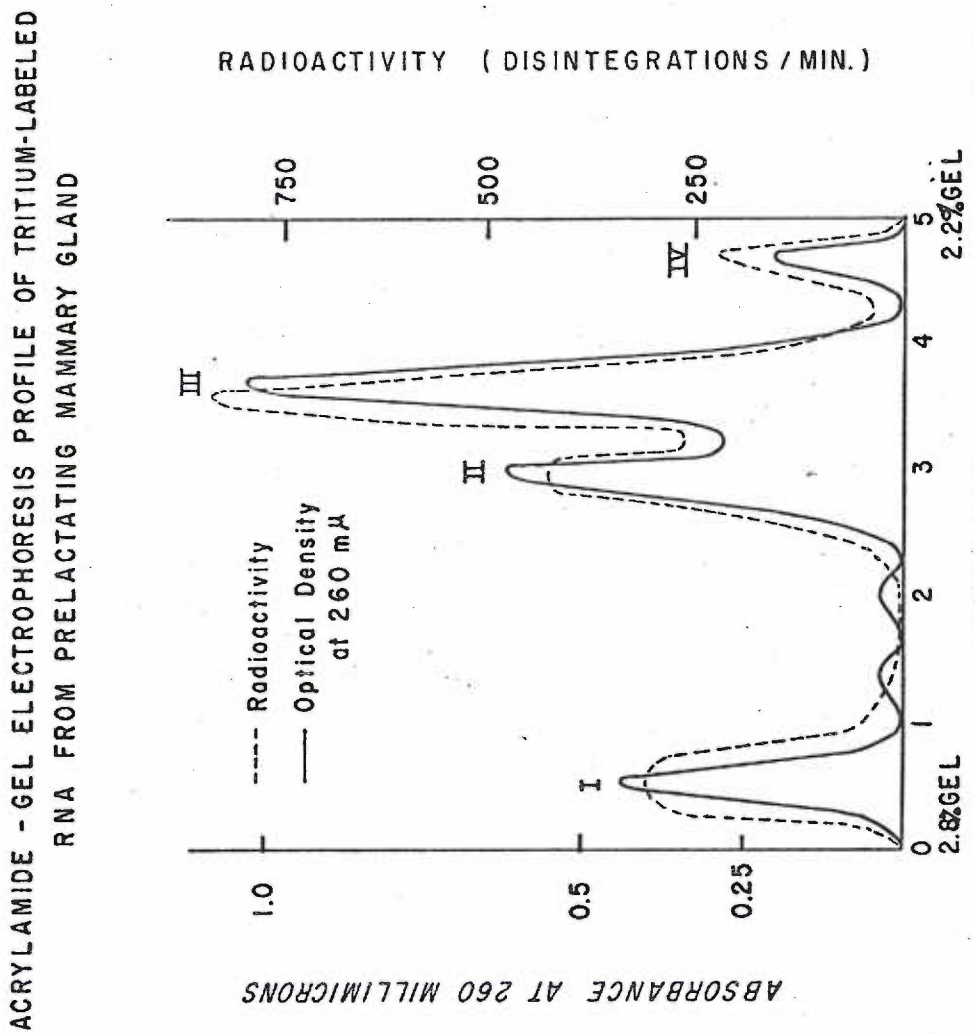

ACRYLAMIDE - GEL ELECTROPHORESIS PROFILE OF TRITIUM-LABELED RNA FROM PRELACTATING MAMMARY GLAND
RADIOACTIVITY (DISINTEGRATIONS / MIN.)
750
500
250
1.0
0.5
0.25
ABSORBANCE AT 260 MILLIMICRONS
---- Radioactivity
—— Optical Density at 260 mμ
I
II
III
IV
2.8% GEL
2.2% GEL
0
1
2
3
4
5
DISTANCE (cm.)

FIGURE 5A

FIGURE 5B

ACRYLAMIDE GEL PROFILE OF TRITIUM-LABELED, PHENOL-EXTRACTED RNA FROM LACTATING MAMMARY GLAND

Six- to seven-day lactating mice were labeled and their mammary gland RNA treated as described for Figure 5A.

Peak I = sRNA (see Figure 3)
Peak II = 18s rRNA (see Figure 3)
Peak III = 28s rRNA (see Figure 3)
Peak IV (see Results, p. 4)

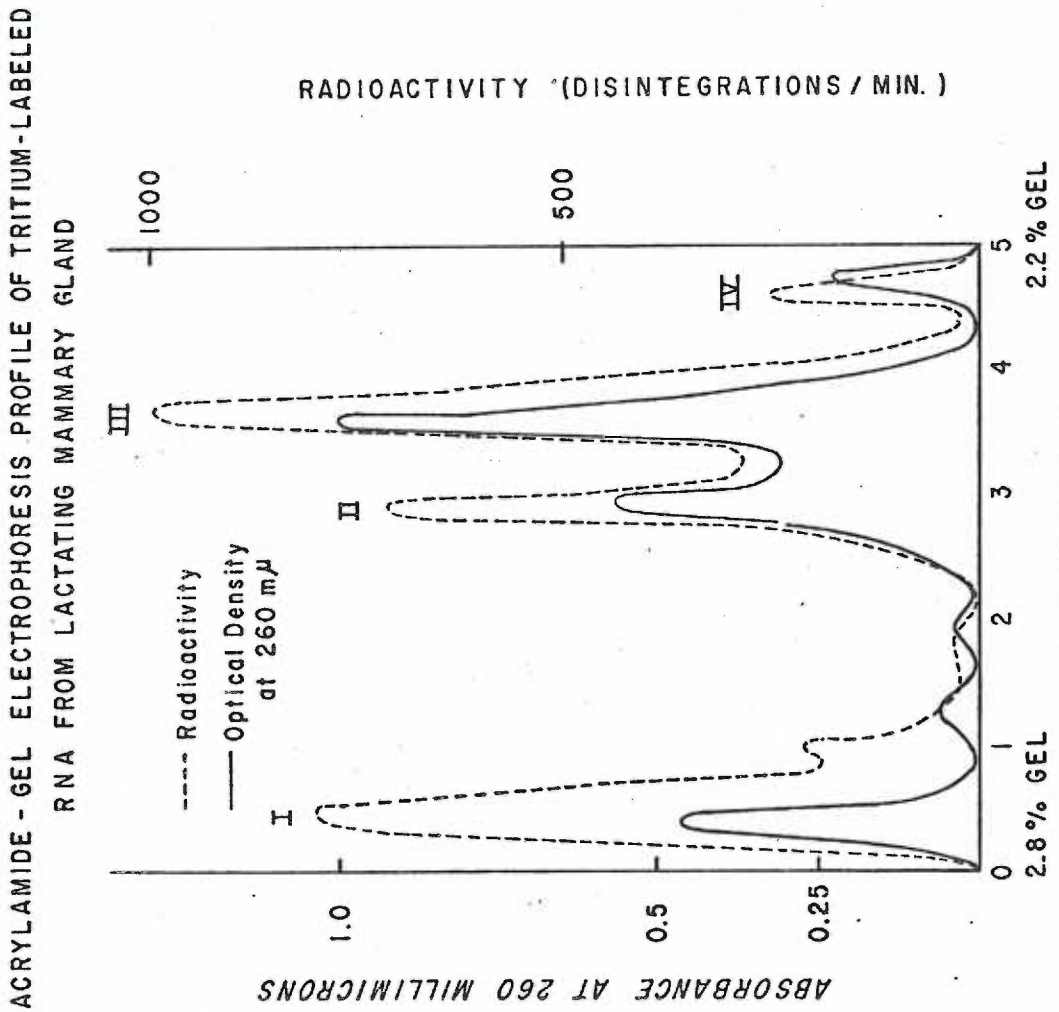

ACRYLAMIDE - GEL ELECTROPHORESIS PROFILE OF TRITIUM-LABELED RNA FROM LACTATING MAMMARY GLAND
RADIOACTIVITY (DISINTEGRATIONS / MIN.)
1000
500
Radioactivity
Optical Density at 260 mμ
I
II
III
IV
2.8% GEL
2.2% GEL
DISTANCE (cm.)
0
1
2
3
4
5
1.0
0.5
0.25
ABSORBANCE AT 260 MILLIMICRONS

FIGURE 5B

TABLE 1

EFFECT OF DNA AND PROTEIN CONTAMINATION
ON OPTICAL DENSITY OF RNA
IN THE ORCINOL TEST

The $O.D._{660}$ of 100µg of Worthington's yeast sRNA was compared to the $O.D._{660}$ of 1) 100µg of yeast sRNA plus 50µg of Worthington's calf thymus DNA, 2) 100µg of calf thymus DNA alone, and 3) 100µg of yeast sRNA plus 50µg of Sigma's bovine serum albumin (BSA). Adding 50µg of DNA to the RNA did not cause any observable increase in $O.D._{660}$ in the orcinol test, but 100µg of DNA alone produced an average $O.D._{660}$ value of 0.064, or 13 per cent of that produced by a like quantity of RNA. The addition of 50µg of BSA elevated the $O.D._{660}$ by 16 per cent, which is significant at $p < .05$, by Student's t-test.

TABLE 1

EFFECT OF DNA AND PROTEIN CONTAMINATION
ON OPTICAL DENSITY OF RNA
IN THE ORCINOL TEST

ORCINOL VALUES

MICROGRAMS RNA	MICROGRAMS DNA	MICROGRAMS BSA	O.D.$_{660}$	AVERAGE O.D.$_{660}$
100	0	0	0.502	
100	0	0	0.498	
100	0	0	0.497	
100	0	0	0.503	
100	0	0	0.499	
100	0	0	0.497	0.500
100	0	0	0.501	
100	0	0	0.501	
100	50	0	0.505	
100	50	0	0.499	
100	50	0	0.510	
100	50	0	0.500	
100	50	0	0.496	0.500
100	50	0	0.495	
100	50	0	0.497	
0	100	0	0.071	
0	100	0	0.066	
0	100	0	0.059	
0	100	0	0.059	
0	100	0	0.060	0.064
0	100	0	0.066	
0	100	0	0.072	
0	100	0	0.071	
100	0	50	0.589	
100	0	50	0.585	
100	0	50	0.580	
100	0	50	0.577	0.580
100	0	50	0.581	
100	0	50	0.582	
100	0	50	0.579	
100	0	50	0.578	

TABLE 2

CONTAMINATION OF RNA HYDROLYSATES BY DNA

Undiluted frozen samples of mammary gland RNA were randomly selected and thawed. For RNA determinations an aliquot of the sample was diluted 1:10 with distilled water and assayed for RNA by the orcinol test. For measuring DNA contamination the undiluted sample was tested by the diphenylamine method.

TABLE 3

CONTAMINATION OF RNA HYDROLYSATES BY PROTEIN

Undiluted frozen samples of mammary gland RNA were randomly selected and thawed. For RNA determinations an aliquot of the hydrolysate was diluted 1:10 with dilstilled water and assayed for RNA by the orcinol test. For measuring protein contamination, the sample was diluted 1:2 with 0.1N sodium hydroxide and the Oyama-Eagle (1956) procedure was performed. The values on the table are calculated in terms of the concentrations of RNA and protein in the original, undiluted samples. The column of the far right compares micrograms of protein to micrograms of RNA as a percentage figure.

TABLE 2

CONTAMINATION OF RNA HYDROLYSATES BY DNA

SAMPLE NUMBER	MICROGRAMS RNA PER ML.	MICROGRAMS DNA PER ML.	DNA:RNA AS PER CENT
1	1600	20	1
2	1420	70	5
3	1100	20	2
4	1330	20	1
5	1500	100	7
6	1340	80	6
7	1350	20	1
8	1300	30	2

TABLE 3

CONTAMINATION OF RNA HYDROLYSATES BY PROTEIN

SAMPLE NUMBER	MICROGRAMS RNA PER ML.	MICROGRAMS PROTEIN PER ML.	PROTEIN:RNA AS PER CENT
1	1350	50	4
2	1560	80	5
3	1540	100	6
4	1540	70	5
5	1450	90	6
6	1540	90	6
7	1590	60	4
8	1260	110	9

TABLE 4
EFFECT OF RNA CONTAMINATION OF THE OPTICAL DENSITY OF DNA IN THE DIPHENYLAMINE TEST

DIPHENYLAMINE VALUES

MICROGRAMS DNA	MICROGRAMS RNA	O.D.$_{600}$	AVERAGE O.D.$_{600}$
100	0	0.243	
100	0	0.239	
100	0	0.242	
100	0	0.242	0.241
100	0	0.243	
100	0	0.240	
100	0	0.241	
100	100	0.240	
100	100	0.244	
100	100	0.241	
100	100	0.241	0.242
100	100	0.242	
100	100	0.240	
100	100	0.245	

The O.D.$_{600}$ produced by 100 g of Worthington's calf thymus DNA in the diphenylamine test is compared to that produced by 100µg of DNA plus 100µg of Worthington's yeast sRNA. The addition of RNA has no significant effect on color development in the amounts used here.

TABLE 5

EFFECT OF PROTEIN CONTAMINATION OF THE COLOR DEVELOPMENT IN THE DIPHENYLAMINE TEST

Worthington's calf thymus DNA was made up to concentration of 50µg/ml and 100µg/ml. The $O.D._{600}$ in the diphenylamine test was measured at these concentrations and was compared to samples which had added, respectively, 50µg and 100µg of BSA. The $O.D._{600}$ of the BSA group was significantly higher ($p < .01$ by Student's t-test) than that of the DNA groups.

TABLE 5

EFFECT OF PROTEIN CONTAMINATION ON THE COLOR DEVELOPMENT IN THE DIPHENYLAMINE TEST

MICROGRAMS DNA	MICROGRAMS BSA	DIPHENYLAMINE VALUES O.D.$_{600}$	AVERAGE O.D.$_{600}$
50	0	0.115	
50	0	0.112	
50	0	0.109	
50	0	0.117	0.114
50	0	0.115	
50	0	0.116	
50	0	0.114	
50	50	0.138	
50	50	0.139	
50	50	0.131	
50	50	0.132	0.135
50	50	0.140	
50	50	0.130	
50	50	0.137	
100	0	0.219	
100	0	0.214	
100	0	0.215	
100	0	0.214	0.216
100	0	0.216	
100	0	0.220	
100	0	0.213	
100	100	0.264	
100	100	0.271	
100	100	0.279	
100	100	0.277	0.271
100	100	0.266	
100	100	0.270	
100	100	0.271	

TABLE 6

LEVEL OF PROTEIN CONTAMINATION
IN THE DNA HYDROLYSATES

MICROGRAMS DNA BY DIPHENYLAMINE DETERMINATION	MICROGRAMS PROTEIN BY OYAMA-EAGLE DETERMINATION	PROTEIN:DNA AS PER CENT
225	20	9
180	110	61
135	10	7
215	10	5
200	15	8
110	5	5
260	5	2
95	5	5

Randomly chosen frozen DNA hydrolysates were thawed, diluted 1:2 with 3 per cent PCA, and their DNA content measured by the diphenylamine method. Aliquots were diluted 1:2 with 0.1N sodium hydroxide, also, for use in the Oyama-Eagle (1956) protein determination. Results are presented in terms of the percentage ratio of protein to DNA.

TABLE 7

EFFECT OF THE NUMBER OF WASHES ON
REMOVAL OF ACID-INSOLUBLE RADIOACTIVE LABEL

PCA from the initial precipitation and the washes were saved during the Schmidt-Thannhauser procedure. One tenth ml of each wash was solubilized and counted as described in section VIC, 1-3. The CPM for each sample is presented. Also recorded (the last group of the table) are the CPM for 0.1 ml of the RNA hydrolysates themselves. Each successive wash appears to reduce the CPM to about 10 per cent that of the previous wash.

TABLE 7

EFFECT OF THE NUMBER OF WASHES ON
REMOVAL OF ACID-SOLUBLE RADIOACTIVE LABEL

SOURCE OF SAMPLE	CPM PER 0.1 ML SAMPLE
PCA from 1st wash	1558 2123 1192 757 4036 1106 1152
PCA from 2nd wash	161 236 98 84 424 127 113
PCA from 3d wash	12 15 12 8 38 14 9
RNA Hydrolysate	577 863 442 379 928 455 469

TABLE 8

NORMAL VALUES FOR MOUSE MAMMARY GLAND RNA AND DNA DURING PREGNANCY AND LACTATION

Mice at selected periods of pregnancy (see section I-C of Materials and Methods for staging of pregnancy) and lactation were killed and their mammary gland RNA and DNA fractions were isolated by the Schmidt-Thannhauser method of hydrolysis. RNA was quantitated by the orcinol test and by ultraviolet absorbance at 260mμ. DNA was measured by the diphenylamine test and by $O.D._{260}$ absorbance. The mean values for RNA, DNA, and RNA:DNA ratios of each group were computed.

TABLE 8

NORMAL RNA AND DNA VALUES FOR PRELACTATING AND LACTATING MOUSE MAMMARY GLAND

GRP. NO.	STAGE OF MAMMARY DEVELOPMENT	MICROGRAMS RNA/GRAM MAMMARY GLAND	AVG. RNA/ GROUP	MICROGRAMS DNA/GRAM MAMMARY GLAND	AVG. DNA/ GROUP	RNA:DNA RATIO	AVG. RNA:DNA/ GROUP
1	14-15 d Pregnancy	980 4080 1740 1880 3520 960 1650	2115* ±460	1390 1580 1190 1440 1530 1130 1150	1344 ±71	0.7 2.6 1.5 1.3 2.3 0.8 1.4	1.5 ±0.27
2	15½-17 d Pregnancy	2690 3720 1740 3830 3540 4640 4490	3521 ±383	1340 1230 1890 1480 1350 1440 1890	1517 ±101	2.0 3.0 0.9 2.6 2.6 3.2 2.4	2.4 ±0.29
3	17½-20 d Pregnancy	1530 4720 1850 3800 2240 2850 3270 2300 3250 4340	3015 ±335	1400 1610 1360 1400 1190 1570 1230 1440 1150 1080	1343 ± 56	1.1 2.9 1.4 2.7 1.9 1.8 2.6 1.6 2.8 4.9	2.4 ±0.35

*Standard error of the means

TABLE 8 CONTINUED

GRP. NO.	STAGE OF MAMMARY DEVELOPMENT	MICROGRAMS RNA/GRAM MAMMARY GLAND	AVG. RNA/ GROUP	MICROGRAMS DNA/GRAM MAMMARY GLAND	AVG. DNA/ GROUP	RNA:DNA RATIO	AVG. RNA:DNA/ GROUP
4	0-1 d Lactation	3420		1380		2.5	
		4590		1140		4.0	
		5760		1390		4.1	
		3790	4229	1260	1310	3.0	3.5
		3340	±375	1310	±66	3.8	±0.37
		3410		1610		2.1	
		5290		1080		4.9	
5	1-2½ d Lactation	6180		1080		5.7	
		4740		1290		3.7	
		7370		1700		4.3	
		3340		2020		1.6	
		7420	5896	2360	1579	3.1	3.9
		6090	±588	1330	±148	4.6	±0.38
		4970		1270		3.9	
		4140		1220		3.4	
		8810		1940		4.5	
6	4½-8½ d Lactation	14780		2140		6.9	
		12300		1400		8.8	
		10390		2010		5.2	
		6790	10956	1040	1274	6.5	10.3
		11800	±897	1050	±204	11.2	±1.92
		9720		1290		7.5	
		8850		420		21.1	
		13020		840		15.5	

TABLE 8 CONTINUED

GRP. NO.	STAGE OF MAMMARY DEVELOPMENT	MICROGRAMS RNA/GRAM MAMMARY GLAND	AVG. RNA/ GROUP	MICROGRAMS DNA/GRAM MAMMARY GLAND	AVG. DNA/ GROUP	RNA:DNA RATIO	AVG. RNA:DNA/ GROUP
7	10½-11½ d Lactation	10580 14490 12630 15700 15980 14460 11360 15340	13817 ±724	1300 1180 1360 1580 1550 1520 2290 1800	1573 ±123	8.1 12.3 9.3 9.9 10.3 9.5 4.9 8.5	9.1 ±0.75
8	12-14 d Lactation	12000 16200 14570 10600 11840 16170	13563 ±982	900 1360 1320 1460 960 2430	1405 ±225	13.3 11.9 11.0 7.2 12.3 6.6	10.4 ±1.14
9	16-22 d Lactation	7690 8930 9340 10510 8170 11320 6660 11680	9288 ±629	1230 850 910 2040 1480 1060 780 1200	1194 ±146	6.2 10.5 10.3 5.1 5.5 10.7 8.5 9.7	8.3 ±0.86
10	1½-2½ d Weaning	3650 3840 2420 3810	3430 ±339	440 260 240 640	395 ±93	8.3 14.8 10.1 5.9	9.8 ±1.88

TABLE 9

SPECIFIC ACTIVITY OF TRITIUM-LABELED RNA
OF PREGNANT AND LACTATING MICE

Five- to seven-day lactating mice were injected intraperitoneally with 1 microcurie(μc) per gram of body weight of ^{3}H-6-uridine. Eighteen- to nineteen-day pregnant mice were likewise injected. After three hours, the mice were killed and their mammary glands were removed. RNA and DNA fractions were isolated and measured (DNA values are not shown on this table; they were not significantly different between the two groups). Samples of the RNA hydrolysates were counted in the Packard Tri-Carb liquid scintillation counter. The DPM and specific activity of the samples were calculated as outlined in Materials and Methods section VI-D.

Statistical Analysis (Student's t-test)

	t	df	p
Pregnant vs. Lactating Mice			
A. μg RNA/g gland	8.513	20	<.001
B. DPM/g gland	12.831	20	<.001
C. DPM/μg RNA	5.926	20	<.001

TABLE 9

SPECIFIC ACTIVITY OF TRITIUM-LABELED MAMMARY RNA OF PREGNANT AND LACTATING MICE

PREGNANT MICE

MICROGRAMS RNA/ GRAM GLAND	DPM/GRAM GLAND	DPM/MICROGRAM RNA
3820	31270	8.2
3890	26900	6.9
2600	44350	17.0
4070	52380	12.8
4500	46460	10.3
5860	47930	8.2
4720	57670	12.2
4420	56980	12.9
3380	39120	11.6
3110	27330	9.1
2960	27230	9.2
MEAN 3929 ±280*	41601 ±3594	10.8 ±0.87
	LACTATING MICE	
10650	190330	17.9
9930	200160	20.1
8960	153080	17.1
6270	143630	22.9
17100	209970	12.3
14220	241510	17.0
13950	276430	19.8
14740	273830	18.7
12920	199920	15.4
11380	215520	19.0
13360	221530	16.6
MEAN 12135 ± 921	211446 ±12739	17.9 ±.83

* Standard error of the mean

TABLE 10

EFFECT OF OVARIECTOMY OF PREGNANT BALB/C MICE ON MAMMARY RNA AND DNA LEVELS

Mice were ovariectomized or sham-operated on day 18-19 of pregnancy, and were allowed to nurse a litter of five of their own or foster pups until the time they were killed. They were killed three days after the operation, their mammary glands were removed, and mammary RNA and DNA were measured by the Schmidt-Thannhauser method. Also included as a control group were unoperated mice which had been lactating three days, as had the ovariectomized ones.

Statistical Analysis (Student's t-test)

	t	df	p
Ovariectomy at 18-19 days Pregnancy Compared with Sham operation at 18-19 days Pregnancy			
A. Gland weight	0.797	12	>.4
B. µg RNA/g gland	3.149	12	<.01
C. µg DNA/g gland	0.806	12	>.4
D. RNA:DNA ratio	2.504	12	<.05

	t	df	p
Ovariectomy at 18-19 days Pregnancy Compared with Control at 3 days Lactation			
A. Gland weight	1.217	12	>.2
B. µg RNA/g gland	1.282	12	>.2
C. µg DNA/g gland	0.103	12	<.9
D. RNA:DNA ratio	1.002	12	>.3

	t	df	p
Sham Operation at 18-19 days Pregnancy Compared with Control at 3 days Lactation			
A. Gland weight	0.199	12	>.8
B. µg RNA/g gland	2.457	12	<.05
C. µg DNA/g gland	0.933	12	>.3
D. RNA:DNA ratio	2.204	12	<.05

TABLE 10

EFFECT OF OVARIECTOMY OF PREGNANT BALB/C MICE ON MAMMARY RNA AND DNA LEVELS

PROCEDURE: OVARIECTOMY AT 18-19 DAYS PREGNANCY	WT. MAMMARY GLAND IN GRAMS	MICROGRAMS OF RNA/GRAM OF MAMMARY GLAND	MICROGRAMS OF DNA/GRAM OF MAMMARY GLAND	RNA:DNA RATIO
1	0.92	6260	1180	5.3
2	0.99	9050	1120	8.1
3	0.95	6650	1510	3.6
4	1.01	5510	1560	3.5
5	1.09	5210	1070	4.9
6	0.99	4640	920	5.0
7	0.96	5390	1280	4.2
MEAN	0.99 ±0.02*	6101 ±552	1234 ±88	4.9 ±0.59
SHAM OPERATION AT 18-19 DAYS PREGNANCY				
1	1.01	3400	990	3.4
2	1.09	4590	1180	4.0
3	1.35	5760	1030	4.1
4	1.03	3340	1840	3.0
5	0.91	3410	2240	2.1
6	0.87	3930	1130	3.8
7	0.95	4100	1350	3.0
MEAN	1.03 ±0.06	4076 ±330	1393 ±177	3.3 ±0.27

*Standard error of the mean

TABLE 10 CONTINUED

PROCEDURE: CONTROL AT 3 DAYS LACTATION	WT. MAMMARY GLAND IN GRAMS	MICROGRAMS OF RNA/GRAM OF MAMMARY GLAND	MICROGRAMS OF DNA/GRAM OF MAMMARY GLAND	RNA:DNA RATIO
1	1.31	6180	1080	5.7
2	1.10	4740	1290	3.7
3	1.01	4970	1270	3.9
4	0.93	5370	1380	3.6
5	1.07	6020	1250	4.8
6	0.89	3560	1110	3.2
7	1.00	5990	1190	5.0
MEAN	1.04 ±0.05	5261 ±352	1224 ± 40	4.3 ±0.37

TABLE 11

EFFECT OF OVARIECTOMY ON NUCLEIC ACIDS OF THE MAMMARY GLANDS OF LACTATING MICE

Mice were ovariectomized on the first to second day postpartum or on the seventh to eighth day postpartum. They were allowed to nurse their litters (5 pups) until just before they were killed. They were killed 3 days after ovariectomy, their mammary glands removed, and the mammary gland RNA and DNA measured by the Schmidt-Thannhauser modification.

Control mice were sham-operated on the first to second day postpartum or on the seventh to eighth day postpartum. From that point they were treated as were the ovariectomized animals.

Statistical Analysis (Student's t-test)

	t	df	p
Sham Operated at 1-2 days post partum vs. Ovariectomized at 1-2 days post partum			
A. Gland weight	.800	12	>.4
B. µg RNA/g gland	.146	12	<.8
C. µg DNA/g gland	.444	12	>.6
D. RNA:DNA ratio	.661	12	>.5
	t	df	p
Sham Operated at 7-8 days post partum vs. Ovariectomized at 7-8 days post partum			
A. Gland weight	.115	11	>.9
B. µg RNA/g gland	.031	11	>.9
C. µg DNA/g gland	.031	11	>.9
D. RNA:DNA ratio	.113	11	>.9

TABLE 11

EFFECT OF OVARIECTOMY ON NUCLEIC ACIDS OF THE MAMMARY GLANDS OF LACTATING MICE

PROCEDURE: SHAM-OPERATION AT 1-2 DAYS POSTPARTUM	WT. MAMMARY GLAND IN GRAMS	MICROGRAMS OF RNA/GRAM OF MAMMARY GLAND	MICROGRAMS OF DNA/GRAM OF MAMMARY GLAND	RNA:DNA RATIO
1	1.03	10550	2010	5.2
2	1.01	8420	1320	6.4
3	1.20	11010	1990	5.5
4	0.87	9990	1080	9.2
5	0.94	9580	1190	8.0
6	0.93	11170	1460	7.6
7	1.12	10770	1120	9.6
MEAN	1.01 ±0.04	10213 ±366	1453 ±149	7.4 ±0.65
SHAM-OPERATION AT 7-8 DAYS POSTPARTUM				
1	1.32	13440	1430	9.4
2	0.99	14210	1590	9.0
3	1.19	11700	1410	8.3
4	1.02	13820	1600	8.6
5	0.97	14850	1730	8.6
6	0.95	12150	1760	6.9
7	0.93	12980	1120	11.6
MEAN	1.05 ±0.05	13307 ±423	1520 ± 84	8.9 ±0.54

*Standard error of the means

TABLE 11 CONTINUED

PROCEDURE: OVARIECTOMY AT 1-2 DAYS POSTPARTUM	WT. MAMMARY GLAND IN GRAMS	MICROGRAMS OF RNA/GRAM OF MAMMARY GLAND	MICROGRAMS OF DNA/GRAM OF MAMMARY GLAND	RNA:DNA RATIO
1	0.88	10740	1120	9.6
2	1.02	10310	1980	5.2
3	1.00	8350	1320	6.2
4	0.80	11090	1590	6.9
5	0.96	9310	1200	7.7
6	0.97	9900	1470	6.7
7	1.17	11240	2140	5.2
MEAN	0.97 ±0.04	10134 ±392	1546 ±146	6.8 ±0.58
OVARIECTOMY AT 7-8 DAYS POSTPARTUM				
1	1.21	13900	1310	10.6
2	0.98	12130	1760	6.9
3	0.98	14210	1550	9.2
4	1.06	11980	1440	8.4
5	0.87	13330	1450	9.2
6	1.27	14180	1630	8.7
MEAN	1.06 ±.06	13288 ±411	1523 ±65	8.8 ±0.49

TABLE 12

EFFECT OF ADRENALECTOMY OF PREGNANT MICE ON THEIR MAMMARY GLAND RNA AND DNA LEVELS

BALB/c mice were adrenalectomized at 18-19 days of pregnancy; within 6 hr therafter they aborted their litters. They were given foster pups to nurse for 3 days, at which time they were killed and their mammary glands removed for analysis of RNA and DNA. Pregnant animals (18-19 days) were used as controls (see also Table 10 for 3 day postovariectomy, sham-operated controls and 3 day lactating controls).

Statistical Analysis (Student's t-test)

	t	df	p
Adrenalectomy vs. Pregnant Controls			
A. Gland weight	25.55	11	<.001
B. µg RNA/g gland	2.198	11	>.05
C. µg DNA/g gland	8.280	11	<.001
D. RNA:DNA ratio	.200	11	>.8

TABLE 12

EFFECT OF ADRENALECTOMY OF PREGNANT MICE
ON THEIR MAMMARY GLAND RNA AND DNA LEVELS

PROCEDURE: ADRENALECTOMY AT 18-19 DAYS PREGNANCY	WT. MAMMARY GLAND IN GRAMS	MICROGRAMS OF RNA/GRAM OF MAMMARY GLAND	MICROGRAMS OF DNA/GRAM OF MAMMARY GLAND	RNA:DNA RATIO
1	0.29	1670	810	2.1
2	0.44	2360	1100	2.1
3	0.32	1750	790	2.2
4	0.33	1700	820	2.1
5	0.43	2290	980	2.3
6	0.49	1850	930	1.9
7	0.50	1920	770	2.5
MEAN	0.40 ±0.03*	1934 ±106	886 ±46	2.2 ±0.01
CONTROLS AT 18-19 DAYS PREGNANCY				
1	1.13	1850	1390	1.4
2	1.05	3800	1400	2.7
3	1.01	1530	1400	1.1
4	1.06	4720	1610	2.9
5	0.98	2980	1270	2.3
6	0.91	3330	1460	2.3
MEAN	1.02 ±0.03	3035 ±489	1422 ±45	2.1 ±0.29

*Standard error of the mean

TABLE 13

EFFECT OF ADRENALECTOMY ON MAMMARY GLAND NUCLEIC ACIDS OF LACTATING MICE

Three- to four-day postpartum BALB/c mice were either adrenalectomized or sham-operated. The were allowed to nurse their litters (5 pups) until the experiment was terminated. Three days after the operation the mice were killed, their mammary glands removed, and the RNA and DNA were extracted and quantitated.

Statistical Analysis (Student's t-test)

	t	df	p
Adrenalectomy vs. Sham Operated Mice			
A. Gland weight	8.21	14	<.001
B. µg RNA/g gland	20.08	14	<.001
C. µg DNA/g gland	.90	14	>.3
D. RNA:DNA ratio	7.46	14	<.001

TABLE 13
EFFECT OF ADRENALECTOMY ON MAMMARY GLAND
NUCLEIC ACIDS OF LACTATING MICE

PROCEDURE: SHAM OPERATION	WT. MAMMARY GLAND IN GRAMS	MICROGRAMS OF RNA/GRAM OF MAMMARY GLAND	MICROGRAMS OF DNA/GRAM OF MAMMARY GLAND	RNA:DNA RATIO
1	0.88	12530	1190	10.5
2	1.14	11440	770	17.9
3	0.88	10740	1120	9.6
4	1.10	9760	1580	6.2
5	0.97	13670	1510	9.1
6	1.03	13980	1330	10.5
7	1.07	12850	1520	8.4
8	0.95	11800	1010	11.7
MEAN	1.00 ±.03*	12096 ±511	1254 ±100	10.5 ±1.21
ADRENALECTOMY				
1	0.60	1820	1400	1.3
2	0.55	1510	930	1.6
3	0.60	1300	650	2.0
4	0.42	1190	1180	1.1
5	0.10	1730	870	2.0
6	0.19	1020	990	1.1
7	0.57	2120	1520	1.4
8	0.39	1570	1380	1.1
MEAN	0.43 ±0.07	1533 ±127	1115 ±107	1.4 ±0.13

*Standard error of the mean

TABLE 14

EFFECT OF OVARIECTOMY-ADRENALECTOMY ON MAMMARY NUCLEIC ACID OF PREGNANT AND LACTATING MICE

Two groups of mice were adrenalectomized-ovariectomized: 1)18-19 day pregnant mice, and 2) 3-4 day lactating mice. Following the double operation the mice were killed, their mammary glands were removed, and the RNA and DNA were measured.

Statistical Analysis (Student's t-test)

	t	df	p
Adrenalectomized vs. Ovariectomized Mice at 18-19 days Pregnancy			
A. Gland weight	.031	12	>.7
B. µg RNA/g gland	1.68	12	>.1
C. µg DNA/g gland	1.81	14	>.05
D. RNA:DNA ratio	16.1	14	<.001

TABLE 14

EFFECT OF ADRENALECTOMY-OVARIECTOMY ON MAMMARY NUCLEIC ACID LEVELS OF PREGNANT AND LACTATING MICE

PROCEDURE: ADRENALECTOMY-OVARIECTOMY OF 18-19 DAY PREGNANT MICE	WT. MAMMARY GLAND IN GRAMS	MICROGRAMS OF RNA/GRAM OF MAMMARY GLAND	MICROGRAMS OF DNA/GRAM OF MAMMARY GLAND	RNA:DNA RATIO
1	0.45	2320	970	2.4
2	0.67	1830	1080	1.7
3	0.34	1370	650	2.1
4	0.50	1920	830	2.3
5	0.31	1580	830	1.9
6	0.71	1670	850	2.0
7	0.26	1840	740	2.5
MEAN	0.46 ±0.07*	1790 ±113	850 ±54	2.1 ±0.07
ADRENALECTOMY-OVARIECTOMY OF 3-4 DAY LACTATING MICE				
1	0.62	1400	1080	1.3
2	0.37	1890	1100	1.7
3	0.50	2030	1250	1.6
4	0.55	1360	850	1.6
5	0.51	1220	990	1.2
6	0.44	1150	690	1.7
7	0.39	1490	1110	1.3
MEAN	0.48 ±0.02	1506 ±126	1010 ±69	1.5 ±.08

TABLE 15

EFFECT OF HYDROCORTISONE REPLACEMENT THERAPY ON MAMMARY GLAND RNA AND DNA LEVLES OF LACTATING MICE

Two- to three-day postpartum mice were adrenalectomized and allowed to nurse their litters. Three days later hydrocortisone replacement therapy or placebo (saline) was begun. The hydrocortisone acetate was administered subcutaneously and a dose of 50µg was injected every 12 hours. After 3 days of treatment the animals were killed and their mammary gland nucleic acids quantitated.

Statistical Analysis (Student's t-test)

	t	df	p
Adrenalectomy with Hydrocortisone vs. Adrenalectomy with placebo			
A. Gland weight	10.06	14	<.001
B. µg RNA/g gland	14.77	14	<.001
C. µg DNA/g gland	3.52	14	<.01
D. RNA:DNA ratio	13.54	14	<.001

	t	df	p
Adrenalectomy with Hydrocortisone vs. Sham Controls			
A. Gland weight	.24	14	>.8
B. µg RNA/g gland	2.64	14	<.02
C. µg DNA/g gland	.69	14	>.5
D. RNA:DNA ratio	1.76	14	>.1

TABLE 15

EFFECT OF HYDROCORTISONE REPLACEMENT THERAPY ON MAMMARY GLAND RNA AND DNA LEVELS OF LACTATING MICE

PROCEDURE: ADRENALECTOMY WITH HYDROCORTISONE	WT. MAMMARY GLAND IN GRAMS	MICROGRAMS OF RNA/GRAM OF MAMMARY GLAND	MICROGRAMS OF DNA/GRAM OF MAMMARY GLAND	RNA:DNA RATIO
1	0.91	14940	1110	13.4
2	1.13	13500	1210	11.1
3	1.12	14660	1480	9.9
4	1.00	21290	1580	13.5
5	0.96	13650	1360	10.0
6	1.23	15780	1770	8.8
7	1.02	16500	2040	8.1
8	1.01	12910	1130	11.4
MEAN	1.05 ±0.04*	15403 ±942	1460 ±116	10.8 ±0.70
ADRENALECTOMY WITH PLACEBO				
1	0.56	1610	1220	1.3
2	0.43	1420	890	1.6
3	0.47	1430	1110	1.3
4	0.59	1280	1010	1.3
5	0.19	1490	950	1.3
6	0.20	1110	660	1.7
7	0.16	1740	1170	1.5
8	0.51	1570	960	1.6
MEAN	0.39 ±0.06	1456 ±70	996 ±63	1.5 ±0.06

*Standard error of the mean

TABLE 15 CONTINUED

PROCEDURE: 8-9 DAY POSTPARTUM SHAM-OPERATED CONTROLS	WT. MAMMARY GLAND IN GRAMS	MICROGRAMS OF RNA/GRAM OF MAMMARY GLAND	MICROGRAMS OF DNA/GRAM OF MAMMARY GLAND	RNA:DNA RATIO
1	1.10	10540	1480	7.3
2	1.23	11210	1460	7.7
3	0.96	12390	1190	10.3
4	0.93	10910	920	11.8
5	1.04	15430	1590	9.7
6	1.02	14110	1410	10.0
7	0.95	13660	1540	8.9
8	1.07	10950	1320	8.3
MEAN	1.04 ±0.03	12400 ±639	1364 ±77	9.3 ±0.53

TABLE 16

EARLY EFFECTS OF HYDROCORTISONE TREATMENT OF ADRENALECTOMIZED MICE ON SPECIFIC ACTIVITY OF MAMMARY GLAND RNA

BALB/c mice were adrenalectomized 2 -3 days postpartum. Two days following the operation the mice were injected subcutaneously with either:

1) 100µg og hydrocortisone acetate in saline suspension, or
2) a placebo saline injection of the same volume as the hydrocortisone.

One hour after the hydrocortisone or saline injection all mice were intraperitoneally injected with 1.5µc per gram of body weight of ^{3}H-uridine. The labeling period was 3 hr, after which the animals were killed, their mammary glands removed, and the nucleic acids extracted by the Schmidt-Thannhauser procedure. RNA samples were counted in the Packard-Bell Tri-Carb liquid scintillation counter and the specific activities were computed.

Statistical Analysis (Student's t-test)

	t	df	p
Adrenalectomy with placebo vs. Adrenalectomy with Hydrocortisone			
A. µg RNA/g gland	0.459	14	>.6
B. DPM/g gland	2.737	14	<.02
C. DPM/µg RNA	3.930	14	<.01

TABLE 16

EARLY EFFECT OF HYDROCORTISONE TREATMENT OF ADRENALECTOMIZED MICE ON SPECIFIC ACTIVITY OF MAMMARY GLAND RNA

PROCEDURE: ADRENALECTOMY WITH PLACEBO	MICROGRAMS OF RNA/GRAM OF MAMMARY GLAND	DPM/GRAM OF MAMMARY GLAND	DPM/MICROGRAM OF RNA
1	2290	20840	9.1
2	2110	20260	9.6
3	3170	32330	10.2
4	2530	23530	9.3
5	1980	25200	12.7
6	2300	20470	8.9
7	1870	17020	9.1
8	2440	21960	9.0
MEAN	2336* ±143	22701 ±1619	9.7 ±0.45
ADRENALECTOMY WITH HYDRO-CORTISONE			
1	2540	56640	22.3
2	2010	35980	17.9
3	2030	78760	38.8
4	2930	228250	77.9
5	2610	111450	42.7
6	1760	105420	59.9
7	1950	35490	18.2
8	2120	35680	35.7
MEAN	2244 ±142	85959 ±23052	39.2 ±7.48

*Standard error of the mean

APPENDICES

APPENDIX 1

RECRYSTALLIZATION OF ORCINOL

Fisher's reagent grade orcinol was recrystallized as recommended by Volkin and Cohn (1954):

1) 5 Grams of orcinol was dissolved and boiled at 90° C in 50 ml of reagent grade benzene. The impurities settle as a heavy "tarry" phase.
2) The hot benzene solution was poured off, leaving the tars behind.
3) After the orcinol had recrystallized, it was filtered with a Buchner's funnel and washed several times with a small volume of benzene.
4) The yield was about 2.5 to 3.0 grams of purified orcinol.

APPENDIX 2

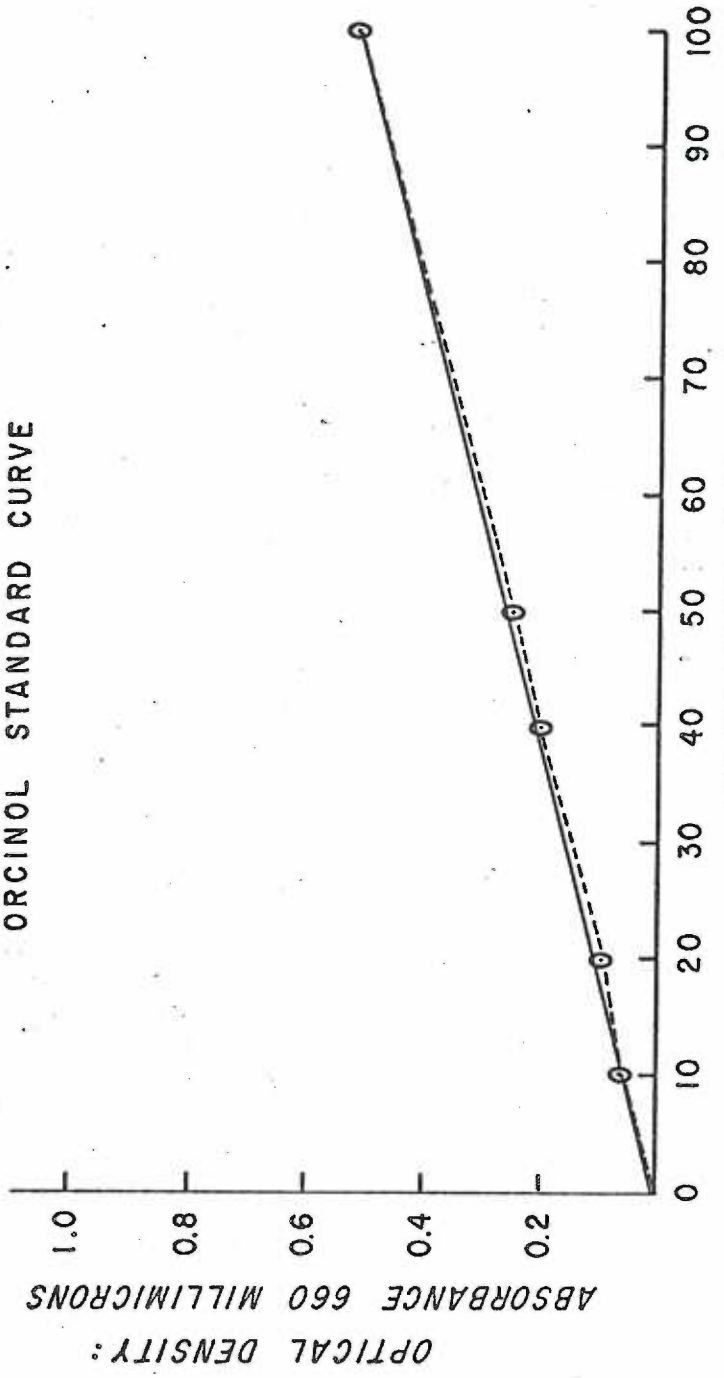
RNA STANDARD CURVE

Worthington's yeast sRNA was diluted to 10, 20, 40, 50, 100 and 200 (not shown on graph) µg per ml with 3 per cent PCA. Standards were prepared in triplicate and treated as described in Materials and Methods, section IV-A2e. The optical density was read to 660mµ and values of the 3 per cent PCA blank subtracted. The three values for each concentration were averaged.

----------(broken line) standard curve drawn by connecting average values for the standards by hand.

_____(solid line) line of best fit, as calculated on the Olivetti-Underwood calculator-computer.

APPENDIX 2


ORCINOL STANDARD CURVE
OPTICAL DENSITY:
ABSORBANCE 660 MILLIMICRONS
1.0
0.8
0.6
0.4
0.2
0
10
20
30
40
50
60
70
80
90
100
MICROGRAMS RNA

APPENDIX 3

DNA STANDARD CURVE

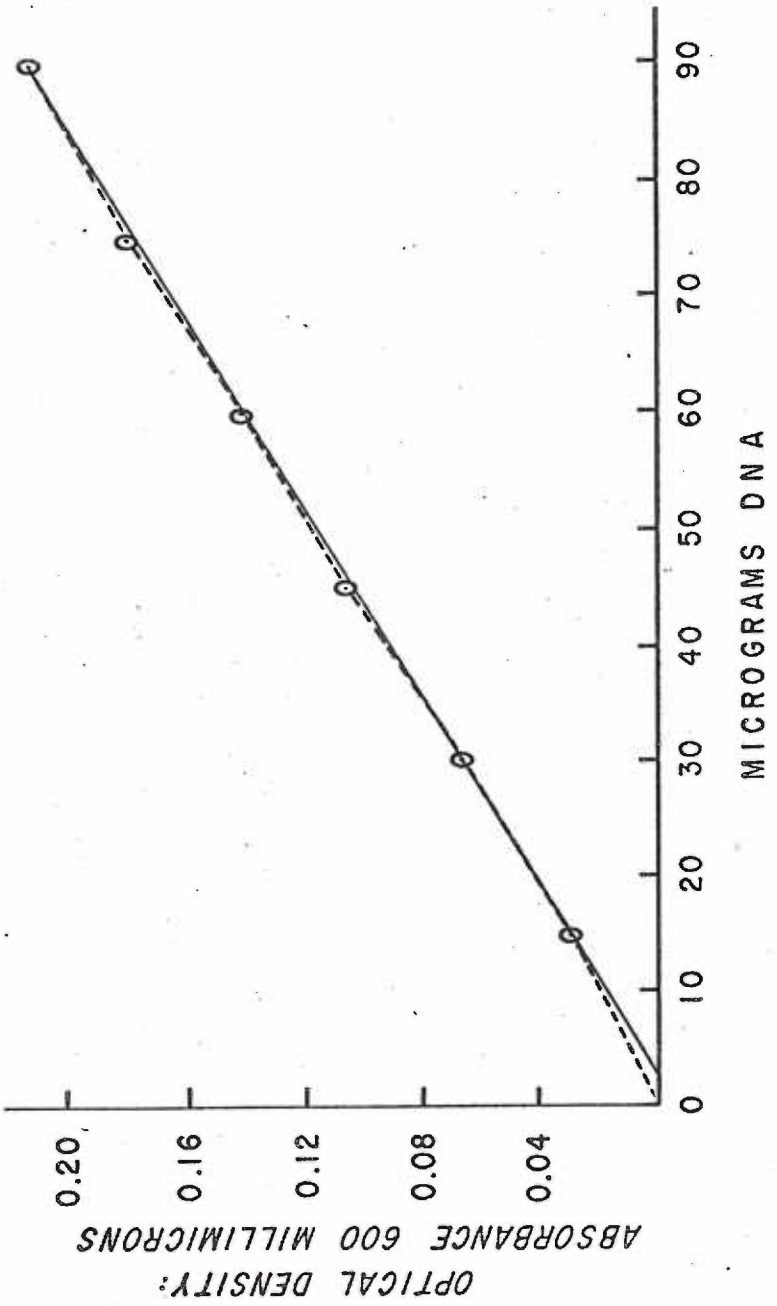
Worthington's calf thymus DNA was diluted to 15, 30, 50, 75, and 90µg per ml with 3 per cent PCA. The diphenylamine reagent was added to the triplicate standards and the tubes incubated as indicated in section IV-B2f of the Materials and Methods. The optical dinsity was read at 600mµ and the values of the 3 per cent PCA blank subtracted. Three values for each standard concentration were averaged.

----------(broken line) standard curve drawn by connecting average values for standards.

_____(solid line) line of best fit as calculated on the Olivetti-Underwood calculator-computer.

APPENDIX 3

DIPHENYLAMINE STANDARD CURVE



APPENDIX 4

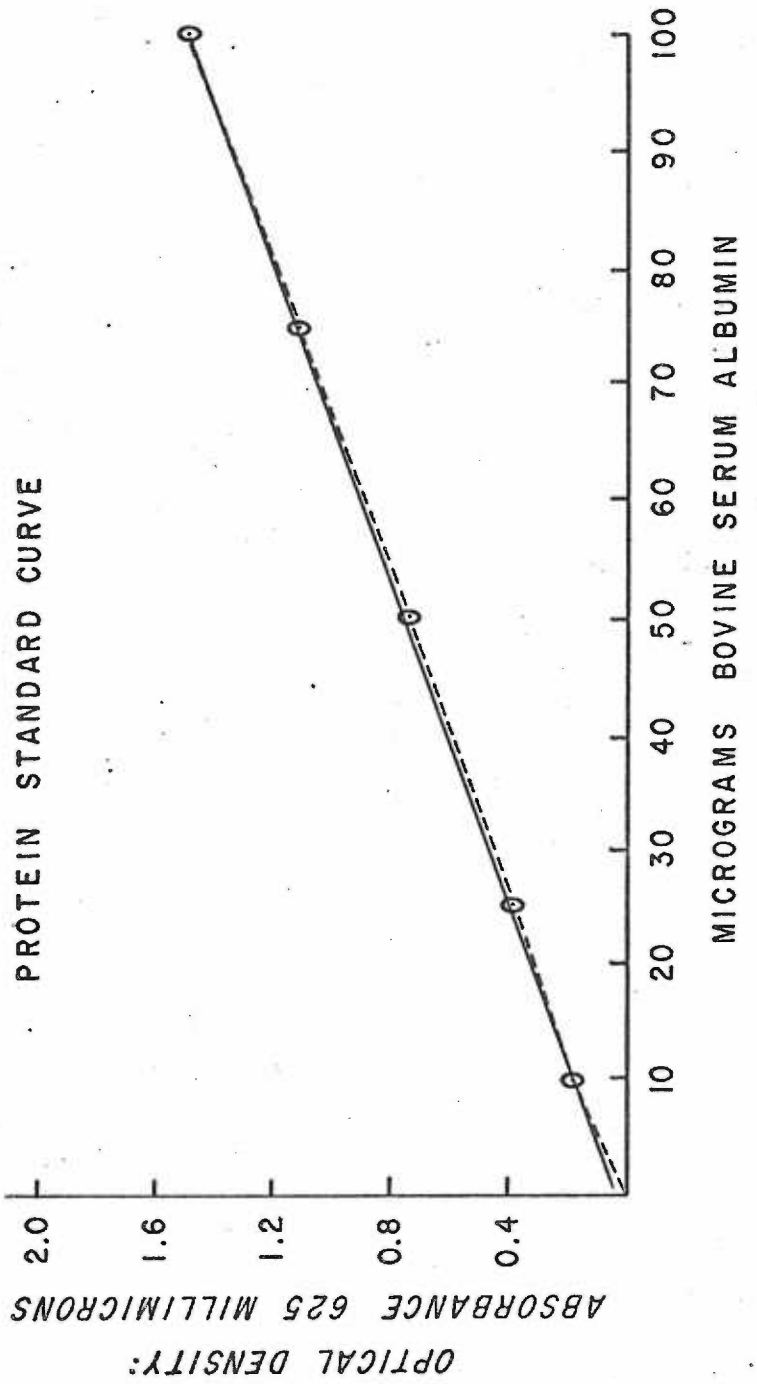
PROTEIN STANDARD CURVE

Bovine serum albumin (BSA) from Sigma Chemical Company was diluted to 10, 25, 50, 75, and 100µg/ml in 0.01 N sodium hydroxide. The standards were pipetted in triplicate and the Oyama-Eagle (1956) test was performed as described in Materials and Methods section V-F. The optical density was read at 625mµ and the 0.01N sodium hydroxide blanks subtracted. The three values for each concentration were averaged.

----------(broken line) standard curve drawn by connecting average values of standards.

_____(solid line) line of best fit, as calculated on the Olivetti-Underwood calculator-computer.

APPENDIX 4

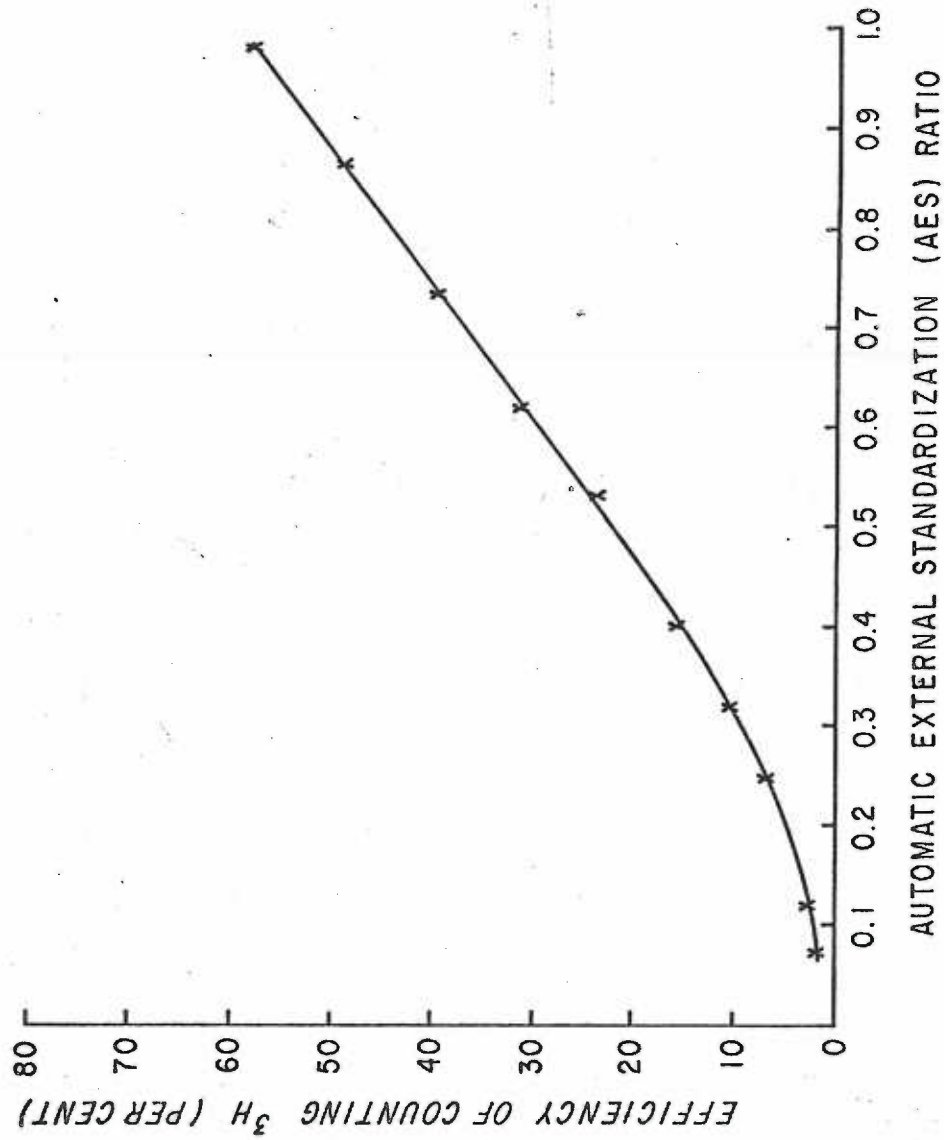

PROTEIN STANDARD CURVE
OPTICAL DENSITY:
ABSORBANCE 625 MILLIMICRONS
2.0
1.6
1.2
0.8
0.4
10
20
30
40
50
60
70
80
90
100
MICROGRAMS BOVINE SERUM ALBUMIN

APPENDIX 5

DETERMINATION OF EFFICIENCY OF TRITIUM COUNTS

A standard curve for the efficiency of counting ^{3}H samples was obtained be determining the ^{3}H counts in a series of 10 quenched ^{3}H standards using the ^{3}H-Quickset on the scintillation counter (window= 50-1000; gain= 50 per cent). Briefly, the automatic external standardization (AES) system exposes any ^{3}H sample to a compound 2-component gamma source, contained within the scintillation counter. The AES ratio expresses the degree of quenching of the sample as a ratio of the counts obtianed between the 2 components of the gamma radiation. The efficiency is determined by calculating the actual ^{3}H counts obtained as a percentage of the known quantity of ^{3}H present in the standard. A standard curve (Appendix 5) is then obtained by graphing the efficiencies obtained with the quenched standards against the AES ratios obtained. This standard curve is valid for all ^{3}H samples which do not contain color quenching. The ^{3}H-labeled tissue samples described in this dissertation did not contain color quenching. The standard curve shown was obtain repeatedly over a several-month period.

APPENDIX 5


EFFICIENCY OF COUNTING ^{3}H (PER CENT)
80
70
60
50
40
30
20
10
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
AUTOMATIC EXTERNAL STANDARDIZATION (AES) RATIO

APPENDIX 6

PREPARATION OF ACRYLAMIDE REAGENTS

A) Buffer: Sigma's Trizma (Tris) 0.04 M

Sodium acetate 0.02 M

EDTA 2 mM

The chemicals were dissolved distilled water and the pH adjusted to 7.1 with acetic acid.

B) Acrylamide, obtained from Matheson, Coleman and Bell, Norwood, Ohio, was recrystallized as follows (Loening, 1967):

1) 70 g of acrylamide was dissolved in 1 liter of chloroform at 50°C.
2) The solution was filtered hot, without suction.
3) The filtered solution was cooled to -20°C, at which temperature crystals reappeared.
4) The crystals were washed several times with cold chloroform and were dried.

C) Bis-acrylamide was purchased from Eastman Organic Chemicals, Rochester, N.Y. It was recrystallized in the following way (Loening, 1967):

1) 10 g of bis-acrylamide were dissolved in 1 liter of acetone at 40 to 50°C.
2) The hot solution was filtered.
3) The solution was cooled to -20°C.
4) After teh crystals reappeared they were washed and allowed to dry.