ROLE OF HORMONES IN CONTROLLING RIBONUCLEIC ACID SYNTHESIS DURING LACTOGENESIS

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HARVEY W. MOHRENWEISER

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ABSTRACT

ROLE OF HORMONES IN CONTROLLING RIBONUCLEIC ACID SYNTHESIS DURING LACTOGENESIS

By Harvey W. Mohrenweiser

The onset of lactation, lactogenesis, is associated with a rapid increase in the RNA, DNA and protein content of the mammary gland and concurrently, the mammary epithelial cell acquires the ability to synthesize the various caseins, whey proteins and lactose. Mammary explants from midpregnant mice have two phases of development. The first phase, which involves epithelial cell division may require only insulin and hydrocortisone. The second phase, which is dependent upon prolactin acting upon cells previously incubated with insulin and hydrocortisone, is the acquisition of the functions of a lactating cell. Prolactin enhances general RNA synthesis in explants and in endocrinectomized lactating rats while cortisol acts in a specific manner in endocrinectomized rats. A 12-15S RNA fraction having properties associated with mRNA has been observed and several species of RNA are detected in differentiated mammary tissue which are not present in mammary tissue from virgin mice. This report deals with the species of RNA synthesized during lactogenesis and the role of hormones in regulating RNA synthesis in mammary tissue slices.

Incorporation of ^{3}H-uridine into trichloroacetic

precipitated RNA during the last hour of 10 hour incubations was about 8-fold higher than the incorporation into RNA fractions isolated by phenol extraction reflecting differences in the fractions of RNA isolated by the two methods. There were also differences in the RNA synthesized during long and short periods of incorporation. More ^{32}P than ^{3}H-uridine was incorporated into specific fractions of RNA, probably reflecting differences in the specific activity of the precursor pool. The incorporation of ^{32}P per g of tissue was 15% less but the incorporation per mg of DNA was 30% higher 3 days prepartum than 3 days postpartum when corrections were made for differences in pool size. The RNA synthesized per gland is twice as much postpartum compared to prepartum rats.

Doubling the glucose concentration and increasing the amino acid concentration 2.5 fold stimulated RNA synthesis 25-50%. The combination of prolactin, hydrocortisone, and insulin stimulated ^{32}P incorporation to the same extent in the enriched and normal medium 199. Histological examination of the tissue after 12 hours of incubation indicated necrosis and degeneration in those samples incubated without hormone while evidence for maintenance of cellular integrity and enhanced secretion was observed in the samples incubated with the 3 hormones. Actinomycin D inhibited protein synthesis 70-80% within 12 hours indicating that some species of RNA in mammary tissue turned over quite rapidly.

The incorporation of ^{3}H-uridine and ^{14}C-amino acids into trichloroacetic acid precipitated material during one hour after the tissue had been preincubated for 0-9 hours was difficult to interpret because it did not agree with the histological examinations. The no hormone control incorporated the most precursor during several time periods. In lactating tissue the incorporation rate declined during the last 3 hours of incubation and insulin was the only hormone treatment which enhanced RNA synthesis compared to the no hormone control. Insulin or insulin, hydrocortisone and prolactin stimulated incorporation in nonlactating tissue.

The effect of hormones on ^{32}P incorporation into phenol extracted RNA was somewhat different from that on ^{3}H-uridine incorporation into RNA precipitated with trichloroacetic acid. Prolactin plus insulin, with or without hydrocortisone, stimulated ^{32}P incorporation into phenol extracted RNA in prepartum tissue while insulin plus hydrocortisone enhanced incorporation only at early times. In postpartum tissue, insulin plus hydrocortisone stimulated ^{32}P incorporation and the addition of prolactin was without an additional effect.

Separation of the various RNA species by polyacrylamide gel electrophoresis did not reveal any fraction from either pre- or postpartum tissue with a specific activity higher than the mRNA fraction, even though a fraction of

approximately 15S could be separated and detected. Similarly, no unique fraction responded to hormonal stimulation but rather the incorporation into all fractions of RNA was enhanced to the same extent. Thus, no species of RNA with the properties expected of mRNA could be detected with the techniques employed in these experiments.

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By

Harvey W. Mohrenweiser

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INTRODUCTION

The onset of lactation, lactogenesis, is associated with the rapid appearance of several components which are unique to milk and the mammary gland. Characteristics of lactating mammary cells are the ability to synthesize and secrete the various caseins, whey proteins and lactose while the nonlactating epithelial cell, which has a histological appearance distinctly different from the lactating cell, does not synthesize these compounds.

From the beginning of pregnancy until attaining the lactational state, changes in the mammary cell seem to involve two distinct phases. The first involves epithelial cell division to form the lobuloalveolar structure while the second is the acquisition of the functions of a lactating mammary cell. These changes have been extensively studied recently in mammary gland explants from the mid-pregnant mouse and the hormonal requirements for development and differentiation have been outlined. These experiments also suggest that at least 2 phases are involved in transforming the tissue derived from the mammary gland of a mid-pregnant mouse into tissue with the functional characteristics of the lactating gland but thus far it has been impossible to study the second phase independently of the cell division phase. Both DNA and RNA synthesis are required

for completion of phase 1 while phase 2 can be completed without concurrent DNA synthesis.

Many hypothesis have been proposed concerning the involvement and role of several hormones in lactogenesis. Although all of the hypotheses involved direct hormone action on the mammary gland, they differ with respect to the function of the hormone and the ultimate site of control.

Extensive increases in the RNA content of the mammary gland occur at the time of parturition. Hormone therapy in endocrinectomized animals indicated that several distinct classes of RNA may be synthesized in response to different hormones. In other tissues also it is unclear whether hormones stimulate the synthesis of all classes of RNA or act in a very specific manner by enhancing the synthesis of an RNA species which could be called messenger RNA.

Thus studies to ascertain the alterations in RNA synthesis which occur during lactogenesis were initiated. It was anticipated that investigations into the classes of RNA synthesized by pre- and postpartum mammary tissue slices and the influence of hormones upon synthesis during short-term incubations (which would be independent of the role of hormones in controlling cell division) could further elucidate the mechanism of lactogenesis and give additional insight into the mechanism of hormone action.

LITERATURE REVIEW

Physiological Changes During Initiation of Lactation

Changes in DNA Content

The initiation of lactation is associated with the newly acquired ability of the mammary gland to secrete milk and this is reflected in changes in many of the measurable parameters of the tissue. The initiation of lactation in the rat is accompanied by an increase in the amount of tissue as well as cell numbers, total DNA, RNA and protein in the mammary gland. Kuhn and Lowenstein (1967) and Baldwin and Milligan (1966) reported a 30-50% increase in wet tissue weight between 2 and 3 days prepartum (5 g) and 1 and 3 days postpartum (8 g). Tissue weights measured by Greenbaum and Slater (1957) were lower and the increase did not occur until the 3rd day of lactation. During this same time period, the water content of the tissue increased from approximately 40%-3 days prepartum-to 65%-5 days later. Conversely, the percent of fat in the tissue decreased by 50% and thus the fat-free dry tissue remains relatively constant at 10-11% of the total wet weight (Kuhn and Lowenstein, 1967).

The DNA content of mammary cells is quite constant (Mumford, 1964) and thus total DNA is routinely utilized as

a measure of total cell numgers and mammary development. The total DNA increase appeared quite variable, ranging from a 50% increase occurring sometime between 3 days prepartum and 2 days postpartum in 3 separate experiments (Kuhn and Lowenstein, 1966), to a 3-fold increase between the 15th day of pregnancy and the first day lactation (Thibodeau and Thayer, 1967). Baldwin and Milligan (1966) observed a doubling of total DNA between the 20th day of pregnancy (10 mg) and 48th hour of lactation (20 mg) as well as a 3-fold increase in the number of nuclei. Tucker and Reece (1963a, 1963b) reported a 2-fold increase in total DNA between the 12th and 18th day of pregnancy and additional 10% increases between the 18th day of gestation and the first day of lactation and also between days 1 and 4 of lactation.

Two other techniques have been used to assay DNA synthesis. Traurig (1967) injected a mouse with ^{3}H-thimidine one hour before sacrificing and counted the percent of intralobular-epithelial alveolar cells which were labeled, assuming that only these cells were undergoing mitosis. Only on day-2 (11%), day-3 (8%) and day-5 (3%) of lactation were more than 1% of the cells labeled. Stellwagen and Cole (1969) assessed cell division by measuring ^{3}H-thimidine incorporation into nuclear DNA. The rate of incorporation (dpm/ug DNA) gradually declined during the period-6 days prepartum to 6 days postpartum-except for a 3-fold increase on the first day of lactation

which is consistent with a burst of cell division immediately after parturition. The rate of histone biosynthesis and the relative amount of the various classes of histones were unchanged during late pregnancy and early lactation, thus detracting from the concept of alteration of classes of histones controlling synthesis of unique species of RNA and lactogenesis (Stellwagen and Cole, 1968, 1969).

Changes in RNA Content

The total RNA content of the mammary gland was highly correlated ($r > 0.93$) with liter weight gain (Tucker, 1966) and thus has been used as an index of protein synthetic activity and secretory ability. The RNA to DNA ratio in mammary tissue of 15-18 day pregnant rats was approximately unity but during the first 2-4 days postpartum a doubling of the total RNA content (15 mg increase) occurred and the RNA-DNA ratio approached 1.5 (Baldwin and Milligan, 1966; Tucker and Reece, 1963a, 1963b; Kuhn and Lowenstein, 1967).

The incorporation of radioactive precursors (glycine and orotic acid) per gland into acid insoluble ribonucleic acid increased approximately 6-8 fold between the 18th day of gestation and the end of the first day of lactation when the rate of incorporation had reached a maximum (Wang and Greenbaum, 1962). The rate of incorporation declined as lactation progressed.

Changes in Protein Content

Changes in total protein content followed closely the changes in DNA and RNA content with a 2-3 fold increase occurring between the 20th day of pregnancy and the 3rd day of lactation (Tucker and Reece, 1963a, 1963b; Greenbaum and Slater, 1957; Slater and Planterase, 1958).

Changes in Enzyme Activity

The rapid increase in metabolic activity associated with lactation was reflected in changes in the various enzyme activities of the cells. Baldwin and Milligan (1966) reported that the patterns of enzyme activity in mammary tissue from rats 3 days and 15 days after parturition were similar. They concluded that the cells acquired an enzyme complement characteristic of fully lactating tissue soon after their formation and furthermore that the apparent changes in enzyme activities between tissue from midpregnant animals and lactating animals were reflections of the low levels of some enzymes in nonsecretory cells, which predominate in tissue from midpregnant animals. Generally, the enzyme activity increased dramatically during the first 3 days of lactation whether expressed as units per g of tissue or per mg of extractable protein. Some enzymes increased in activity after the 3rd day of lactation but these increases could largely be accounted for by the increased extractable protein and remained constant when expressed on a specific activity basis. Kuhn

and Lowenstein (1967) reported similar results for enzymes associated with the biosynthetic pathways for major milk components.

Thibodeau and Thayer (1967) reported that aspartate transcarbamylase, the first unique enzyme in pyrimidine biosynthesis, increased slightly faster but to the same extent as did the RNA content of the gland at the time of parturition. Also the activity of 5'-nucleotidases were at a minimum during early lactation, indicating less degradation of nucleotides when they are in demand for synthetic processes (Wang, 1962).

The enzymes associated with lactose synthesis are particularly interesting because although lactose could not be detected in mammary tissue until the 20th day (2 days prepartum) (Yokoyama, 1969) or 21th day (Kuhn and Lowenstein, 1967) of gestation, the enzymes and substrates necessary for substantial levels of lactose synthesis were present several days prepartum (Glock and McLean, 1954; Kuhn and Lowenstein, 1967). Palmiter (1969) recently demonstrated that the method of assaying lactose synthetase activity, the rate limiting enzyme in lactose biosynthesis, dramatically influenced the detected enzyme activity. Employing a tissue culture assay (whole cells of mouse mammary tissue) the enzyme activity was only 4 times background on the 18th day of pregnancy. A 24- fold increase in activity occurred between the 18th day of pregnancy and

the 6th day of lactation. Enzyme assays in crude homogenates of mammary tissue indicated significant levels of enzyme were present 10 days prepartum with activity beginning to increase at that time. This was also reported by Kuhn and Lowenstein (1967) and would contradict the evidence that the rate limiting B subunit (α-lactalbumin) does not increase until the time of parturition (Turkington et al., 1968; Kuhn, 1968). This may indicate that compartmentalization of cellular components is important in controlling lactose synthesis and regulating other metabolic parameters.

Changes in Metabolite Concentration

Independent of changes in enzyme activity, changes in metabolite levels can dramatically alter cellular metabolism and productivity. Baldwin and Cheng (1969) concluded that prepartum levels of energy substrates and milk precursors in the mammary gland were adequate to support lactation, although some changes in metabolite levels did occur postpartum. The ratio of high energy phosphate bonds to the total adenine pool actually decreased after parturition although the level of adenine nucleotides increased. Wang (1962), although using a limited number of animals, reported a 3-fold increase in ATP and total nucleotides between the 18th day of pregnancy and the first day of lactation but the percent high energy phosphate bonds was unchanged. The molar ratio of ATP to (ADP plus

AMP) reported for mammary tissue decreased from 1.1 prepartum to 0.5 during late lactation. This is much lower than the 3:1 ratio reported for rat liver (Williamson et al., 1969) and chick brain (Kozak and Wells, 1969) indicating a low energy state in the tissue or possibly that anoxia had occurred during the time required to obtain the sample. Similarly the pyridine nucleotide pool increased 4-fold early in lactation but the TPNH:TPN^+ ratio increased only slightly at parturition and the DPNH:DPN^+ ratio decreased (Baldwin and Milligan, 1966).

Glucose concentrations average 0.7-1.2 umoles/g wet mammary tissue and do not change at the time of parturition (Baldwin and Cheng, 1969; Kuhn and Lowenstein, 1967). Stellwagen and Cole (1969) reported a 60% increase in the free amino acid pool between the 20th day of pregnancy and the first day of lactation (10.27 umoles/g wet tissue).

Cell proliferation, secretory cell development, changes in enzyme activity and metabolite levels occur during lactogenesis. The latter two are influenced by hormones and have a role in development of the mammary gland to the lactational state, but changes in the metabolite and enzyme levels do not appear to be the trigger for initiating milk secretion.

Structural and Organizational Changes During Initiation of Lactation

During lactogenesis the cellular structure of alveolar cells undergo marked morphological changes which can be

observed by both the light and electron microscope. Mammary tissue from midpregnant mice had small alveoli. Cellular vacuolation was small and the amount of stainable material in the alveolar lumina was small or totally absent, but lipid droplets were evident. Most fully lactating cells had vacuoles, the alveoli were distended and abundant secretion in the lumen was evident when examined with the light microscope (Elias, 1957; Nandi, 1959).

The use of the electron microscope has increased the resolution of the morphological changes associated with lactogenesis many fold. The nonlactating alveolar cell was characterized by a minimal content of endoplasmic reticulum, a small Golgi apparatus and a minimal number of mitochondia scattered throughout the cytoplasm. Only occasionally were milk protein droplets in evidence but several large milk fat droplets were observed in the lumen (Wellings, 1969). Wellings (1969) summarized the ultrastructural changes associated with the initiation of lactation as follows: a) dramatic increases in the grandular endoplasmic reticulum, b) hypertrophy of the Golgi apparatus and movement to the apical region of the cell, c) increase in number but a diminution in size of the cytoplasmic fat droplets and d) an increase in the number of mitochondia. A 4-fold increase in the relative area of the endoplasmic reticulum and the Golgi apparatus and the number of mitochondria

occurred between the 19th day of pregnancy and 4th day of lactation in C3H/Crgl strain of mice. Hollman (1969) reported similar observations except for mitochondial volume which increased 2.5-fold per cell during the first 5 days postpartum. This increase in the grandular endoplasmic reticulium is in agreement with the data of Gaye and Denamur (1969) where 75% of the polyribosomes in the pseudopregnant rabbit mammary gland are not membrane-bound but prolactin which induces lactation, caused a substantial increase in the aggregation of the polysomes, the amount of microsomes, and their protein synthesizing capacity.

Hormonal Treatments Affecting Initiation of Lactation

Lyons (1958) hypophysectomized and hypophysectomized-ovariectomized-adrenalectomized rats during late pregnancy in experiments to determine the hormonal requirements for initiation of lactation. Prolactin and cortisol acetate together were able to initiate and subsequently sustain a level of lactation equal to 50% of normal (Bintaringsih *et al.*, 1958). Similar results were obtained by Cowie and Tindal (1961).

Ovariectomization of rats during midpregnancy resulted in induced mammary secretion (Lin and Davis, 1967). A rapid elevation of the RNA content of the gland was evident after 8 hours and new proteins were detected within 24 hours, associated with an increased rate of ^{14}C-leucine incorporation. The level of DNA remained constant indicating that

no extensive cell proliferation was necessary for initiation of lactation. The authors suggested that the lack of milk secretion prepartum was due to the inhibitory influence of progesterone which was removed by ovariectomy or parturition.

Similarly, estrogen administration to the lactating mouse blocked lactation and protein and RNA synthesis by mammary tissue slices *in vitro* declined to a level comparable to that observed in the 14th day postweaning mouse (Sirakov and Rychlik, 1968).

Talwaker *et al.* (1961) administered hydrocortisone acetate during the 5th-9th or 10th-13th days of pregnancy and were able to induce lactation. No other hormone was active alone or enhanced the activity of hydrocortisone. They concluded that low levels of active adrenal glucocorticoids were responsible for the absence of lactation.

Injection of cortisol into the pregnant mouse initiated lactation and slightly stimulated the rate of RNA synthesis by tissue slices but the incorporation of ^{14}C-leucine into protein was less than in tissue from pregnant mice (Sirakov and Rychlik, 1968). Changes in the free amino acid pool as observed by Stellwagen and Cole (1969) could bias the measurements.

Cowie (1969) has outlined several of the general hypotheses for the initiation of lactation. First, the secretory activity of the mammary epithelium may be

inhibited by high levels of estrogen and/or progesterone. Also the release of prolactin from the anterior pituitory could be inhibited by these hormones. The decline in estrogen and progesterone at parturition releases the inhibition. Secondly, high levels of prolactin may be responsible for initiating lactation. High levels of oxytocin during parturition could cause a large amount of prolactin to be released from the anterior pituitory. Thirdly, during pregnancy the level of active adrenal corticoid is too low and either the level or activity is increased at parturition.

Hormonal Requirements for Maintaining Lactation

Earlier, Cowie (1957) had hypophysectomized rats on the 4th day of lactation in order to study the hormones required for maintenance of lactation. The combination of adrenalcorticotrophic hormone and prolactin was able to maintain lactation at approximately 50% of normal. High levels of prolactin alone had a small effect while adrenalcorticotrophic hormone alone was ineffective. Talwalker *et al.* (1960) administered adrenal glucocorticoids to intact rats daily from the 4th-18th day of lactation and were able to increase molk secretion.

Baldwin and Martin reported the effects of hypophysectomy on the first (1968a) or 10th day of lactation (1968b) and subsequent hormone therapy upon various

parameters of mammary metabolism. Mammary gland weight, RNA and DNA per g of tissue, ^{32}P incorporation into nucleic acids and ^{14}C-leucine incorporation into protein were decreased by hypophysectomy on the first day of lactation. Mammary gland weight and RNA and DNA content were increased by administration of either cortisol or prolactin but both cortisol and prolactin were required to increase these parameters to levels comparable to sham operated controls. Prolactin alone was as effective as the combination of cortisol and prolactin in stimulating ^{32}P incorporation into all of the various nucleic acid fractions. It was concluded that prolactin was controlling DNA synthesis and stimulated RNA synthesis in a general manner while cortisol, which only affected ^{32}P incorporation into a RNA fraction defined as DNA-like, acted in a specific manner. Cortisol did not enhance amino acid incorporation into total extractable protein while both cortisol and prolactin were necessary for retention of the capacity for casein synthesis. Two types of enzyme activity response were noted. The activity of most enzymes was independent of hormone treatment and was a reflection of cell numbers, while glucose-6-P dehydrogenase responded differently in that the combination of cortisol and prolactin caused a 4-fold enhancement of activity.

Hypophysectomy on the 10th day of lactation caused decreases in mammary gland weight but the RNA and DNA con-

centration were affected only slightly (Baldwin and Martin, 1968b). Prolactin and prolactin plus cortisol administration increased the RNA and DNA content of the tissue to almost 30% above sham-operated controls. Similarly prolactin increased ^{32}P incorporation into nuclear RNA also 2-fold over control values. Other results were similar to those obtained with rats hypophysectomized on the first day of lactation. Additionally, it should be noted that although ^{14}C-leucine incorporation into casein by the prolactin-cortisol groups was comparable to the sham operated control group, the low growth rate of the pups in the hormone group indicated a drastic reduction in synthetic activity.

Baldwin et al. (1969) investigated the effects of endocrinectomy on the fifth day of lactation and subsequent hormone therapy for 5 days upon RNA synthesis by isolated mammary gland nuclei. The results are outlined as follows: 1) alloxan diabetes did not affect either of the RNA polymerase activities (high or low salt) or the type of RNA synthesized as analyzed by nearest neighbor frequency and polyacrylamide gel electrophoresis, 2) ovariectomy followed by estrogen administration did not effect RNA synthesis but progesterone treatment caused alteration of the base composition and nearest neighbor frequency in the synthesized RNA which is consistent with the results of Lin and Davis (1967), 3) adrenalectomy and treatment with hydrocortisone did not effect RNA polymerase activity, RNA base

composition or nearest neighbor frequency but did alter the pattern of RNA synthesis as analyzed by gel electrophoresis, thus supporting the concept of a specific effect of cortisol upon RNA synthesis (Baldwin and Martin, 1968a, 1968b), 4) both cortisol and prolactin administration to hypophysectomized rats increased RNA polymerase activity in the low salt assay and changed the base composition and nearest neighbor patterns of the RNA synthesized. The addition of hormone *in vitro* did not affect the extent or class of RNA synthesized. The fractions of RNA synthesized *in vitro* appeared to correspond to those characterized by Sirakov *et al.* (1968) as being messenger RNA. The authors concluded that these data, in conjunction with the observation that prolactin and cortisol had specific effects on several synthetic enzymes (Kursrud and Baldwin, 1969), were further support that cortisol and prolactin exert specific regulatory influence on the rates and patterns of RNA synthesis in mammary secretory cells. Hypophysectomy causes drastic changes in cell number thus the changes in patterns of RNA synthesis could have resulted from changing cell population. This change in cell population was used to explain differential changes in enzyme activity in pre- and postpartum tissue (Baldwin and Milligan, 1966).

RNA and Protein Synthesis by Mammary Tissue Slices

Sirakov and Rychlik (1968) reported that mammary tissue slices from 16 day lactating mice incorporated 56 umole of ^{14}C-leucine per mg DNA per hour into trichloroacetic acid-precipitable protein. Tissue from 18 day pregnant mice was only 30% as active and was only as active as normal resting mammary tissue from 14 day postweaning mice. Incorporation per mg dry weight increased 10-fold between the pregnant and lactating periods (609 umole/mg dry weight). Expressed as incorporation per mg RNA the mammary tissue from pregnant mice was as active as tissue from lactating mice (16 uumole/mg RNA).

The incorporation of ^{3}H-uridine into trichloroacetic acid-precipitated material increased 7-fold in the lactating tissue compared to tissue from pregnant mice. The incorporation per cell doubles between the pregnant and lactating state but the specific activity of the RNA synthesized by tissue slices from pregnant mice was 50% higher (0.9 uumole/mg RNA) than RNA from lactating tissue. The lactating tissue slices maintained a linear rate of ^{3}H-uridine and ^{14}C-leucine incorporation for only 90 minutes, while the incorporation in slices from nonlactating glands was linear throughout the total incubation period (150 minutes). Addition of various hormones *in vitro* did not affect the rate or extent of incorporation.

Mayne *et al.*, (1966) reported that insulin stimulated

^{14}C-adenine incorporation into RNA in tissue slices from 12-17 day pregnant rats during the first 3 hours of incubation. The response of tissue slices from 12-15 day lactating rats was very variable (3-40% stimulation) and in 2 of 6 experiments the insulin stimulation was not statistically significant. Insulin also stimulated ^{14}C-leucine incorporation (38%) into protein by slices from midpregnant rat mammary tissue but no consistent stimulation was detected in similar experiments with slices from lactating tissue. Actinomycin D inhibited RNA synthesis 97% in mammary slices from pregnant rats and inhibited ^{14}C-leucine incorporation 25% but did not prevent the insulin stimulation of protein synthesis, thus suggesting that insulin does not stimulate protein synthesis only via increased RNA synthesis.

Sirakov et al. (1968) characterized the RNA synthesized by mammary tissue slices from pregnant and lactating mice during a 90 minute in vitro incubation. Very little radioactive material was isolated from the cytoplasm of lactating tissue. Analysis of the cytoplasmic RNA from nonlactating tissue on methylated-albumin (MAK) columns indicated the sRNA had the highest specific activity with a broad radioactive region corresponding to the rRNA being of lower specific activity. The RNA which bound tightly to the column had only 25% the specific activity of the rRNA fractions (calculated from Figure 1-b) even though

the tightly bound RNA was referred to as rapidly labeled, DNA-like-RNA. Contrary to the author's conclusions all of the nuclear RNA separated by MAK column chromatography are equally labeled if the specific activity of each fraction is calculated rather than being expressed as total radioactivity per peak. Sucrose gradient analysis of cytoplasmic RNA from nonlactating tissue indicated that a heterodisperse fraction, slightly smaller than 18S and largely overlapping the sRNA peak, was extensively labeled.

The ability of isolated RNA to stimulate amino acid incorporation in a bacterial cell-free protein-synthesizing system has been used as an assay for mammalian mRNA but it has been shown that rRNA also has this property, presumably because it inhibits ribonuclease degradation of mRNA by acting as an alternative substrate (Hunt and Wilkinson, 1967). Sirakov et al. (1968) employed this technique to further characterize the fractions of RNA isolated from mammary tissue. Crude preparations of nuclear RNA from nonlactating tissue had the greatest stimulatory ability and cytoplasmic RNA from lactating tissue had the lowest activity (3%). Conversely, assaying the fractions isolated by sucrose density centrifugation indicated the 12-15S fraction of cytoplasmic RNA from lactating tissue had the greatest stimulatory activity. A similar fraction from nonlactating tissue was much less active. Several very high molecular weight species

of nuclear RNA from nonlactating tissue had stimulatory activity which was absent in comparable fractions from lactating tissue. Thus the authors concluded that the 15S RNA fraction of lactating tissue was the major species of RNA synthesized in the nucleus but it did not enter the cytoplasm as measured by specific activity of this fraction in the cytoplasm and therefore cytoplasmic mRNA turned over very slowly and had a long half life.

Palmiter (1969a) observed that within 1 hour of the addition of insulin, hydrocortisone and prolactin, the incorporation of uridine, amino acids and galactose into TCA precipitated material was enhanced in midpregnant mouse mammary slices which had been previously incubated for 17-21 hours without hormones. Insulin alone was partially active indicating it may have a passive role by simply restoring normal metabolic function.

Mammary Tissue Differentiation In Vitro

Since the early experiments of Elias (1957), demonstrating the maintenance of cell features, and Elias and Rivera (1959), demonstrating the induction of secretory response in explants (mammary epithelial tissue from a mouse 10-14 days pregnant) incubated in a chemically defined medium containing prolactin, insulin and hydrocortisone, many advancements have been made in elucidating the hormonal response mechanism.

Protein Synthesis in Explants

During the first 3 hours of incubation, mammary tissue from the midpregnant mouse incorporates ^{32}P into a protein which is identical to authentic mouse casein (Turkington et al., 1965). This synthesis appears to occur in some cells which have already differentiated into a lactating type cell. Explants incubated without insulin, hydrocortisone and prolactin lost most of the ability to synthesize casein while explants cultured in the presence of the 3 hormones for 48 hours synthesized casein at a 3-5 fold stimulated rate and incorporated ^{32}P into noncasein phosphoprotein at a 50% increased rate (Juergens et al., 1965).

Insulin alone was able to maximally stimulate the synthesis of soluble nonmilk protein while all 3 hormones were necessary for the induction of α-lactalbumin and β-lactoglubulin synthesis (Stockdale et al., 1966). The synthesis of the whey proteins was not coordinate but after 48 hours of culture the ratio of whey protein to casein approached that observed in mouse milk.

Mayne and Barry (1970) reported insulin and prolactin stimulated ^{32}P incorporation into casein 4-fold within 24 hours and 5-fold at 48 hours over the level attained with explants incubated in the presence of insulin or insulin and corticosterone which were only slightly more effective than no hormone. Corticosterone acted synergistically

with the other 2 hormones to stimulate ^{32}P incorporation an additional 50-100%. The incorporation of ^{14}C-lysine into total protein responded in a similar but less dramatic manner to the various hormone combinations.

Role of Hormones in Controlling RNA Synthesis

Total RNA synthesis during the last 4 hours of a 48 hour incubation was increased 4-fold when explants were cultured for 48 hours in the presence of all 3 hormones. An initial basal rate of ^{3}H-uridine incorporation was maintained with no hormone addition while insulin, insulin plus prolactin and insulin plus hydrocortisone were as effective as the 3 hormone combination during the first 24 hours. No increase in RNA synthesis was observed with these latter combinations though during the second 24 hours (Stockdale et al., 1966).

Mayne et al. (1966) and Mayne and Barry (1967) noted a decreased rate of incorporation (68%) of ^{14}C-adenine into RNA within 3 hours in explants incubated in the absence of insulin and an increasing rate of decline in incorporation in total RNA during the first 12 hours of incubation. Mayne et al. (1968) reported that all 3 hormones were necessary to maintain a basal level of RNA synthesis for 48 hours. The RNA content of explants incubated 24 and 48 hours in insulin and corticosterone containing medium declined 3.5 and 22.2% and they incorporated 14.1 and 56.4% less ^{14}C-adenine during the last 3 hours of the respective

incubation periods than during the initial measurements. The RNA content of explants cultured in the presence of all 3 hormones was increased 8.0 and 10.3% at 24 and 48 hours and the rate of ^{14}C-adenine incorporation was enhanced 20 and 18%. Further investigation (Mayne and Barry, 1970) indicated insulin plus prolactin and insulin, prolactin and corticosterone stimulated ^{14}C-uridine incorporation 2-fold during 48 hours of incubation when compared to insulin alone or insulin and corticosterone. Time course studies indicated that insulin-stimulated RNA synthesis reached a maximum after 12 hours of incubation and then declined while RNA synthesis by explants incubated in the presence of all 3 hormones was stimulated an additional 50% during the next 12 hours.

Recently, Green and Topper (1970) reported that hydrocortisone does not stimulate RNA synthesis. Prolactin stimulated RNA and casein synthesis in epithelial cells previously incubated with insulin and hydrocortisone but also stimulated RNA synthesis (as measured by uridine incorporation into TCA precipitated material) in explants incubated with insulin which are unable to synthesize casein.

Turkington (1970) using RNA-DNA hybridization techniques detected species of RNA in explants incubated in presence of 3 hormones which were not detected in virgin mouse mammary epithelial cells.

Role of Hormones in Controlling DNA Synthesis

DNA synthesis by mammary epithelial cells was reported to be maximally stimulated by insulin alone (Turkington and Topper, 1967). Contrary to this report, Mayne and Barry (1970) observed that insulin plus prolactin and insulin, prolactin and corticosterone stimulated ^{3}H-thymidine incorporation into DNA 3- and 4-fold, respectively, when compared to insulin alone or insulin and corticosterone. Time course studies indicated that insulin stimulated DNA synthesis for only 12 hours before it returned to basal levels while DNA synthesis remained at the 12 hour level for 24 hours if prolactin and corticosterone were also present.

Sequence and Role of Hormones

Lockwood *et al.* (1967) were able to partially resolve the sequence of hormone requirements for explant differentiation. Insulin appeared to be involved in epithelial cell proliferation but cells formed during the influence of insulin alone would not synthesize casein after prolactin addition unless one cycle of cell division occurred in the presence of hydrocortisone. The data supporting this sequence and the role of each hormone will be outlined.

Role of Insulin

Insulin stimulated glucose uptake (2x) and the conversion of ^{14}C-glucose to $^{14}CO_2$ (10x) and fatty acids (10x)

by mouse mammary explants although the extent of conversion both with and without insulin decreased 50% during 48 hours of incubation (Mayne and Barry, 1970). Insulin stimulated amino acid incorporation into protein (Wool and Manchester, 1962) and also enhanced amino acid incorporation in the presence of actinomycin D (Mayne et al., 1966).

Glucose-6-P dehydrogenase activity was increased in explants incubated 22 hours in insulin-containing medium (Leader and Barry, 1968). Further addition of other hormones was without effect at 22 hours but the addition of prolactin or serum from lactating mice was stimulatory in cultures continued for 45 hours. Inhibitor studies indicated RNA synthesis was not necessary for the insulin stimulation and RNA and protein synthesis were not necessary after 3.5 and 12 hours respectively for the increased activity measured at 22 hours.

Turkington and Ward (1969) measured RNA polymerase activity in nuclei isolated from mammary explants. Insulin caused a 3-fold increase in activity while hydrocortisone was without a direct effect. Insulin also promoted formation of daughter cells within the alveolar epithelium which were ultrastructurally indistinguishable from parent cells (Mills and Topper, 1969, 1970). The daughter cells still contained a minimal amount of endoplasmic reticulium and only a few mitochondria.

Role of Hydrocortisone

Addition of hydrocortisone to the insulin medium resulted in cells with an intermediate level of ultrastructural development. The rough endoplasmic reticulium increased extensively and the lateral paranuclear Golgi apparatus increased (Mills and Topper, 1969, 1970).

Most of the remaining evidence concerning the role of hydrocortisone is indirect in that it is dependent upon subsequent action by prolactin. Only the prolactin stimulation of RNA synthesis occurs in cells which have been previously incubated in insulin alone as well as insulin and hydrocortisone even though casein synthesis does not occur in the insulin treated cells (Green and Topper, 1970), thus the exact role of hydrocortisone is unclear.

Hydrocortisone had an effect on induction of RNA polymerase activity in that only covertly differentiated cells (cells previously incubated in insulin and hydrocortisone) responded to prolactin stimulation. Colchicine inhibited enzyme activity only if added during the period of covert differentiation (Turkington and Ward, 1969).

Corticosterone may stimulate glucose metabolism in the presence of insulin (Mayne and Barry, 1970). This could indicate that at least part of the action of the adrenal corticoids is to stimulate metabolism rather than to directly affect cell replication.

Role of Prolactin

The addition of prolactin to postmitotic, covertly differentiated mammary cells stimulated RNA synthesis 2-fold and resulted in casein synthesis (Turkington, 1968). Either colchicine or hydroxyurea added at zero time prevented the stimulatory effect of prolactin added 12 hours later indicating that prior cell division and DNA synthesis are necessary before prolactin may act (Mayne and Barry, 1970).

Prolactin could initiate the secretory response in covertly differentiated cells even though colchicine was added simultaneously, indicating that prolactin acted after hydrocortisone and insulin and also that the action of prolactin did not require DNA synthesis (Turkington et al., 1967; Turkington and Ward, 1969). The addition of actinomycin D before or simultaneously with prolactin resulted in complete inhibition of casein synthesis (Mayne and Barry, 1970). Addition of actinomycin D 16 hours after prolactin addition (Turkington, 1968) or during the last 8 hours of the 48 hour incubation in the presence of all 3 hormones (Stockdale et al., 1966) depressed casein synthesis indicating that a rather short-lived RNA species is involved in casein biosynthesis. Inhibition of protein synthesis at the time of prolactin addition, also inhibited casein synthesis (Mayne and Barry, 1970; Turkington and Ward, 1969).

The prolactin stimulation of casein synthesis in the

presence of insulin and corticosterone did not occur during the first 12 hours of incubation but the addition of prolactin within the first 12 hours of incubation to explants incubated in corticosterone and insulin-containing medium resulted in a stimulation of casein synthesis 24 hours later. Prolactin was without effect if not added during the first 12 hours of incubation.

Prolactin addition resulted in completion of the ultrastructural differentiation of hydrocortisone-insulin treated cells. There was translocation of the rough endoplasmic reticulium, the Golgi apparatus and the nucleus and the appearance of secretory granules in the cytoplasm (Mills and Topper, 1970).

Explants incubated in the presence of insulin and hydrocortisone and subsequently transferred to prolactin-containing medium exhibited dramatically increased lactose synthetase activity (Palmiter, 1969) and specifically increased the level of α-lactalbumin which is the rate-limiting subunit of lactose synthetase (Turkington et al., 1968). Thus it would appear that acquisition of the lactational state was dependent upon prolactin acting on cells previously incubated in insulin and hydrocortisone.

The events which occur during lactogenesis can be briefly summarized as follows: 1) most parameters measured, including DNA, RNA, enzyme activity and metabolite concentration increased; 2) the data from endocrinectomized rats

indicated that hydrocortisone and/or prolactin were necessary for initiation and maintenance of lactation; 3) mammary explants required insulin, hydrocortisone and prolactin for differentiation into lactating cells. The exact stimulus for initiation of lactation is still unknown.

Mammalian RNA Metabolism

Several features of mammalian cells give rise to unique problems in RNA synthesis. The majority of the cellular DNA is located in the nucleus while the bulk of the RNA is in the cytoplasm, thus the relationship of transcription to translation is much more complex than in bacterial systems. Secondly the slow turnover of mammalian cells indicates that control of RNA and protein degradation may be an important regulatory phenomena.

Over 90% of the radioactive precursor incorporated into RNA by HeLa cells during 30 minutes of incubation was retained in the nucleus (Perry, 1962). Evidence exists that this rapidly-labeled material was composed of: 1) larger molecules which were precursors to ribosomal RNA (r-pre-RNA); 2) a class of RNA comprising about 1% of the total RNA which was constantly being synthesized and degraded within the nucleus (HnRNA) (Darnell, 1968).

Pathway of RNA Synthesis

Darnell (1968) has summarized the pathway of ribosomal RNA formation as follows: 1) within the nucleus a 45S

ribosomal RNA precursor (r-pre-RNA) is synthesized which is subsequently cleaved to a 41S and then to a 36S molecule. The 45S r-pre-RNA molecule is a polynucleotide which contains sequences destined to become 28S and 16S ribosomal RNA and some unmethylated regions of unknown function (Brown, 1968); 2) the 36S particule gives rise to an 18S ribosomal RNA molecule in the nucleoplasm and a 32S particle, probably still in the nucleus; 3) the 32S particle is degraded to yield the 28S ribosomal RNA species; 4) the 28S and 18S ribosomal subunits are transported into the free ribosomal subunit pool of the cytoplasm; 5) a 5S ribosomal RNA molecule is associated with the 45S precursor molecule and is processed in conjunction with the 28S ribosomal RNA molecule although a free cytoplasmic pool of 5S molecules exists (Knight and Darnell, 1967); 6) ribosomal proteins appear to become associated with the 45S RNA molecule either during or immediately after its synthesis (Warner and Soeiro, 1967); and 7) about 50% of the 45S r-pre-RNA is precursor to the 28S and 18S ribosomal RNA molecules but the remainder appears to be degraded within the nucleus (Jeanteur et al., 1968; Weinberg and Penman, 1970).

Rapidly-Labeled RNA

Recently it has been suggested that eukaryotic cells transcribe more genetic information than is translated into protein (Church and McCarthy, 1970) and that this extra RNA

is associated with a class of RNA, DNA-like in composition, which is confined to the nucleus (Attardi et al., 1966; Penman et al., 1968). After 20 minutes exposure to ^{32}P over 75% of the radioactive material in the nucleus was associated with this fraction rather than the r-pre-RNA (Soeiro et al., 1968). Aronson and Wilt (1969) have calculated that only 6% of the nuclear RNA synthesized was transferred out of the nucleus. Soeiro et al. (1968) and Church and McCarthy (1967) calculated that less than 10% of the nuclear RNA was precursor to cytoplasmic RNA. Indications are that 60-90% of this material was not transferred to the cytoplasm even hours after it was synthesized, but eventually decayed to the acid soluble pool (Amaldi and Attardi, 1968; Attardi et al., 1966). The half life of this RNA fraction in HeLa Cells was calculated to be less than 3 minutes (Soeiro et al., 1968) while the half life of mRNA in HeLa cells was greater than 3 or 4 hours (Penman et al., 1963). Most suggestions as to the function of this RNA fraction indicate a possible relationship to mRNA and regulation of cell function but the reasons for a rapidly turning over species confined to the nucleus and how it functions in regulation are not evident.

These observations, that much of the rapidly labeled RNA never became involved in protein synthesis and also that most of the rapidly labeled RNA in the cytoplasm was in the soluble RNA fraction (Latham and Darnell, 1965), would

suggest that data implying that DNA-like-RNA and rapidly labeled RNA were synonymous with mRNA, needs to be reevaluated.

General Mechanisms of Hormone Action

It has often been observed that many hormones can regulate the rate of protein synthesis in their respective target organs but are without effect when added directly to a cell-free protein synthesizing system *in vitro*. Studies with inhibitors have implicated RNA synthesis in the mechanism of hormone action. These observations gave credence to a hypothetical mechanism, based upon the repressor-inducer model of bacteria, involving hormonal modulation through selective control of mRNA synthesis (Karlson, 1963). Tata (1968) has outlined several observations which are inconsistent with this being the only mechanism of hormone action: 1) ribosomal RNA is the major product of gene transcription; 2) there is a high degree of selective restriction of RNA to the nucleus; and 3) much of the mRNA in the animal cell is long-lived suggesting that the entire protein-synthesizing unit may turn over at similar rates (Omura *et al.*, 1967). Thus several theories concerning the mechanism of hormone action have been proposed.

Translational Control

The anabolic action of some hormones such as insulin

(Wood and Cavicchi, 1967) and adrenalcorticotrophic hormone (Garren et al., 1965) may be the result of a form of translational control of protein synthesis, namely increased metabolic rate rather than a direct effect on RNA synthesis. Recent reports that insulin acts only at the cell membrane and is involved in membrane transport are consistent with a nontranscriptional role for insulin (Minimura and Crofford, 1969; Kono, 1969).

Means and Hamilton (1966a, 1966b) detected increased RNA synthesis by rat uterus when estrogen was injected but also noted increased ^{3}H-uridine uptake in the presence of estrogen. Agarwal et al. (1969) measured a 20% increase in the acid soluble nucleotide pool of rat liver 3 hours after cortisone treatment and 30 minutes after ^{3}H-orotic acid injection. Billing et al. (1969) were unable to detect any increased RNA synthesis by the immature rat uterus until at least 5 hours after estradiol administration when corrections were made for increased uptake of RNA precursors. Thus it would seem that one mechanism of hormone action involves transport across the cell membrane and increased metabolite concentration.

A more elaborate translational control mechanism involving a translation repressor has been proposed by Thompson et al. (1966) to explain the superinducibility by actinomycin D of dexamethasone-induced tyrosine transaminase. Gelehrter and Tompkins (1967) were unable to detect

any increase in total cellular RNA or its rate of synthesis from labeled precursors by hepatoma cells in tissue cultures when dexamethasone was inducing a 3-15 fold increase in enzyme activity suggesting that only very small changes in RNA synthesis are required for hormone action. Reel and Kenney (1968) have suggested that the superinduction was a result of actinomycin D inhibiting transaminase degradation before it affected the rate of synthesis, thus the existence and nature of translation repressors are unclear and controversial.

Another possible source of translational control revolves about changes in cellular ultrastructure. Palmiter et al. (1970) used physical and electron microscope assays to observe organization of ribosomes into polysomes in the chick oviduct when either estrogen or progesterone was administered. The early assembly of polysomes occurred from preexisting ribosomes, before new ribosomes entered the cytoplasm. This was similar to the observations of Gaye and Denamur (1969), that prolactin caused increased polysome assembly in rabbit mammary tissue (and induced lactation), and of Mills and Topper (1970) detailing ultrastructural changes in response to hormones in differentiating explants. It has been proposed that membrane bound ribosomes are more active than free ribosomes (Henshaw et al., 1963) or may actually be a mechanism for controlling rates and types of proteins synthesized (Pitot, 1969).

Another type of nontranscriptional control involves transport of RNA from the nucleus to the cytoplasm. Spirin (1967) proposed that mRNA may be transported from the nucleus to the cytoplasm complexed with ribonuclear protein as particles which have been termed informsomes and which have the potential to control translation.

Church and McCarthy (1967) reported a modification of this selective transport of nuclear RNA in regenerating rat liver. Willems et al. (1969) observed an increased transfer of nuclear RNA into the cytoplasm during compensatory renal growth. Total RNA synthesis was not accelerated but the quantity of heterodisperse rapdily turning over RNA in the nucleus declined. Church and McCarthy (1970) using RNA-DNA hybridization techniques were able to detect many species of RNA molecules which were confined to the nucleus of the uterus of both estrogen treated and control rabbits. Some RNA molecules restricted to the nucleus of uterine cells from control animals were detected in the cytoplasm of uterine cells from treated animals. The spectrum of molecules retained in the nucleus seemed more dramatic in animals before hormone treatment. These observations would suggest that increased protein synthesis could occur via increased processing of RNA into the cytoplasm rather than increased RNA synthesis.

Transcriptional Control

The most often observed result of hormone administra-

tion is a dramatic increase in RNA synthesis. The type of RNA synthesized during hormonal stimulation remains a point of contention. Tata (1968) views hormone action as resulting from synthesis of all types of RNA and especially new-highly active ribosomes while others (Kidson and Kirby, 1964) argue for hormone action through selective stimulation of new mRNA.

The data supporting the first theory are 2-fold. Namely, no increase in synthesis of an RNA fraction known to be mRNA has been observed without similar increases in all classes of RNA (Jackson and Sell, 1968), thus suggesting mRNA and rRNA increase in a parallel manner, and secondly, in many hormone-target organ interactions including the effect of estrogen on rat uterus, growth hormone and hydrocortisone on rat liver and thyroid hormone on toadpole liver, newly formed microsomes have been isolated which have increased protein synthesizing ability (Tata, 1968).

Much data has also been accumulated indicating mRNA is synthesized in response to hormone administration (Kidson and Kirby, 1964; Fujii and Vallee, 1967; Drews and Brawerman, 1967). The RNA which is rapidly labeled (Kidson and Kirby, 1964), extracted by hot detergent phenol (Greenman *et al.*, 1965) or high pH (Drews and Brawerman, 1967), is DNA-like in composition and/or has the ability to stimulate amino acid incorporation in a cell-free system

(Cartouzou et al., 1965) has been implied to be mRNA. Hydrocortisone stimulated ^{32}P incorporation into the hot phenol extracted RNA (Greenman et al., 1965) and ^{3}H-orotic acid incorporation into the high pH extracted RNA (Drews and Brawerman, 1967).

Schmid et al. (1967) demonstrated increased synthesis of RNA which had characteristics of mRNA when cortisol was added in vitro to isolated nuclei from rat liver. The fraction of RNA extracted with phenol at 55° was most rapidly labeled and was more effective in stimulating amino acid incorporation in a cell-free system than a similar fraction from nonhormone stimulated nuclei. Subsequently, Lang et al. (1968) were able to isolate an RNA fraction from livers of cortisol treated rats which increased the tyrosine-α-keto-glutarate transaminase activity when added to an in vitro protein synthesizing system. Inhibitor studies indicated that the enzyme was synthesized in vitro by de novo synthesis.

Recently, many investigators have employed the RNA-DNA hybridization technique to detect new RNA species synthesized during hormone influence. O'Malley and McGuire (1968, 1969) were able to detect unique nuclear RNA species in the chick oviduct 6 hours after progesterone administration and before avidin synthesis. Hahn et al. (1969) detected new rapidly labeled RNA species in the liver of the oviparous lizard prior to estrogen induced vitellinogenesis.

The nature of the new RNA species are unknown although the observations are consistent with a specific early hormone effect on mRNA synthesis. But with the large amounts of RNA synthesized having no known function, it is not possible to make a priori assumptions as to the nature of the new RNA species.

MATERIALS AND METHODS

Materials

Primiparous Sprague Dawley rats were obtained from Spartan Research Animals Incorporated, Haslett, Michigan. Uridine-5-^{3}H, L-leucine-^{14}C, L-amino acid-^{14}C-mixture, and $H_3{}^{32}PO_4$-carrier free-were obtained from New England Nuclear. Hydrocortisone acetate, insulin (bovine pancreas-26 units/mg), actinomycin D, lactalbumin hydrolyzate (tissue culture grade) and bovine liver robosomal RNA were from Mann Research Laboratories. Prolactin (ovine-B grade) and agarose (B grade) were from Calbiochem. Medium 199-Hanks base (10x) was purchased from Difco Laboratories and Medium 199-Earles base (10x) was from Baltimore Biological Laboratories. Deoxyribonuclease (bovine pancreas-electrophoretically purified) was obtained from Worthington Biochemical Corporation. Tri-iso-propylnaphthalene sulfonate and m-cresol were obtained from Eastman Organic Chemicals. Sodium lauryl sulfate was from Sigma. Acrylamide and N, N'-methylene-bis-acrylamide were prep grade and obtained from Canalco Industries.

Methods

Tissue Slices

Rats (normally 3 per experiment) were usually

sacrificed within 30 minutes after the pups were removed (if lactating). The tissue from the inguinal glands was removed as rapidly as possible and placed in cold Krebs-Ringer bicarbonate buffer (DeLuca and Cohen, 1964) containing 0.1% glucose and 0.1% lactalbumin hydrolyzate. The tissue was washed several times, cut free-hand into 10-25 mg pieces and washed several more times with buffer. The slices were quickly blotted and weighed before being transferred to the incubation flasks.

Incubation

Tissue slices were incubated in a modified Medium 199 (Table 1 and 2) at 37° under an atmosphere of 95% O_2-5% CO_2. The flasks were gently shaken (40 cycles/minute) in a Dubnoff shaker. The incubations were stopped by removing the tissue from the incubation medium and either freezing or adding 10 ml of acetone.

Total Isolation of RNA and Protein

Total RNA and protein were isolated by the methods of Mayne et al. (1966) with some modifications. The slices were washed in 10 ml of acetone, homogenized in a minimal amount of H_2O and reprecipitated with 20 ml of acetone. Two subsequent TCA (7%) washes were done before the precipitate was dissolved in 1 ml of 1 N NaOH and then precipitated twice more with TCA. The precipitate was dried by washing with ethanol, ethanol-ether (1:1) and finally

Table 1. Tissue Slice Incubation Medium for Total RNA Isolation Experiments (Medium 199-H)

Usually 350 mg of tissue slices were incubated in 12 ml of buffer under an atmosphere of 95% O_2-5% CO_2 at 37° in a shaking H_2O bath.

1	part	Medium 199-Hanks base (10x) w/o $NaHCO_3$ Appendix Table 1
9	part	Autoclaved triple distilled H_2O
0.01	M	N-2-Hydroxyethyl Piperazine-N-2-Ethane Sulfonic acid (HEPES)
0.035	M	$NaHCO_3$
0.1%		Glucose
0.15%		Lactalbumin Hydrolyzate
50	ug/ml	Penicillin G, 1585 units/mg
50	ug/ml	Streptomycin Sulfate
pH 7.4		

Table 2. Tissue Slice Incubation Medium for Phenol Extracted RNA Experiments (Medium 199-E)

One g of tissue slices was incubated in 25 ml of buffer under an atmosphere of 95% O_2-5% CO_2 at 37° in a shaking H_2O bath.

250	ml	Modified Krebs-Ringer Bicarbonate buffer
75	ml	Medium 199-Earles base (10x) w/o $NaHCO_3$
675	ml	Autoclaved triple distilled H_2O
2.2	g	$NaHCO_3$
50	ug/ml	Penicillin G, 1585 units/mg
50	ug/ml	Streptomycin Sulfate
0.15%		Glucose
0.15%		Lactalbumin Hydrolyzate

pH 7.4

Modified Krebs-Ringer Bicarbonate Buffer:

80 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 2.5 mM $MgSO_4$

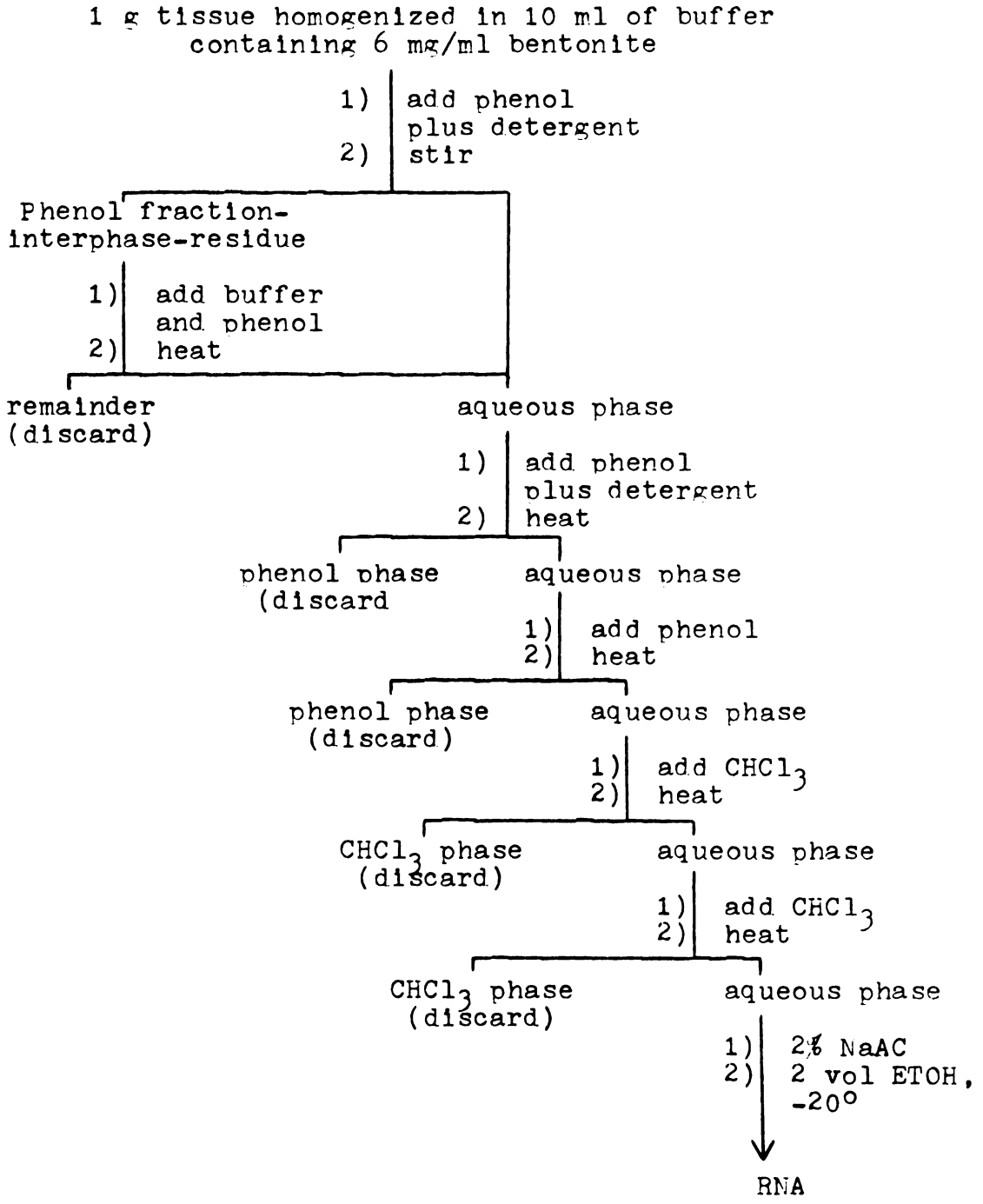
ether. The dried precipitate was dissolved in 0.5 N KOH and aliquots taken for determination of RNA, DNA, protein and radioactivity. RNA and DNA were determined by the orcinol and diphenylamine methods respectively (Schneider, 1957) and the method of Lowry et al. (1951) was used for protein determination. Radioactivity was determined with a 3 channel Packard Tri-Carb liquid scintillation counter employing the XDC scintillation solvent of Burno and Christian (1961). Efficiency (18% for ^{3}H and 39% for ^{14}C) was determined by the "spiking" technique of Okita et al. (1956) and correction was made for overlap of ^{14}C into the ^{3}H channel (12% of ^{14}C). Control experiments indicated less than 0.5% of the uridine was incorporated into the nonbase hydrolyzed (DNA) fraction, thus RNA and DNA were not separated.

Extraction of RNA

RNA was extracted by modification of the methods of Scherrer and Darnell (1962), Penman (1966) and Loening (1967). Phenol was distilled and washed with 0.001 M EDTA shortly before use. Bentonite was prepared by the method of Frankel-Conrat and Singer (1961). The homogenization buffer was 0.05 M sodium acetate 0.01 M EDTA, 0.1 M KCl at pH 6.0. This isolation scheme is shown in Figure 1.

The tissue was gently homogenized in 10 volumes of buffer containing 6 mg of purified bentonite per ml. Twelve ml of phenol containing 10% m-cresol and 2% tri-

Figure 1. Scheme for Phenol Extraction of RNA



iso-propylnaphthalene sulfonate was added and the sample stirred for 20 minutes at room temperature. The samples were cooled to 4° and the phases separated by centrifugation for 2 minutes at 8,000 x g.

Fifty mg of tri-iso-propynaphthalene sulfonate was added to the aqueous phase after it was removed. Five ml of buffer and 5 additional ml of phenol were added to the remaining phases which were then heated to 55° for 4 minutes. The phases were separated as before and the aqueous phases combined.

The combined aqueous phase was extracted twice with 12 ml of phenol at 55° for 2 minutes. The phases were separated by centrifugation at 4° for 2 minutes at 8,000 x g.

The resultant aqueous phase was extracted twice with chloroform at 55° for 2 minutes. Each time the phases were separated by centrifugation at 500 x g at room temperature for 30 seconds.

The final aqueous phase was made 2% with sodium acetate (pH 5.1) and the RNA precipitated by the addition of 2 volumes of ethanol at -20° for 8 hours. The precipitate was collected by centrifugation, dissolved in several ml of 2% sodium acetate (pH 5.1) and reprecipitated with cold ethanol. After centrifugation, the pellet was dissolved in enough gel buffer to yield an RNA concentration of 1-2 mg per ml. RNA was determined by the method of Warburg and Christian (1942) and recovery was usually 80% of expected and the 260/280 ratio was about 2.0.

Polyacrylamide Gel Electrophoresis of RNA

The procedure described is a modification of the system of Weinberg et al. (1967) and Peacock and Dingman (1968). Gels of 2.2 and 2.4% acrylamide and .6% agarose were routinely used. The gels were cast in round, high quality quartz tubes (0.5 cm I.D. x 10 cm). The gel buffer was 40 mM Tris base, 20 mM sodium acetate and 3 mM EDTA at pH 7.6. Ten ml of aceylamide solution, adequate for 4 gels, were prepared at one time.

A mixture of 60 mg of agarose, 7.5 ml of buffer and 0.5 ml of glycerol was heated until the agarose was in solution.

Two hundred and forty (or 220/mg of acrylamide, 10 mg N,N'-methylene-bis-acrylamide and 8 µl of N, N, N', N'-tetra-methylethylenediamine were dissolved in 2 ml of buffer. The temperature of the solutions was adjusted to 40° and they were mixed. Six mg of ammonium persulfate was added and the gels were cast. The gels were cooled at 4° for 2 minutes in order to assure that the agarose would set before the acrylamide polymerized.

After 1 hour of polymerization, a small layer of material which had formed above the gel proper was removed, leaving a gel 9.5 cm long, with a flat surface and little 260 mµ absorbance at the top. The gels were preelectrophoresed for 1 hour at 5 mA per tube. The electrophoresis buffer was the same as the gel buffer except it contained 0.3% sodium lauryl sulfate.

The sample (10-80 µg of RNA) containing 5% sucrose was layered on the gel and a current of 3 mA per tube was applied for 10 minutes followed by a current of 5 mA per tube for an additional 80 minutes. Some samples contained larger than normal amounts of DNA and were incubated with an equal volume of deoxyribonuclease solution (200 ug of enzyme/ml of 200 mM NaCl, 1 mM KH_2PO_4 and 3 mM $MgCl_2$ at pH 7.0) for 30 minutes at 37° immediately before being electrophorised.

The gels were scanned without being removed from the tubes at 260 mµ with a Gilford recording spectrophotometer equipped with a linear transport attachment. The area of the various peaks was measured with a plamineter. The repeatibility of the area determination and the relationship of area to amount of RNA applied to the gel is shown in Table 3.

Gels containing ^{32}P were cut into 2 mm slices and counted using a scintillation fluid of 7 g of 2,5-diphenyloxazole and 0.6 g of 1,4-bis-2-(5-phenyloxazolyl)-benzene per liter of toluene. Determination of ^{32}P in gel slices by Cerenkov radiation (suggested by L. L. Beiber) indicated very little quenching of ^{32}P by the acrylamide. Gels containing ^{3}H were counted according to the techniques of Peacock and Dingman (1967).

Specific Activity of ^{32}P

Acid soluble material for ^{32}P specific activity

Table 3. Relationship of Amount of RNA in Sample to Area of Absorbance in Various Peaks

The amount of RNA in several samples was determined by the method of Warburg and Christian (1942). The area of the peaks was determined with the use of a planineter. Several samples containing only sucrose were electrophoresed so the base line absorbance could be accurately determined.

ug of RNA in Sample	Area Under Peaks	sq cm/10 ug
30	18.1	6.03
37.5	23.3	6.21
37.5	24.5	6.53
45	27.7	6.15
48	31.1	6.47
52.5	31.0	5.90

Average 6.21 sq cm/10 ug of RNA.

determination was prepared by the method of Baldwin and Cheng (1969). The tissue was powdered under liquid nitrogen and then homogenized in 4 volumes of cold perchloric acid-ethanol-water (8:50:42). After centrifugation at 4° for 10 minutes at 35,000 x g the supernatant was removed and neutralized with 3 M K_2CO_3 in 0.5 M triethanolamine. The $KClO_4$ was removed and the supernatant analyzed for free phosphate.

The phosphate determinations were by the method of Peter (1963). Two ml of isobutanol-benzene (1:1), 4 ml of acid molybdate (1.5 g of ammonium molybdate in 100 ml of 0.5 N H_2SO_4) and 1 ml of sample were vigorously shaken for 30 seconds before the phases were separated. The absorbance of the upper layer was determined at 410 mμ. Radioactivity was measured with a Nuclear Chicago low background planchet counter at 30% efficiency.

RESULTS AND DISCUSSION

The results will be presented in two major sections. The first section will deal with development of the various systems. The optimal incubation conditions for RNA synthesis were developed and changes in the composition of the tissue between 3 days prepartum and 3 days postpartum were examined. Also in this section, characterization of the RNA by polyacrylamide gel electrophoresis and the discrepancies between RNA synthesis as assayed by phenol extraction and TCA precipitation will be discussed.

The second section will treat the effect of hormones upon RNA and protein synthesis and will also discuss the interrelationship of the several methods of assessing hormonal effects. Throughout these sections the abbreviations, I for insulin, P for prolactin, and HC for hydrocortisone acetate will be used in the tables.

Development of Systems

Development of Incubation Techniques

Preliminary experiments to define the optimal operating parameters of the systems utilized bovine as well as rat mammary tissue. Bovine tissue, which had been frozen in several different buffer systems including glycerol, never incorporated radioactive precursors as well as unfrozen

tissue which was subsequently used in all experiments.

The first experiments involved development of the buffer system necessary for maximum rate and extent of incorporation. The results presented in Table 4 indicate that both buffering capacity and metabolite levels are important in maintaining maximal levels of incorporation in bovine mammary tissue slices. The enhanced incorporation in the incubation systems containing higher buffer concentrations is evident within the first hour of incubation. The effect of the higher buffer concentration is undoubtedly a reflection of the low sodium bicarbonate level used in the Hank's base medium and the resultant inability to maintain proper pH when large amounts of very metabolically active tissue was incubated rather than a stimulatory effect of bicarbonate. The higher buffer concentration was able to maintain pH 7.4 as was the Medium 199-Earles base used in later experiments.

The inclusion of additional glucose and amino acids in the complex incubation medium had an even greater stimulatory effect on ^{3}H-uridine and ^{32}P incorporation into RNA by tissue slices from rat mammary gland (Table 5) than was observed with bovine mammary tissue. A stimulatory effect of additional amino acids and glucose was not observed with bovine tissue during the first hour of incubation but the combination of glucose and amino acids stimulated ^{3}H-uridine incorporation during the first 30 minutes in rat mammary tissue slices.

Table 4. Effect of Buffer Systems and Metabolite Concentration on ^{3}H-Uridine Incorporation into RNA by Tissue Slices from Lactating Bovine Mammary Gland

One g of bovine mammary tissue slices were incubated in 10 ml of Medium 199 (Appendix Table 1) with the indicated additions and 10 uc of uridine-5-^{3}H (24.7 C/mmole as outlined in Methods. RNA was precipitated with TCA as outlined in Methods.

Buffer System	dpm/mg RNA	
	60 Min	180 Min
Medium 199 + 35 mg $NaHCO_3$	1000	2330
Medium 199 + 350 mg $NaHCO_3$	1300	3325
Medium 199 + 35 mg $NaHCO_3$ and 0.01 M HEPES[1]	1070	1955
Medium 199 + 350 mg $NaHCO_3$ and 0.01 M HEPES	1370	2610
Medium 199 + 350 mg $NaHCO_3$-0.01 M HEPES and 0.1% glucose	1420	3135
Medium 199 + 350 mg $NaHCO_3$-0.01 M HEPES-0.1% glucose and 0.1% lactalbumin hydrolyzate	1390	4325

[1]N-2-Hydroxyethyl Piperazine-N-2-Ethane Sulfonic acid

Table 5. Effect of Added Glucose and Amino Acids on Incorporation of Precursor into RNA by Mammary Tissue Slices

a) 300 mg of mammary tissue slices from rats 72-84 hours postpartum were incubated in 5 ml of Medium 199-H (Table 1) with and without added amino acid and glucose and containing 5 μc of uridine-5-^{3}H (24.7 C/mmole. The RNA was precipitated with TCA as outlined in Methods.

	Uridine Incorporated, dpm/100 μg RNA		
Treatment	30 Min	60 Min	180 Min
Medium 199	282	487	1842
Medium 199 + 0.1% glucose + 0.1% lactalbumin hydrolyzate	732	2346	4135

b) One g of tissue from mammary glands of rats 3 days prepartum and 72-84 hours postpartum was incubated in Medium 199-E (Table 2) or Medium 199 (Appendix Table 1) with 50 μc of ^{32}P as outlined in Methods. RNA was extracted and fractionated and the specific activity of the 30S, 18S, and sRNA determined. Insulin (I), hydrocortisone (HC) and prolactin (P) were present at a concentration of 40 μg/100 mg of tissue.

Treatment	^{32}P Incorporated, cpm/10 μg RNA/12 hours
Prepartum	
Medium 199-E	3713
Medium 199	2255
Medium 199-E + HC-P-I	4518
Medium 199 + HC-P-I	2686
Postpartum	
Medium 199-E	823
Medium 199	545
Medium 199-E + HC-P-I	1166
Medium 199 + HC-P-I	667

Similarly the increased level of amino acids and glucose stimulated the incorporation of ^{32}P into phenol extracted RNA from both lactating and nonlactating slices. The data in Table 5b is biased in that the specific activity of the ^{32}P in the Medium 199-Earles base (Appendix Table 1) medium is approximately 20% lower than in the Medium 199-E (enriched medium, Table 3) but the data in Table 7 indicates that the specific activity of the tissue phosphate pool is proportional to the specific activity of the ^{32}P in the incubation medium, thus approximately 25% of the increased incorporation would be due to increased specific activity of the precursor pool. Based on this assumption, the increased amino acids and glucose caused a 50% increase in the amount of RNA synthesized. The addition of hydrocortisone, prolactin and insulin did not affect the response of the tissue to added metabolites.

Previously, Wool and Manchester (1962) had observed that amino acid incorporation by lactating mammary tissue slices was stimulated by the addition of oxidizable substrates or insulin, but the addition of oxidizable substrates prevented the insulin stimulation of incorporation which occurred when slices were incubated in minimal medium. This suggested that insulin increased the available energy supply to the tissue. Although no direct effect of glucose alone was detected, the increased level of glucose in the incubation medium was retained because of the observation

of Wool and Manchester (1962) and also because of the report that glucose (10 um/ml) prevented many of the extreme morphological modifications observed when mammary tissue was dissociated with trypsin (Pitelka et al., 1969).

The level of glucose in the enriched incubation medium in the experiments in Tables 4 and 5 is 11 um/ml while a level of 14 μm/ml as indicated in Tables 2 and 3 was used in all subsequent experiments. The glucose concentration of the buffer was 2.5 fold higher than normally observed in rat blood (Long, 1961) and 10 times the level reported for mammary tissue (Kuhn and Lowenstein, 1967; Baldwin and Cheng, 1969).

Schingoethe et al. (1967) reported that the addition of amino acids, including levels 10 times higher than normally contained in Medium 199, stimulated the synthesis of α-lactoglubulin and β-casein. The addition of 0.15% lactalbumin hydrolyzate increases the amino acid concentration of the incubation medium 2-fold (final concentration 2.2 mg/ml) compared to normal Medium 199. This is approximately 2.5-fold higher than the free amino acid level in blood (Long, 1961) but only 40% and 120% higher than the free amino acid concentration in lactating and nonlactating rat mammary tissue.

The stimulation of synthesis by added metabolites would indicate that although sufficient levels of metabolites for substantial amounts of synthesis are available, the

metabolite level of the cell may limit productivity. Baldwin and Cheng (1969) reported that the level of metabolites in prepartum mammary tissue was adequate to sustain milk synthesis but that at high levels of production, metabolite concentration may become limiting. Similarly Kuhn and Lowenstein (1967) detected all the precursors for lactose several days before lactose was detected. Thus, although metabolite level may be important in determining the level of production it would not appear to be the key to initiation of lactation. It is also possible that *in vitro* concentrations are the result of a decline in metabolite level during preparation of the tissue slices or simply that uptake is more limiting *in vitro* than *in vivo*.

Composition of Pre- and Postpartum Tissue

The relative changes in RNA, DNA and protein content of the tissue as reported in Table 6 are similar to those reported by other workers (Baldwin and Milligan, 1966; Tucker and Reece, 1963a, 1963b) but the content per 100 mg of wet tissue is 20-30% less. This is probably a reflection of the amount of buffer weighed out with the tissue but may also result from a lower RNA content of the gland. The protein content of the tissue increased 28%, the DNA content, which indicates cell number, increased 50% and the RNA content increased 125%. The RNA-DNA ratio increased 55% indicating increased synthetic activity per cell as well as an increased number of cells.

Table 6. Protein, RNA and DNA Content of Mammary Tissue of Rats 3 Days Prepartum and 3 Days Postpartum

The RNA, DNA and protein content of the TCA precipitated tissue was determined as outlined in Methods. The values are the average of 20 observations from 6 postpartum rats and 10 observations from 3 prepartum rats.

	Content per 100 mg Wet Tissue	Ratio[1]
3 Days Prepartum		
Protein	2.46 mg	3.7
RNA	87 ug	1.3
DNA	66 ug	1.0
3 Days Postpartum		
Protein	3.17 mg	3.2
RNA	193 ug	2.0
DNA	99 ug	1.0

[1]Ratio of mg of protein and ug of RNA to ug of DNA per 100 mg of tissue.

Changes in Phosphate Pool Size

The phosphate pool size in mammary tissue (Table 7) increases 50% between 3 days prepartum and 2 days postpartum which is reflected in a 30% decrease in the specific activity of the free phosphate pool after 12 hours of incubation. The difference in specific activity between the samples incubated with and without hormone is probably an indication of the experimental error of the method. The ratio in ^{32}P specific activity between the lactating and nonlactating tissue was quite repeatable (77 and 72%) in two experiments. In each experiment, the specific activity of the phosphate pool in lactating tissue was 40% of the specific activity of the buffer while in nonlactating tissue it was 60% of the specific activity calculated for the ^{32}P in the buffer.

Similar changes in pool size at the time of initiation of lactation have been reported for total nucleotides and uridine nucleotides (Wang, 1962) adenine nucleotides (Baldwin and Cheng, 1969) and amino acids (Stellwagen and Cole, 1969).

The specific activity of the intracellular phosphate pool is only 32% at one hour and 70% at 3 hours of the maximum attained at 12 hours indicating that incorporation during short periods of incubation would be low because of the low specific activity of the precursor pool. Also any effect on uptake which results in increased specific activity of the precursor pool, particularly during short term

Table 7. Specific Activity of Free Phosphate in Mammary Tissue from Lactating and Nonlactating Rats

One g of tissue was incubated as outlined in Methods. In experiment I and II 50 uc of ^{32}P was added to 25 ml of Medium 199-E (100 mg NaH_2PO_4/l) (6.4×10^6 dpm/umole) and in experiment III, 12.5 uc was added (1.4×10^6 dpm/umole). All values are the average of 2 determinations made on the same sample.

	umole Pi/g Wet Tissue	cpm/umole Pi
I 18th Day of Gestation		
1 hour	1.8	3.7×10^5
12 hours	1.6	10.5×10^5
12 hours + HC-P-I	1.7	11.7×10^5
12 hours*	1.6	7.8×10^5
12 hours + HC-P-I*	1.7	7.5×10^5
II 2nd Day of Lactation		
3 hours	3.4	5.4×10^5
12 hours	2.3	9.3×10^5
12 hours + HC-P-I	2.3	8.1×10^5
III 12 Hours of Incubation		
18th day of gestation	---	2.9×10^5
10 hours postpartum	---	2.1×10^5
48-60 hours postpartum	---	2.1×10^5

*^{32}P present only during last 3 hours of incubation.

incubations will be immediately reflected in increased incorporation into RNA, which, if changes in specific activity of the precursor pool were not accounted for, would be interpreted as increased RNA synthesis.

Several previous reports of hormonal stimulation appear to be due to increased uptake of precursor. Means and Hamilton (1966a) concluded that the early increases in uterine RNA synthesis in response to estrogen was due to increased uridine uptake and Billings et al. (1969) could detect no increased RNA synthesis in uterine tissue until 5 hours after estrogen administration if corrections were made for changes in the specific activity of the uridine pool. The changes in specific activity of the intracellular pool may be more important with ^{3}H-uridine than with ^{32}P because of the observation that uptake is the rate limiting step in the incorporation of uridine into acid soluble phosphorylated products (Plagemann and Roth, 1969).

Histological Evidence of Changes in Tissue During Incubation

Histological examination was undertaken to determine the extent to which gross morphological changes were occurring during the extended periods of incubation. Photomicrographs of the tissue are shown in Figure 2.

The tissue incubated for 9 or 12 hours without hormone exhibited more necrosis than tissue incubated with insulin, hydrocortisone and prolactin. The tissue incubated without hormone was often grossly degenerated, did not have well

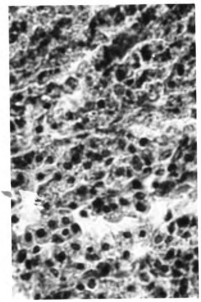
Figure 2. Photomicrograph of Lactating and Nonlactating Mammary Tissue Slices Incubated in the Presence and Absence of Hormones

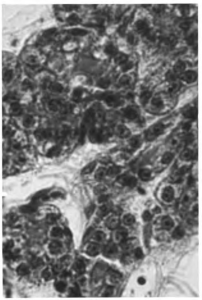
Upper, left: Tissue slice from rat mammary gland 3 days prepartum incubated 9 hours without hormones. (x400)

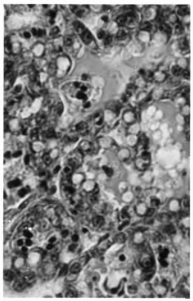
Upper, right: Tissue slice from rat mammary gland 3 days prepartum incubated 9 hours in presence of insulin, hydrocortisone and prolactin. (x400)

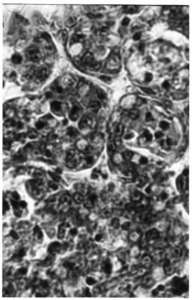
Lower, left: Tissue slice from rat mammary gland 3 days postpartum incubated 12 hours without hormone. (x400)

Lower, right: Tissue slice from rat mammary gland 3 days postpartum incubated 12 hours with insulin, hydrocortisone and prolactin. (x400)









defined alveoli, and more pycnotic nuclei were observed. More stainable material was normally observed in the lumen of tissue slices incubated with the 3 hormone combination.

Lesser amounts of stainable material were observed in the alveolar lumen of nonlactating tissue incubated 12 hours in the presence of hormones than in lactating tissue slices under similar conditions. In general, the 3 hormone combination appeared to maintain the tissue for 12 hours in a condition similar to that observed at the beginning of the incubation period.

Characterization of RNA in Pre- and Postpartum Tissue

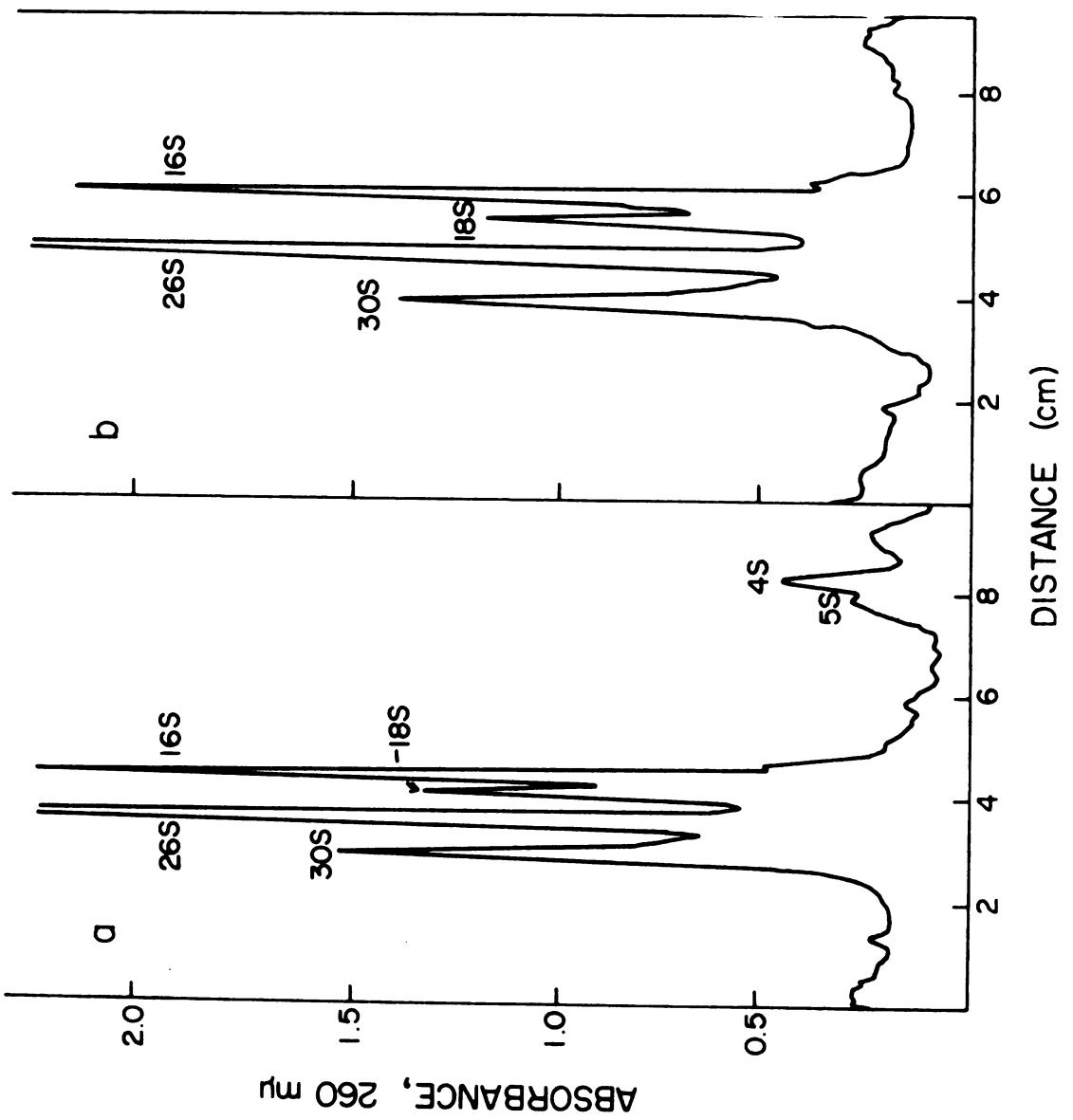
In order to investigate the possibility that specific minor fractions of RNA are synthesized in response to hormones or that the profile of RNA species synthesized changed at the time of parturition, a polyacrylamide gel electrophoresis system was developed for separating the various RNA species according to size.

Sirakov *et al.* (1968) identified a 12-15S RNA species as mRNA and Baldwin *et al.* (1969) reported that isolated mammary nuclei *in vitro* synthesized mainly RNA's of 12-15S and thus presumably mRNA. In order to ascertain that the electrophoresis system would separate a 12-15S RNA species from the 18S rRNA species, a mixture of rRNA from *Escherichia coli* (26S and 16S) and RNA from lactating mammary tissue was electrophorised. It is evident from Figure 3a that a 15S species of RNA would be readily resolved by the electrophoresis system employed. Electrophoresis for longer

Figure 3. Electrophoretic Analysis of a Mixture of E. coli Ribosomal RNA and Total RNA from Lactating Mammary Tissue

a) RNA was extracted from lactating mammary tissue as outlined in Methods. The mixture of E. coli and mammary RNA was electrophoresed for 90 min in 2-4% acryalamide - 0.6% agarose gels.

b) The same gel as shown in (a) was electrophorised for an additional 35 min.


ABSORBANCE, 260 mμ
2.0
1.5
1.0
0.5
a
26S
16S
30S
18S
4S
5S
b
26S
16S
30S
18S
2
4
6
8
DISTANCE (cm)

periods of time (Figure 3b) resolved a peak which was slightly smaller than 16S. The peaks were designated as 30S, 18S and sRNA on the basis of their migration relative to the values of Peacock and Dingman (1968). The individual peaks were identical to appropriate fractions isolated by sucrose density gradient centrifugation (Figure 4) and to liver rRNA purchased from Mann Research Laboratories.

The separation attainable with large amounts of RNA from lactating tissue is shown in Figure 5a and the effect of applying dilute samples is illustrated in Figure 5b. The same material was applied to both gels except a smaller amount of material which was 4-fold diluted was applied to the second gel. Better separation was generally achieved with small (<50 ug) concentrated (2 mg/ml) samples.

The resolution of RNA species from nonlactating tissue (Figure 6a) was normally not as good as with RNA from lactating tissue (Figure 6b) although the separation was adequate. This poorer resolution did not appear to be due to degradation of the RNA but was possibly a result of DNA contamination. The peak denoted as DNA was the only one to disappear when the sample was incubated with DNase. The peak migrating faster than sRNA was only observed in those samples previously incubated with DNase. A similar peak was observed to migrate slightly faster than sRNA, when ATP or ADP were added to the sample. Thus, material migrating faster than sRNA was normally not assayed.

Figure 4. Sucrose Density-Gradient of Mammary RNA

The sample was the total phenol extracted RNA from lactating tissue. The gradient was 5-30% (w/v) sucrose in tris-acetate buffer (0.01 M tris, 0.01 M sodium acetate, 0.14 M KCl). The volume was 12 ml and was run for 7 hours at 30,000 rpm at 17° in the SB 283 rotor of the B-60 International ultracentrifuge. Analysis was with a Gilford recording spectrophotometer equipped with a flow cell.

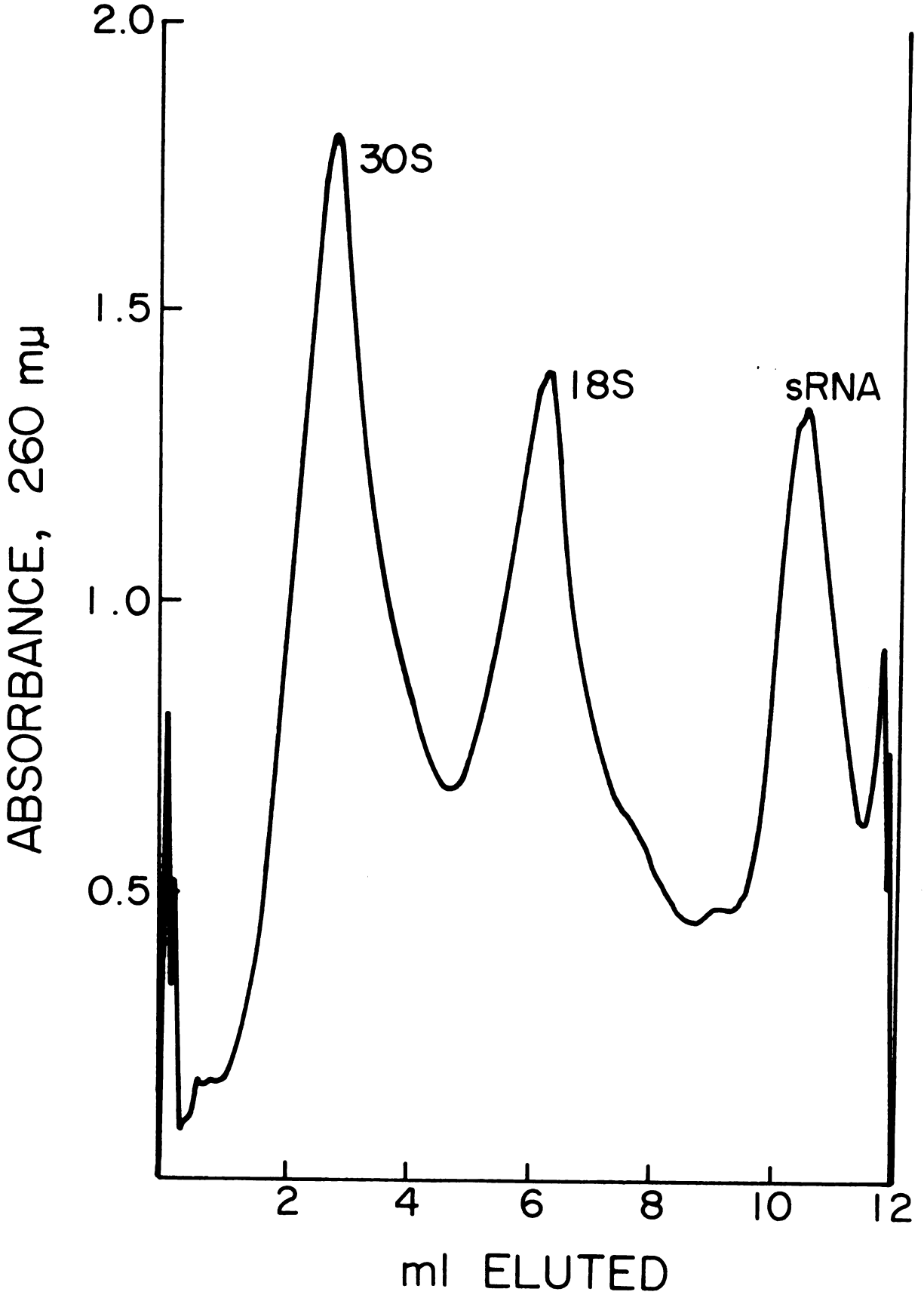

2.0
1.5
1.0
0.5
ABSORBANCE, 260 mμ
30S
18S
sRNA
2
4
6
8
10
12
ml ELUTED

Figure 5. Effect of Sample Size and RNA Concentration on Separation Attained by Electrophoresis

a) 52 ug of RNA (2 ug/ul) from lactating mammary tissue was electrophorised for 90 min at 5 ma in 2-4% acrylamide - 0.6% agarose gels.

b) 30 ug of RNA (0.7 ug/ul) from lactating mammary tissue was electrophorised for 90 min at 5 ma in 2.5% acrylamide - 0.6% agarose gels.

The dashed line is the background absorbance observed with a blank gel.

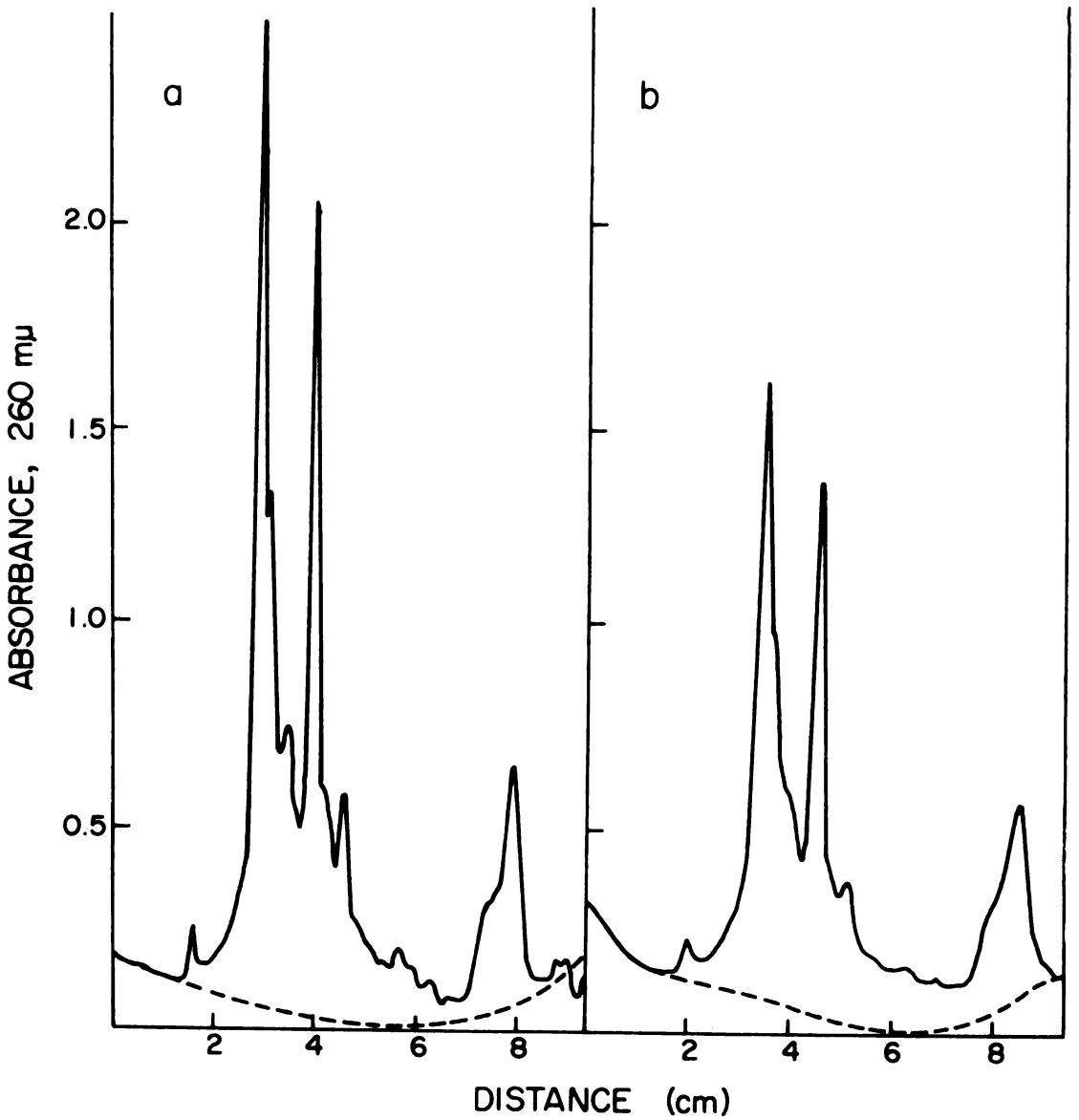
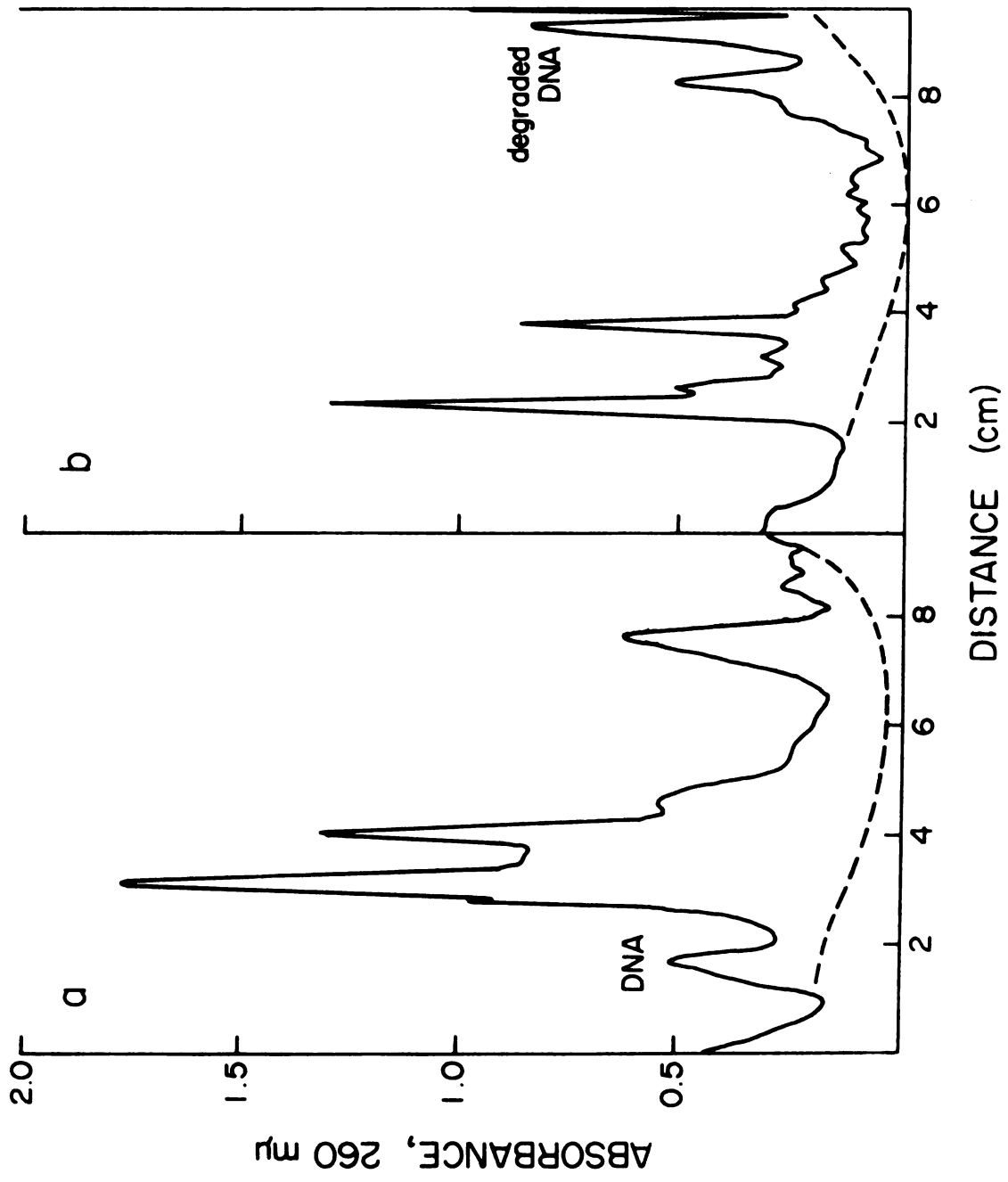

a
b
2.0
1.5
1.0
0.5
ABSORBANCE, 260 mμ
2
4
6
8
2
4
6
8
DISTANCE (cm)

Figure 6. Electrophoretic Analysis of RNA from Prepartum and 6-18 Hours Postpartum Mammary Tissue

a) 30 ug of RNA (1.5 ug/ul) extracted from mammary tissue of rats 2-3 days prepartum as outlined in Methods, was electrophorised for 90 min at 5 ma in 2.4% acrylamide - 0.6% agarose gel.

b) 15 ug of RNA (2.0 ug/ul) (incubated with DNase prior to application) from mammary tissue of rats 6-18 hours postpartum was electrophorised for 90 min at 5 ma in 2.4% acrylamide - 0.6% agarose gel.

The dashed line is the background absorbance observed with a blank gel.


a
b
DNA
degraded DNA
2.0
1.5
1.0
0.5
ABSORBANCE, 260 mμ
2
4
6
8
2
4
6
8
DISTANCE (cm)

A peak absorbing at 260 mμ always migrated faster than the 18S rRNA species and as shown in Figure 3a and 3b, it was somewhat smaller than 16S. Thus, a species with precedent for being mRNA-like can be resolved, and was observed in both pre- and postpartum mammary tissue. A similar species was also detected in RNA isolated from mammary tissue of the midpregnant mouse, (E. Cummins, personal communication).

The coincidence of the ^{3}H-uridine labeling pattern and the absorbancy profile is illustrated in Figure 7. Similar profiles were obtained when ^{32}P labeled RNA was electrophorised. There was no evidence of any radioactive material being excluded from the gel.

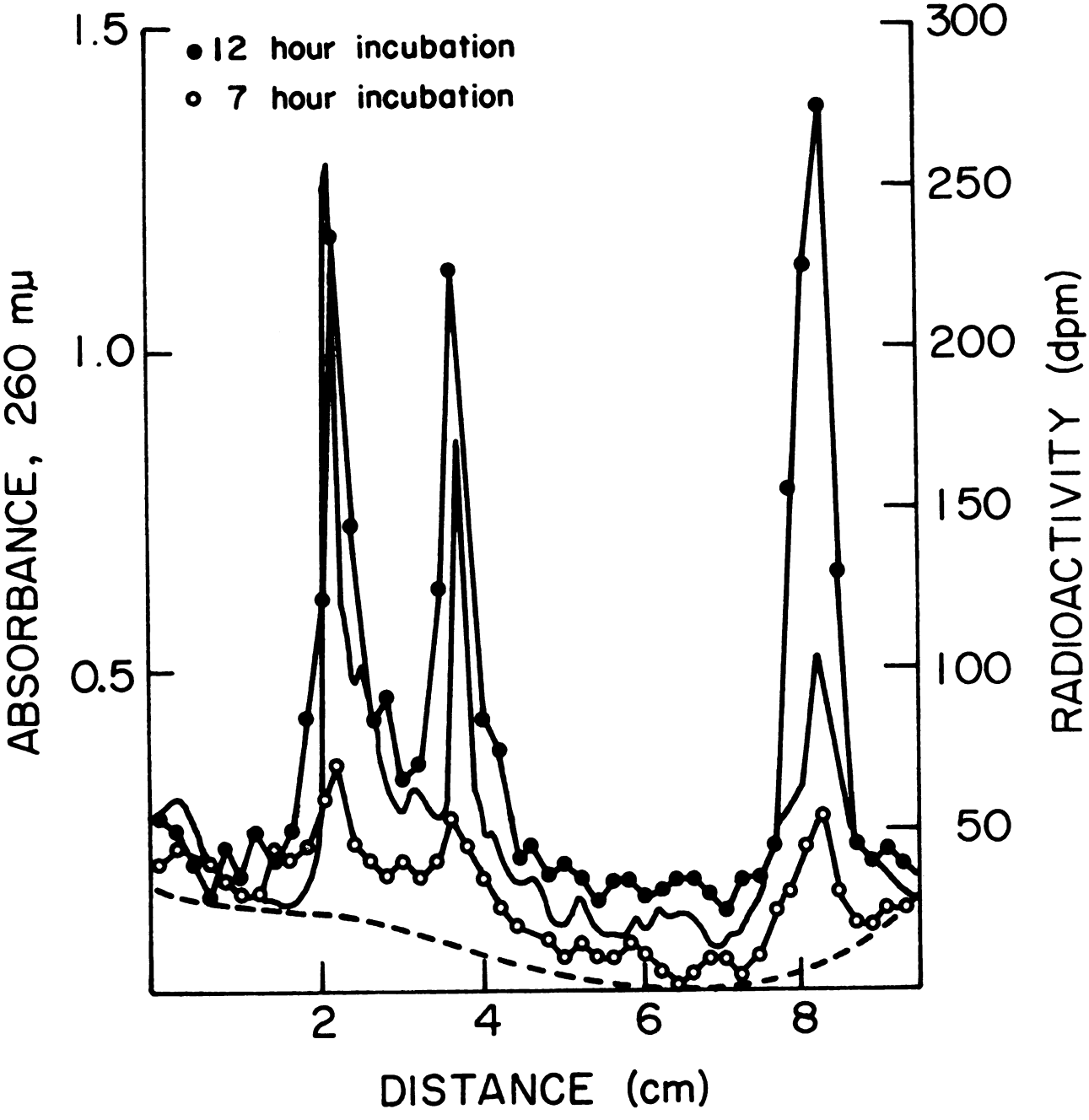
Evidence for mRNA

Two lines of evidence indicate that the 15S RNA species isolated by the techniques utilized in these experiments was not the mRNA proposed by Sirakov et al. (1968) and Baldwin et al. (1969). The amount of 15S RNA present (although because the peak was small, accurate quantitation was difficult) relative to rRNA was constant between midpregnancy and late lactation. The data of Gaye and Benamur (1969) and Mills and Topper (1970) indicated the number of polysomes increased during lactogenesis thus suggesting an increased amount of mRNA postpartum.

Secondly, as presented in Appendix Tables 3-7 no species of RNA was detected, which had a specific activity

Figure 7. Electrophoretic Analysis of RNA from Mammary Tissue of Rats 6-18 Hours Postpartum Labeled with ^{3}H-Uridine

One g of tissue slices was incubated in Medium 199-E with 50 uc of ^{3}H-uridine (Sp. Act. 24.7 C/umole) for 7 and 12 hours. The RNA was extracted as outlined in Methods. Twenty ug of RNA was electrophorised for 90 min at 5 ma in 2.2% acrylamide - 0.6% agarose gel.


● 12 hour incubation
○ 7 hour incubation
ABSORBANCE, 260 mμ
RADIOACTIVITY (dpm)
DISTANCE (cm)
1.5
1.0
0.5
300
250
200
150
100
50
2
4
6
8

higher than rRNA, with the possible exception of sRNA from prepartum tissue. This increased rate of sRNA synthesis prepartum is consistent with Turkington's (1969) observation that the amount of sRNA relative to rRNA increases during late pregnancy. Also hormones did not significantly affect the 15S RNA species in any manner distinct from the other classes of RNA as might be expected if hormones have a specific effect on mRNA synthesis. Thus, utilizing the techniques available, no species of RNA with characteristics associated with mRNA or uniquely influenced by hormones could be detected.

Discrepancies in RNA Synthesis as Assayed by Two Procedures

Two distinctly different methods were used to assess the extent and rate of incorporation of labeled precursor into RNA. The TCA precipitation method measured ^{3}H-uridine incorporation into all acid insoluble, base-labile products while the phenol extraction method measured the incorporation of ^{3}H-uridine or ^{32}P into 3 defined species of RNA which accounted for over 90% of the radioactivity observed, namely, the 30S, 18S and sRNA fractions.

The time period during which the radioactive precursor was present influenced the calculated level of synthesis (Table 8). It would have been anticipated that the calculated cummulative synthesis would have been less, rather than greater as was observed. The reason for this being, that when the isotope was present for only the last

Table 8. Effect of Length of Labeling Period Upon Amount of ^{3}H-Uridine Incorporated Into RNA by Mammary Tissue Slices

350 mg of tissue from the mammary gland of rats were incubated in Medium 199-H (Table 1) with ^{3}H-uridine[1] as outlined in Methods. The RNA was precipitated with TCA and the radioactivity determined.

	Uridine Incorporation[2] µµmole/mg RNA		
	5 Hrs	7 Hrs	10 Hrs
Prepartum			
Zero time[3]	170	232	---
Cummulative[4]	254	372	---
Postpartum			
Zero time[3]	182	226	458
Cummulative[4]	333	497	644

[1]Prepartum tissue - Uridine-5-^{3}H - 15 µc/incubation (Sp. Act. 143 uc/µmole).
Postpartum tissue - Uridine-5-^{3}H - 15 µc/incubation (Sp. Act. 125 uc/µmole).

[2]Calculations based on specific activity of uridine in the incubation medium.

[3]Isotope was added at beginning of incubation (zero time).

[4]Isotope was present only for last hour of incubation, thus isotope added at the end of 4, 6, or 9 hours of preincubation. The values were obtained by summing the series of such incubation.

hour of incubation (cummulative), the time required to establish a labeled precursor pool should have been magnified because during each hour a new precursor pool was established rather than only once as when the isotope was added at zero time. Instead these results indicate a rapid burst of ^{3}H-uridine incorporation immediately after the precursor is added. It is also possible that non-specific binding of the isotope was occurring or that large amounts of uridine were being incorporated at the end of existing polynucleotides. It does not seem to involve a decrease in the specific activity of the precursor pool during longer incubation periods either because the rate of incorporation in these experiments is quite linear. Also the calculated uptake of uridine, based upon RNA synthesis is less than 0.1% of the uridine in the medium.

The method of isolating the RNA and labeled precursor used also influenced the calculated levels of synthesis (Table 9). The TCA precipitation method indicated over 7 times more uridine was incorporated than was detected by the phenol extraction method. This would indicate that the fractions of RNA isolated by the two methods were quite dissimilar. The amount of ^{32}P incorporated into phenol extracted RNA is almost 200-fold higher than the amount of ^{3}H-uridine incorporated (assuming that RNA is 25% uridine, Baldwin and Martin, 1968a, 1968b) which is undoubtedly a

Table 9. Effect of Assay Method and Isotope Upon Calculated Amount of RNA Synthesized by Mammary Tissue Slices

Values for phenol extracted uridine are from Figure 7, for TCA precipitated uridine are from Table 8, and for phenol extracted ^{32}P are from Table 14. All calculations are based on the specific activity of the precursor in the incubation medium.

	Precursor Incorporated, umole/mg RNA During Incubation Period		
	7 Hrs	10 Hrs	12 Hrs
Phenol extracted uridine	30	---	50
Total precipitated uridine	226	458	---
Phenol extracted ^{32}P	21,000	---	39,000

reflection of the specific activity of the intracellular precursor pool.

The extent to which actinomycin D inhibits incorporation was also dependent upon the assay method (Table 10). The incorporation of ^{3}H-uridine into TCA precipitated RNA was only inhibited about 65% by actinomycin D while ^{32}P incorporation into phenol extracted RNA was almost completely inhibited in lactating tissue and in nonlactating tissue during 12 hours but not 3 hours of incubation. The lack of complete inhibition of uridine incorporation is not in agreement with the results of Mayne and Barry (1967) where actinomycin D (at slightly higher concentrations) completely inhibited incorporation.

The almost complete lack of ^{32}P incorporation into phenol extracted RNA (Tables 11 and 14) during one hour of incubation was noted. Although only one experiment is reported utilizing ^{3}H-uridine incorporation into phenol extracted RNA (Figure 7) the same lack of incorporation is noted indicating that differences in uptake of isotope would not explain all of the discrepancies between the two systems. Also, preliminary experiments indicated significant levels of ^{32}P were incorporated into TCA precipitated RNA during the first 30 and 60 minutes of incubation. This would again indicate that the fractions of RNA isolated by the two methods are not identical. Yet the recovery of RNA by the phenol extraction method was almost equal (80%) to

Table 10. Effect of Actinomycin D on Incorporation of Precursor Into RNA by Mammary Tissue Slices

a) 350 mg of tissue from mammary glands of rats 2-3 days prepartum or 3 days postpartum were incubated in Medium 199-H (Table 12) with ^{3}H-uridine[1] as outlined in Methods. The RNA was precipitated with TCA and the radioactivity determined.

Treatment	Uridine Incorporation umole/mg RNA/60 Min[2]			
	1 Hr	3 Hrs	5 Hrs	7 Hrs
Prepartum				
No addition	36	--	67	58
+ 7.1 ug actinomycin D[3]	27 (74)[4]	--	22 (33)	19 (33)
+ 4.3 ug actinomycin D	--	--	22 (33)	21 (36)
Postpartum				
No addition	48	--	69	80
+ 12 ug actinomycin D	43 (89)	30	27 (39)	27 (34)

[1]Prepartum tissue - Uridine-5-^{3}H - 15 uc/incubation (Sp. Act. 143 uc/umole)
Postpartum tissue - Uridine-5-^{3}H - 10 uc/incubation (Sp. Act. 125 uc/umole)

[2]Isotope was only present during last hour of incubation.

[3]Actinomycin D was present at the concentration/100 mg tissue as indicated (4.3 ug/100 mg = 1.2 ug/ml).

[4]% of normal.

Table 10. (Continued)

b) One g of tissue from mammary glands of rats 3 days prepartum or 72-84 hours postpartum was incubated in Medium 199-E (Table 2) with 50 uc of ^{32}P as outlined in Methods. RNA was extracted and fractionated and the specific activity of the 30S, 18S and sRNA fractions determined.

Treatment	^{32}P Incorporation cpm/10 ug RNA	
	3 Hrs	12 Hrs
Prepartum		
No addition	543	3713
+ 10 ug actinomycin D	423	80
Postpartum		
No addition	131	881
+ 10 ug actinomycin D	34	21

[1]3 hour sample was preincubated for 1 hour with actinomycin D before ^{32}P was added.

[2]Actinomycin D was present at a concentration of 10 ug/100 mg tissue (4 ug/ml).

the total precipitated RNA and the recovery of phenol extracted material would probably be higher except for the relatively large losses (percentage) due to small sample size. Thus, the difference must be due to a small fraction of RNA which turns over very rapidly.

In order to ascertain that the difference observed between the short term ^{3}H-uridine incorporation experiments and the longer labeling periods with ^{32}P were not associated with the fractionation of the RNA species on acrylamide gel an isolation precedure involving phenol extraction followed by TCA precipitation was used (Table 11). The RNA extracted by this procedure was comparable to the fraction I-RNA (rRNA) of Baldwin and Martin (1968a, 1968b). The small amount of tissue used in these experiments prevented accurate measurements of the specific activity of the fraction II-RNA although generally it was similar to the specific activity of the fraction I-RNA. The lack of incorporation during the first hour is in agreement with the data obtained when the phenol extracted RNA was fractionated by electrophoresis. This would indicate that the isotope incorporated during a one hour labeling period is predominatedly into an RNA fraction which is not isolated by the two phenol extraction methods employed.

The discrepancies can be explained by the observations of Perry (1962) that most of the radioactive precursor incorporated into RNA during short incubation periods is retained

Table 11. ^{32}P Incorporation Into Phenol Extracted RNA by Tissue Slices from Mammary Glands of Rats 2 Days Postpartum

400 mg of tissue from mammary gland of rats 3 days prepartum were incubated in Medium 199-H with 50 uc of ^{32}P as outlined in Methods. RNA was twice extracted at room temperature with phenol containing 1% sodium deoxycholate and then precipitated with TCA as outlined in Methods. Insulin (I), prolactin (P) and hydrocortisone acetate (HC) were present at a concentration of 40 ug/100 mg[1] of tissue.

	^{32}P Incorporation cpm/100 ug RNA
1 hour	0
3 hours	336
5 hours	912
7 hours	1724
10 hours	2137
1 hour + I	66
5 hours + I	895
10 hours + I	2129
1 hour + HC-P-I	30
5 hours + HC-P-I	1002
10 hours + HC-P-I	2989

[1]13.5 ug/ml.

in the nucleus and that a large portion of this incorporation is into a small class of RNA (HnRNA) (1% of total RNA) which is degraded within the nucleus with a half life of less than 3 minutes (Soerio et al., 1968). Thus it would seem that the ^{3}H-uridine incorporated into TCA precipitated RNA was predominately into HnRNA while ^{32}P incorporation into phenol extracted RNA was predominately a measure of cytoplasmic RNA synthesis.

Calculations suggest that greater than 90% of the isotope incorporated into RNA does not enter the cytoplasm and at the present time has no known function (Church and McCarthy, 1967; Aronson and Wilt, 1969). This is not to indicate that it has no function nor that the incorporation is not meaningful but until it can be shown that this synthesis is reflected in changes within the cytoplasm, these types of studies will be difficult to interpret. Thus, if most of the precursor was incorporated into the HnRNA fraction during short incubation periods and the HnRNA was not isolated by the phenol extraction method, this could explain the discrepancies between the two systems.

Results of Hormone Treatment

Effect of Hormones Upon RNA Synthesis

The results of experiments to ascertain the effect of short term incubation in the presence of hormone upon ^{3}H-uridine incorporation are presented in Table 12. All of

Table 12. Effect of Various Hormones Upon ^{3}H-Uridine Incorporation Into TCA Precipitated RNA by Tissue Slices From Mammary Glands of Rats

400 mg of tissue from the mammary glands of rats 3 days postpartum was incubated in Medium 199-H (Table 1) with 10 uc of Uridine-5-^{3}H (Sp. Act. 153 uc/umole) as outlined in Methods. The RNA was precipitated with TCA and the radioactivity determined. Insulin (I), hydrocortisone acetate (HC) and prolactin (P) were present at a level of 25 ug/100 mg of tissue (9 ug/ml).

	Expressed as % of ^{3}H-Uridine Incorporated by Slices Incubated Without Hormone[1]	
Treatment	60 Min	180 Min
No hormone[2]	100	100
+ I	116	105
+ HC	108	74
+ P	128	80
+ HC-I	112	110
+ P-I	115	155
+ HC-P-I	146	106

[1] $\frac{\text{dpm/mg RNA + hormone}}{\text{dpm/mg RNA - hormone}}$ x 100, average of 3-6 observations.

[2] 100% = 25 uumole/mg RNA during 1 hour and 80 uumole/mg RNA during 3 hours.

the hormones stimulated ^{3}H-uridine incorporation during the first 60 minutes but only the combinations containing insulin were stimulatory during 3 hours of incubation. The very large variations in incorporation, as were also observed by Mayne et al. (1966), made interpretation of the results qualitative at best.

In an attempt to compensate for the variability during short term experiments, the incubation period was extended to 10 or 12 hours. The uridine incorporation during the last hour of these incubations is presented in Table 13. Irrespective of the hormonal conditions, lactating tissue slices incorporated less ^{3}H-uridine during the last hour of a 10 hour incubation than during the last hour of a 7 hour incubation. Insulin was the only hormone treatment which was as effective as no hormone in lactating tissue while in nonlactating tissue, insulin alone, the 3 hormone combination, and prolactin plus twice the level of insulin and hydrocortisone stimulated uridine incorporation when compared to the no hormone control. The incorporation by lactating tissue slices incubated with insulin alone decreased between 5 and 7 hours and a significant decrease in incorporation between 5 and 7 hours was also observed in nonlactating tissue slices incubated with insulin or the 3 hormone combination indicating that tissue slices with the most synthetic activity during early incubation periods were unable to maintain that rate during extended incubation.

Table 13. Effect of Various Hormones Upon ^{3}H-Uridine Incorporation Into TCA Precipitated RNA by Mammary Tissue Slices

350 mg of tissue from the mammary glands of rats 2-3 days prepartum or 3 days postpartum was incubated in Medium 199-H (Table 1) with ^{3}H-Uridine[1] as outlined in Methods. The RNA was precipitated with TCA and the radioactivity determined. Insulin (I), hydrocortisone acetate (HC) and prolactin (P) were present at a basal level of 50 ug/100 mg of tissue (15 ug/ml).

	Uridine Incorporation uumoles/mg RNA/60 Min[2]				
Treatment	1 Hr	3 Hrs	5 Hrs	7 Hrs	10 Hrs[3]
Prepartum[4]					
No hormone	35	--	59	54	--
+ I	--	--	88	68	--
+ P	--	--	57	55	--
+ P-I	--	--	51	62	--
+ P-HC	--	--	31	35	--
+ P-HC-I	35	--	73	62	--
+ 2x(HC-I)P	--	--	82	79	--
Postpartum[5]					
No hormone	51	72	75	84	70
+ I	47	70	95	85	71
+ HC	--	--	74	71	63
+ P-I	--	--	62	74	63
+ HC-P-I	50	73	75	72	59

[1]Prepartum tissue - Uridine-5-^{3}H - 15 uc/incubation (Sp. Act. 143 uc/umole).
Postpartum tissue - Uridine-5-^{3}H - 10 uc/incubation (Sp. Act. 125 uc/umole).

[2]Calculations were based on specific activity of uridine in incubation Medium.

[3]Isotope was present only during last 60 minutes of incubation.

[4]Data was from only 1 experiment.

[5]Data is average of 2 or 3 experiments.

The combination of prolactin and hydrocortisone without insulin inhibits uridine incorporation 30-50% in nonlactating tissue.

The effect of hormones upon ^{32}P incorporation into phenol extracted RNA is summarized in Table 14. The incorporation into various fractions of RNA is presented in Appendix Tables 3-7. Instead of being present for only the last hour of incubation as was the ^{3}H-uridine, ^{32}P was present during the entire incubation period. Contrary to the results obtained with ^{3}H-uridine incorporation, several hormone combinations stimulated ^{32}P incorporation into RNA when compared to the no hormone control. Generally differences of less than 10% were considered insignificant because of variation in the specific activity of the precursor pool and also errors in measuring the area of the various peaks and in slicing the gels. Some liberty was taken with these criteria when small but consistent differences were evident in several observations.

Although only three direct measurements were made, insulin appears to stimulate ^{32}P incorporation, both by itself and in conjunction with other hormones. The stimulation by insulin alone was about 10% while generally the stimulation by the 3 hormone combination was 20-50%. This indicates that at least one of the other two hormones had a stimulatory effect in the presence of insulin. Although only a limited number of observations were made; prolactin

Table 14. Effect of Various Hormones Upon ^{32}P Incorporation Into Phenol Extracted RNA by Mammary Tissue Slices

One g of tissue from mammary glands of rats 2-3 days prepartum or 6-18 or 72-84 hours postpartum was incubated in Medium 199-E 9Table 2) with 50 µc of ^{32}P except in experiment prepartum I when 30 µc was used, as outlined in Methods. RNA was extracted, fractionated and the specific activity of the 30S, 18S and sRNA determined. Insulin (I), hydrocortisone acetate (HC) and prolactin (P) were present at a level of 40 ug/100 mg of tissue (15 ug/ml).

	^{32}P Incorporation cpm/10 ug RNA					
Treatment	1 Hr	3 Hrs	5 Hrs	7 Hrs	10 Hrs	12 Hrs
3 Days Prepartum						
No hormone	105	475	868	---	1841	----
+ HC-P-I	---	470	858	---	2153	----
+ HC-I	212	759	---	---	2270	----
+ P-I	---	435	---	---	3496	----
2 Days Prepartum						
No hormone	---	---	---	---	----	3713
+ HC-P-I	---	---	---	---	----	4518
+ HC-I	---	---	---	---	----	3664
6-18 Hours Postpartum I						
No hormone	---	---	---	1355	----	2316
+ HC-P-I	---	---	---	1844	----	2646

6-18 Hours Postpartum II						
No hormone	---	---	431	---	----	2396
+ HC-P-I	---	---	---	---	----	3268
+ I[1]	---	---	479	---	----	----
72-84 Hours Postpartum I						
No hormone	0	131	198	---	881	----
+ HC-P-I	20	115	339	---	----	----
+ I	---	107	---	---	1091	----
+ P-I	---	190	---	---	----	----
+ HC-I	10	---	---	---	1224	----
48-60 Hours Postpartum II						
No hormone	---	70	249	---	----	819
+ HC-P-I	---	---	506	---	----	1162
+ HC-I	---	---	---	---	----	1178
+ P	---	---	---	---	----	753
No hormone[2]	---	115	---	---	----	----
+ HC-P-I[2]	---	139	---	---	----	----

[1]Isotope was present only during last 5 hours of incubation.

[2]Isotope was present only during last 3 hours of incubation.

plus insulin were as active as the 3 hormone combination at longer incubation times with prepartum tissue, while at shorter times hydrocortisone and insulin stimulate synthesis. In postpartum tissue the combination of hydrocortisone and insulin were as stimulatory as the 3 hormone combination and enhanced incorporation to a greater extent than insulin alone. The 3 hormone combination stimulated ^{32}P incorporation in all tissues examined, the enhanced rate of incorporation being detected at 5 hours in the lactating tissue but not until 10 hours in the nonlactating tissue. It is possible that this delay was due to the time required for cell division but it is more likely a reflection of the time required to deplete the endogenous hormone pool of the cell. The enhanced rate of synthesis in the presence of the 3 hormone combination was also observed if the sample was preincubated for 9 hours and the isotope was present for only the last 3 hours.

Role of Hormones in Regulating Amino Acid Incorporation in Tissue Slices

Amino acid incorporation by tissue slices from prepartum mammary tissue measured during the last hour of incubation (after preincubation for various lengths of time) is enhanced by insulin alone or insulin plus prolactin and hydrocortisone (Table 15). Prolactin plus insulin was the only treatment which caused increased incorporation of amino acids during the 7th hour of incubation relative to

Table 15. Effect of Various Hormones Upon ^{14}C-Amino Acid Incorporation Into TCA Precipitated Protein by Mammary Tissue Slices

350 mg of tissue from the mammary glands of rats 2-3 days prepartum or 3 days postpartum was incubated in Medium 199-H (Table 1) with 1 μc of ^{14}C-amino acid mixture (Sp. Act. approximately 0.5 uc/μg in each experiment) as outlined in Methods. The protein was precipitated with TCA and the radioactivity determined. Insulin (I), hydrocortisone acetate (HC) and prolactin (P) were present at a basal level of 50 ug/100 mg of tissue (15 ug/ml).

	Amino Acid Incorporation, ug[1]/mg Protein/60 Min[2]				
Treatment	1 Hr	3 Hrs	5 Hrs	7 Hrs	10 Hrs
Prepartum[3]					
No hormone	14	--	25	21	--
+ I	--	--	25	25	--
+ P	--	--	21	20	--
+ P-I	--	--	22	26	--
+ P-HC	--	--	15	19	--
+ HC-I-P	12	--	25	25	--
+ 2x(HC-I)P	--	--	28	25	--
Postpartum[4]					
No hormone	18	21	28	35	32
+ I	18	32	32	35	28
+ HC-I	--	--	27	34	27
+ P-I	--	--	23	28	29
+ HC-P-I	18	24	25	24	20

[1]Calculations are based on specific activity of amino acid incubation medium.

[2]Isotope was present only during last 60 minutes of incubation.

[3]Data is from only 1 experiment.

[4]Data is average of 2 or 3 experiments.

the incorporation during the 5th hour of incubation. This was also observed when uridine incorporation in prepartum tissue was measured indicating that the response by prepartum tissue to prolactin may be delayed.

Only insulin alone stimulated amino acid incorporation in lactating tissue slices and then only at 3 and 5 hours of incubation (Table 15). Again prolactin plus insulin stimulated amino acid incorporation 10 hours relative to the rate of incorporation during shorter periods of incubation. In no case was prolactin plus insulin more active than insulin alone, thus, indicating that the response was a reflection of lower levels of incorporation during shorter times of incubation.

Evidence that all of the hormone stimulation was not mediated via RNA synthesis was indicated in experiments involving actinomycin D inhibition of protein synthesis. As previous discussed RNA synthesis was inhibited 70-100% by actinomycin D depending upon the assay system and the extent of inhibition was not influenced by hormones. The results presented in Table 16 indicate that protein synthesis is also inhibited by actinomycin D, but in nonlactating tissue, hormones retarded the inhibitory effect of actinomycin D. The 3 hormone combination enhanced amino acid incorporation in the presence of actinomycin D approximately 30% compared to the no hormone sample with actinomycin D. No effect of hormone in the presence of actinomycin D

Table 16. Effect of Actinomycin D on ^{14}C-Amino Acid Incorporation Into TCA Precipitated Protein by Mammary Tissue Slices

350 mg of tissue from the mammary glands of rats 2-3 days prepartum or 3 days postpartum was incubated in Medium 199-H (Table 1) with 1 uc of ^{14}C-amino acid mixture (Sp. Act. approximately 0.5 uc/ug in each experiment) as outlined in Methods. The protein was precipitated with TCA and the radioactivity determined. Insulin (I), hydrocortisone acetate (HC) and prolactin (P) were present at a basal level of 50 ug/100 mg of tissue (15 ug/ml).

	Amino Acid Incorporation ug/mg Protein/60 Min[1]			
Treatment	1 Hr	3 Hrs	5 Hrs	7 Hrs
Prepartum				
No hormone	14	--	25	21
+ HC-P-I	12	--	25	25
+ 7.1 actinomycin D[2]	11 (78)[3]	--	9 (33)	6 (28)
+ 7.1 actinomycin D + HC-P-I	12 (97)	--	12 (49)	9 (36)
+ 4.3 actinomycin D	--	--	8 (33)	8 (36)
+ 4.3 actinomycin D + HC-P-I	--	--	12 (47)	12 (48)
Postpartum				
No hormone	16	22	25	32
+ 12 ug actinomycin D	20	14 (65)	10 (40)	6 (17)

[1]Isotope was present only during last hour of incubation.

[2]Actinomycin D was present at the concentration/100 mg tissue as indicated (4.3 ug/100 mg = 1.2 ug/ml).

[3]% of normal.

was observed with tissue from postpartum rats. Since RNA synthesis did not appear to be required for this hormonal stimulation, some degree of hormonal regulation at a non-transcriptional level must occur. Several possibilities for control exist. Namely, increased uptake of precursor, increased translation of stable mRNA, decreased rate of RNA turnover (degradation) or possibly some combination of these events. Mayne et al. (1966) observed a similar stimulation of the same order of magnitude when mammary tissue slices from prepartum rats were incubated with insulin and actinomycin D.

The inhibition of protein synthesis by actinomycin D indicates that a species of RNA (presumably mRNA) is turning over quite rapidly. This species of RNA turns over faster in lactating tissue, where amino acid incorporation is inhibited 83% after 6 hours preincubation, than in non-lactating tissue where amino acid incorporation is inhibited only 60-70%. This increased inhibition by actinomycin D would indicate that mRNA has a relatively short half life and the mRNA for the milk proteins may be degraded faster than mRNA for cellular proteins. This inhibition data contradicts the conclusions of Sirakov et al. (1966) that mRNA turns over very slowly in the cytoplasm of lactating mammary tissue. The extent of inhibition of protein synthesis is similar to that reported by Mayne et al. (1966) and Stockdale et al. (1966).

Integration of Results from Several Methods of Assessing Hormonal Effects on Mammary Tissue Slices

The results of histological examination of both pre- and postpartum tissue indicated that during 9-12 hours of incubation, hormones were necessary for maintenance of cellular integrity. The specific hormone combination necessary for maintenance was not ascertained because only the 3 hormone combination was compared to no hormone at all. Similar results were obtained if the incorporation of labeled precursor during 5-12 hours of incubation into phenol extracted RNA was measured. When incorporation of ^{3}H-uridine or ^{14}C-amino acids was measured during the last hour of 5, 7 and 10 hours of incubation, only in nonlactating tissue were hormone combinations containing insulin able to stimulate synthesis. In the same type of experiment with lactating tissue slices insulin alone was most effective in maintaining uridine incorporation at a level comparable to the no hormone control. Insulin alone stimulated amino acid incorporation by lactating tissue slices at 3 and 5 hours but could not maintain incorporation at a level comparable to the no hormone control at 10 hours.

At least two possible explanations occur for the seemingly paradoxical inhibition of RNA and protein synthesis by hormones which are necessary for preventing necrosis of the tissue. Haddad *et al.* (1969) have reported that increased RNA synthesis occurred in degenerated nerve tissue compared to normal nerve tissue. Also isolated

tadpole tail pieces undergoing regression incorporated more amino acids into protein and uridine into RNA than similar tail slices prior to metamorphosis (Tata, 1966) but a decreased incorporation of leucine and no change in uridine incorporation were observed in in vivo experiments (Tonoue and Frieden, 1970). Thus it would seem that in vitro, degenerating tissue incorporates more precursor into RNA and protein than does normal tissue. This could be a mechanism to retard or repair degeneration or possibly the permeability of the cell membrane is increased and the only change is in the specific activity of the precursor pool and this does not occur in vivo. The other possibility is that the rate of synthesis and accumulation of secretory products in hormone treated tissue is sufficient to either inhibit uptake of precursor or decrease the rate of synthesis as is observed in vivo when intramammary pressure increases. The incorporation of uridine and amino acids into TCA precipitated products, because it was a short term incubation, would be very responsive to these influences. Thus the data in Tables 13 and 15 are probably a reflection of hormonal stimulation, state of degeneration and synthetic activity of the tissue. The incorporation when the isotope was present for longer periods of time would be less susceptible to the latter two influences because the precursor pool is probably well established before degeneration and synthetic activity become important

and also the rate of uptake of precursor is somewhat less important in the longer term experiments.

Thus, ^{32}P incorporation into phenol extracted RNA is probably a better measure of hormone influence on RNA synthesis than is the measurement of ^{3}H-uridine incorporation into TCA precipitated RNA.

Calculation of Amount of RNA Synthesized by Mammary Tissue Slices

The amount of ^{32}P incorporated by tissue slices *in vitro* into RNA per mg of tissue is greater in postpartum tissue than in prepartum tissue when corrections are made for the differences in specific activity of the precursor pool and the increased RNA content (2.2-fold) of the tissue. The incorporation per mg of DNA is still greater prepartum even when these corrections are made.

The calculations based on the measured specific activity of the isolated RNA, the specific activity of the precursor pool and the RNA content of the tissue indicate 100% more RNA/gland is synthesized postpartum than prepartum (Table 17). Similar calculations of ^{3}H-uridine incorporation indicated about 2.5 times more RNA was synthesized per g of lactating tissue than per g of nonlactating tissue if no corrections are made for changes in pool size. Sirakov and Rychlik (1968) reported that the specific activity of the isolated RNA was higher from prepartum tissue than from postpartum tissue as was also observed in these experiments

Table 17. Calculation of Amount of RNA Synthesized by Lactating and Nonlactating Mammary Tissue

18th Day of Gestation

$$\frac{300 \text{ cpm}}{10 \text{ ug RNA/hr}} \div \frac{3.5 \times 10^6 \text{ dpm}}{\text{umole Pi}} = \frac{86 \text{ uumole Pi incorporated}}{10 \text{ ug RNA/hr}}$$

$$\frac{87 \text{ ug RNA}}{100 \text{ mg tissue}} \times \frac{5 \text{ g mammary tissue}}{\text{rat}} \times \frac{86 \text{ uumole Pi incorporated}}{10 \text{ ug RNA/hr}}$$

$$= .0374 \text{ umole Pi incorporated/gland/hr}$$

$$\frac{.0374 \text{ umole Pi}}{\text{gland/hr}} \times 339 \text{ (average MW of nucleotide monophosphate)}$$

$$= 12.7 \text{ g ug RNA synthesized/gland/hr}$$

3rd Day of Lactation

$$\frac{120 \text{ cpm}}{10 \text{ ug RNA/hr}} \div \frac{2.5 \times 10^6 \text{ cpm}}{\text{umole Pi}} = \frac{48 \text{ uumole Pi incorporated}}{10 \text{ ug RNA/hr}}$$

$$\frac{193 \text{ ug RNA}}{100 \text{ mg tissue}} \times \frac{8 \text{ g mammary tissue}}{\text{rat}} \times \frac{48 \text{ uumole Pi incorporated}}{10 \text{ ug RNA/hr}}$$

$$= .0741 \text{ umoles Pi incorporated/gland/hr}$$

$$\frac{.0741 \text{ umole Pi}}{\text{gland/hr}} \times 339 \text{ (average MW of nucleotide monophosphate)}$$

$$= 25.1 \text{ ug RNA synthesized/gland/hr}$$

if changes in precursor pool size and RNA content were not taken into account. The level of incorporation in their mouse mammary tissue slice system was about 1% of the level reported in Table 10. Wang (1962) reported the uridine nucleotide pool doubled at the time of the initiation of lactation. If this is reflected in a decreased specific activity of the precursor pool, the RNA synthesis per g of tissue by lactating tissue could be 5 times higher than in nonlactating tissue. The calculations outlined in Table 18 suggest that differences in the amount of RNA synthesized per mammary gland between prepartum and postpartum tissue may account for about 5% of the increase in RNA content of the gland although it could be probably 2-fold higher if the time required for the extracellular ^{32}P to establish an intracellular precursor pool is accounted for. Also the lower amount of RNA/g of tissue (note Table 6) would also result in lower values, thus the calculated level of synthesis could possibly account for 25% of the increased RNA of the mammary tissue. The discrepancy between 25% and 100% would indicate that either *in vitro* synthesis is not an accurate measure of *in vivo* synthesis or the rate of RNA degradation *in vivo* decreases at the time of parturition. It is still impossible to separate the increased RNA content from the increases in DNA and tissue because they all increased, although to differing extents, during the initiation of lactation.

Table 18. Relation of RNA Synthesis to the Increased RNA Content in Postpartum Tissue

3rd day of lactation	15.7 mg RNA/rat mammary gland
18th day of gestation	4.4 mg RNA/rat mammary gland
	11.3 mg increase in RNA
3rd day of lactation	25.1 ug RNA synthesized/gland/hr
18th day of gestation	12.7 ug RNA synthesized/gland/hr
	12.4 ug more RNA synthesized/hr 3 days postpartum

1) Assume there was an 156 hour time interval between measurements. Also, assume that there was no change in amount of RNA degraded/hour, and the rate of increase was constant throughout interval.

2) $\frac{12.4 \text{ ug more RNA synthesized}}{2 \text{ (for average increase)}} \times 156 \text{ hours} = .97 \text{ mg increase in RNA}$

3) $\frac{.97 \text{ mg}}{11.3 \text{ mg}} = 8.6\%$ of increase could come from increased rate of synthesis.

Calculation of Amount of Protein Synthesis

Calculations pretaining to the amount of amino acids incorporated into protein are in Table 19. Assuming a 3 day postpartum rat secretes 10 ml of 12% protein milk (1.2 g of protein/day), the minimum rate of synthesis in tissue slices is 20% of the *in vivo* rate. Any difference in specific activity between the free amino acid pool and the amino acids in the medium would increase the calculated amount of synthesis. Although the calculations on amount of synthesis are approximations, they indicated the tissue slices were very active during the period of incubation.

Table 19. Calculation of Amount of Protein Synthesized by Tissue Slices from Mammary Gland of Rats 3 Days Prepartum or 3 Days Postpartum

3 Days Postpartum

1) Amino acids incorporated = 35 ug/mg protein[1]

2) 3.17 mg protein/100 mg wet tissue

3) Thus 106 ug protein synthesized/100 mg wet tissue/hour

4) Assume mammary tissue of rat weighs 8 g

5) Thus 8.5 mg of protein produced/rat/hour = 204 mg of protein synthesized/day

3 Days Prepartum

1) Amino acids incorporated = 25 ug/mg protein[1]

2) 2.46 mg protein/100 wet tissue

3) 62 ug protein synthesized/100 wet tissue/hour

[1]Calculation based on assumption that specific activity of free amino acid pool in tissue is same as specific activity of amino acids in incubation Medium, thus calculation is minimum value.

DISCUSSION

The results of this study which attempted to dissociate the process of cell division from initiation of lactation are consistent with other observations concerning a role for hormones during lactogenesis.

The stimulation observed when the glucose and amino acid concentrations in the medium were increased would indicate that energy metabolism or general metabolite levels may have a role in controlling synthetic processes. The rather general effect of the combination of all 3 hormones in enhancing uridine incorporation during the first 3 hours of incubation as well as the stimulatory effect of insulin alone and the necessity for insulin to be present for other hormones to act could be the result of a general increase in metabolism and/or membrane permeability. Also the enhancement of amino acid incorporation by the 3 hormones in the presence of actinomycin D presumably occurs without RNA synthesis, and thus must reflect either increased uptake or increased metabolism. Mayne et al. (1966) reported that insulin alone had this same effect. Insulin has been shown to stimulate uptake and metabolism (Mayne and Barry, 1970; Louis and Williams, 1970) which would be compatible with it being stimulatory in the presence of actinomycin D.

These data would be consistent with the suggestion

of Linzell (1970) that although increased blood flow does not cause the initiation of lactation, it is associated with it and may increase the flow of metabolites necessary for milk synthesis to the gland immediately after parturition. The stimulation of synthesis by increasing metabolites would be compatible with the suggestion of Baldwin and Cheng (1969) that metabolites may be limiting synthesis at high levels of milk production. All of these observations would be consistent with a stimulation of synthesis at the level of translation rather than transcription during lactogenesis.

More definitive conclusions concerning the role of hormones can be reached by examination of the synthesis of RNA species isolated by phenol extraction and electrophoresis. The longer time period of incorporation would mean a larger portion of the labeled RNA would be located in the cytoplasm while RNA isolated from samples incubated shorter times would give information about rapidly labeled RNA.

Prolactin and insulin are effective in stimulating ^{32}P incorporation into the 30S, 18S and sRNA fractions isolated from mammary tissue of rats 2 days prepartum while hydrocortisone, except possibly during short term incubation, was without effect as also reported by Mayne and Barry (1970). Hydrocortisone and insulin were most effective in stimulating RNA synthesis in lactating tissue slices. The addition of prolactin did not enhance incorporation.

The stimulatory effect of hydrocortisone is consistent with the report of Talwaker et al. (1961) that hydrocortisone administration to lactating rats increased milk secretion. These combinations were also the most effective in maintaining amino acid incorporation into protein in the respective tissue.

The major species of RNA appear to turnover at similar rates in lactating tissue while the specific activity of the sRNA is higher than the specific activity of the rRNA in mammary tissue prepartum. This is consistent with increasing sRNA relative to rRNA during late pregnancy as was observed by Turkington (1969).

The inability to detect any species of RNA which was rapidly labeled relative to rRNA, even at short time intervals, was disturbing expecially since the reports of Sirakov et al. (1968) and Baldwin et al. (1970) of RNAs with characteristics ascribed to mRNA being isolated from mammary tissue and the suggestion of Baldwin and Martin (1969a, 1969b) that hydrocortisone stimulates synthesis of specific RNA species. The few times when high specific activity species were observed in the 12-15S region it appeared to reflect the problem of accurately determining the area of the small peak rather than extremely high levels of radioactivity. Actually the calculations of Soeiro et al. (1969) indicate that the rate of synthesis of mRNA in HeLa cells, with a mRNA half life of 3 hours, would only be one sixth

the rate of synthesis of rRNA indicating that specific activity rather than rate of synthesis measurements are necessary for detecting rapidly labeled RNA species. Also calculations indicate that over 75% of the ^{32}P incorporated during the first 20 minutes of incubation of HeLa cells is into HnRNA, thus if the rates of RNA synthesis in mammary nuclei are similar to HeLa cells, less than 10% of the RNA synthesized in the studies of Baldwin et al. (1969) could have been mRNA. Similarly the observations of Sirakov et al. (1968) that much of the rapidly labeled material they called mRNA did not leave the nucleus would suggest it was actually HnRNA. The inhibition of protein synthesis in lactating tissue slices by actinomycin D is also inconsistent with the conclusion that cytoplasmic RNA turns over at a very slow rate (Sirakov, et al., 1968).

An RNA species of 15S has been observed in all mammary tissue samples examined including midpregnant mouse and late pregnant rat. Although quantitation of the area of this peak is difficult no change in the ratio of the area of this peak to the ribosomal peaks was observed as would be expected from that data of Gayne and Denamur (1969) and Mills and Topper (1970) that the number of polysomes increases during lactogenesis. Thus although a species of RNA which has hitherto been described as having characteristic of mRNA could be isolated, characteristics such as rapid turnover, change in amount with functional changes of

the tissue or differential response to hormone treatment could not be detected with the methods employed.

Mammary tissue slices from rats in late gestation respond to increased metabolite availability and prolactin plus insulin administration by increasing the incorporation of labeled precursor into all classes of RNA while hydrocortisone plus insulin and increased metabolite concentrations stimulate RNA synthesis in tissue slices from mammary glands of rats 2 days postpartum. The appropriate hormone combination complements the increased metabolite availability. These observations coupled with the inability to isolate species of RNA which responded in a rapid and specific manner to hormones would indicate that lactogenesis is an inherent property of the differented mammary epithelial cell.

SUMMARY

Mammary tissue slices incubated *in vitro* incorporated labeled precursors of RNA and protein at a rate calculated to be comparable with rates of synthesis *in vivo*. Several discrepancies in rate of incorporation and response to hormones are observed when defined RNA fractions are assayed rather than total precipitated RNA. Increased metabolite concentrations and insulin stimulated incorporation of both protein and RNA precursors. The combination of hydrocortisone, prolactin and insulin was also able to overcome a portion of the actinomycin D inhibition of protein synthesis, thus some regulation at the level of translation occurs in mammary cells.

Prolactin plus insulin stimulated RNA synthesis in prepartum tissue while hydrocortisone plus insulin stimulated synthesis in tissue slices from postpartum rats. All of the classes of RNA isolated by phenol extraction and electrophoresis were synthesized at equal rates and no specificity in response to hormones was noted. No evidence was observed for an RNA species with characteristics normally associated with mRNA.

REFERENCES

Agarwal, M. K., Hanoune, J., Yu, F. L., Weinstein, I. B., and Fergelsen, P. (1969), Biochemistry 8, 4806.

Amaldi, F., and Attardi, G. (1968), J. Mol. Biol. 33, 737.

Aronson, A. I., and Wilt, F. H. (1969), Proc. Nat. Acad. Sci. U.S. 62, 186.

Attardi, G., Parnas, H., Hwang, M., and Attardi, B. (1966), J. Mol. Biol. 20, 145.

Baldwin, R. L., and Cheng, W. (1969), J. Dairy Sci. 52, 523.

Baldwin, R. L., Kursrud, G. O., Martin, R. J., Cheng, W., and Schober, N. D. (1969), Biol. Reprod. 1, 31.

Baldwin, R. L., and Martin, R. J. (1968a), Endocrinology 82, 1209.

Baldwin, R. L., and Martin, R. J. (1968b), J. Dairy Sci. 51, 748.

Baldwin, R. L., and Milligan, L. P. (1966), J. Biol. Chem. 241, 2058.

Bintaringsih, Lyons, W. R., Johnson, R. E., and Li, C. H. (1958), Endocrinology 63, 640.

Billing, R. J., Barbiroli, B., and Smellie, R. M. S. (1969), Biochem. Biophys. Acta 190, 60.

Brown, D. D. (1968), Current Topics Develop. Biol. 2, 47.

Bruno, G. A., and Christian, J. E. (1961), Anal. Chem. 33, 1216.

Cartouzou, G., Mante, S., and Lessitzky, S. (1965), Biochem. Biophys. Res. Commun. 20, 212.

Church, R. B., and McCarthy, B. J. (1967), Proc. Nat. Acad. Sci. U.S. 58, 1548.

Church, R. B., and McCarthy, B. J. (1970), Biochem. Biophys. Acta 199, 103.

Cowie, A. T. (1957), J. Endocrin. 16, 135.

Cowie, A. T. (1970), in Lactogenesis, Reynolds, M., and Folley, S. J., Ed., Philadelphia, Pa., University of Penn. Press, pp. 65-71.

Cowie, A. T., and Tindal, S. J. (1961), J. Endocrin. 22, 403.

Darnell Jr., J. E. (1968), Bact. Rev. 32, 262.

DeLuca, H. F., and Cohen, P. P. (1964), in Monometric Techniques, Umbreit, W. W., Burris, R. H., and Stauffer, J. F., Ed., Minneapolis, Minn., Burgess Publishing Co., pp. 132.

Dingman, C. W., and Peacock, A. C. (1968), Biochemistry 7, 659.

Drews, J., and Brawerman, G. (1967), Science 156, 1385.

Elias, J. J., (1957), Science 126, 842.

Elias, J. J., and Rivera, E. M. (1959), Cancer Res. 19, 505.

Fujii, T., and Villee, C. (1967), Proc. Nat. Acad. Sci. U.S. 57, 1468.

Garren, L. D., Ney, R. L., and Davis, W. W. (1965), Proc. Nat. Acad. Sci. U.S. 53, 1443.

Gaye, P., and Denamur, R. (1969), Biochem. Biophys. Acta 186, 99.

Gelehrter, T. D., and Tomkins, G. M. (1967), J. Mol. Biol. 29, 59.

Glock, G. E., and McLean, P. (1954), Biochem. J. 56, 171.

Green, M. R., and Topper, Y. J. (1970), Biochem. Biophys. Acta 204, 441.

Greenbaum, A. L., and Slater, T. F. (1957), Biochem. J. 66, 155.

Greenman, D. L., Wicks, W. D., and Kenney, F. T. (1965), J. Biol. Chem. 240, 4420.

Haddad, A., Salua, I., and Crus, A. R. (1969), J. Neurochem. 16, 865.

Hahn, W. E., Church, R. B., and Gordman, A. (1969), Endocrinology 84, 738.

Henshaw, E. C., Bojaishi, T. B., and Hiatt, H. H. (1963), J. Mol. Biol. 7, 122.

Hollman, K. H. (1970), in Lactogenesis, Reynolds, M., and Folley, S. J., Ed., Philadelphia, Pa., University of Penn. Press, pp. 27-41.

Hunt, J. A., and Wilkinson, B. R. (1967), Biochemistry 6, 1688.

Jackson, C. D., and Sells, B. H. (1968), Biochem. Biophys. Acta 155, 417.

Jeanteur, P., Amaldi, F., and Attardi, G. (1968), J. Mol. Biol. 33, 757.

Juergens, W. G., Stockdale, F. E., Topper, Y. J., and Elias, J. J. (1965), Proc. Nat. Acad. Sci. U.S. 54, 605.

Karlson, P. (1963), Perspect. Biol. Med. 3, 318.

Kidson, C., and Kirby, K. S. (1964), Nature 203, 599.

Knight, E., and Darnell Jr., J. E. (1967), J. Mol. Biol. 28, 491.

Kono, T. (1969), J. Biol. Chem. 244, 1772.

Kozak, L. P., and Wells, W. W. (1969), Arch. Biochem. Biophys. 135, 371.

Kuhn, N. J. (1968), Biochem. J. 106, 743.

Kuhn, N. J., and Lowenstein, J. M. (1967), Biochem. J. 105, 995.

Kursrud, G. O., and Baldwin, R. L. (1969), Biol. Reprod. 1, 21.

Lang, N., Henlick, P., and Sekeris, C. E. (1968), Acta Endocrinology 57, 33.

Latham, H., and Darnell, Jr., J. E. (1965), J. Mol. Biol. 14, 1.

Leader, D. P., and Barry, J. M. (1969), Biochem. J. 113, 175.

Leake, R. E., and Barry, J. M. (1967), Biochem. J. 104, 41P.

Lin, T. M. Y., and Davis, J. W. (1967), Endocrinology 80, 1043.

Lockwood, D. H., Stockdale, F. E., and Topper, Y. J. (1967) Science 156, 945.

Loening, U. E. (1967), Biochem. J. 102, 251.

Long, C. (1961), in Biochemists Handbook, Princeton, N.J., D. Van Nostrand Co., Inc., pp. 853.

Lowry, O. H., Rosebrugh, N. J., Farr, A. L., and Randall, R. V., J. Biol. Chem. 193, 265.

Lyons, W. R. (1958), Proc. Roy. Soc., Ser. B. 149, 303.

Mayne, R., and Barry, J. M. (1967), Biochem. Biophys. Acta 138, 195.

Mayne, R., and Barry, J. M. (1970), J. Endocrin. 46, 61.

Mayne, R., Barry, J. M., and Rivera, E. M. (1966), Biochem. J. 99, 688.

Mayne, R., Forsyth, A., and Barry, J. M. (1968), J. Endocrin. 41, 247.

Means, A. R., and Hamilton, T. H. (1966a), Proc. Natl. Acad. Sci. U.S. 56, 1594.

Means, A. R., and Hamilton, T. H. (1966b), Biochem. Biophys. Acta 129, 432.

Mills, E. S., and Topper, Y. J. (1969), Science 165, 1128.

Mills, E. S., and Topper, Y. J. (1970), J. Cell. Biol. 44, 310.

Minemura, T., and Crofford, O. B. (1969), J. Biol. Chem. 244, 5181.

Mumford, R. E. (1964), Dairy Sci. Abs. 26, 293.

Nandi, S. (1958), Science 128, 772.

Okita, G. R., Spratt, J., and Leroy, G. V. (1956), Nucleonics 14, 76.

O'Malley, B. W., and McGuire, W. L. (1968), Proc. Nat. Acad. Sci. U.S. 60, 1527.

O'Malley, B. W., and McGuire, W. L. (1969), Endocrinology 84, 63.

Omura, T., Siekevitz, P., and Palade, G. E., J. Biol. Chem. 242, 2384.

Palmiter, R. D. (1969a), Endocrinology 84, 747.

Palmiter, R. D. (1969b), Biochem. J. 113, 409.

Palmiter, R. D., Christensen, A. K., and Schimke, R. T. (1970), J. Biol. Chem. 245, 833.

Peacock, A. C., and Dingman, C. W. (1967), Biochemistry 6, 1818.

Penman, S. (1966), J. Mol. Biol. 17, 117.

Penman, S., Scherrer, K., Becker, V., and Darnell, J. E. (1963), Proc. Nat. Acad. Sci. U.S. 49, 654.

Penman, S., Vesco, C., and Penman, M. (1968), J. Mol. Biol. 34, 49.

Perry, R. P. (1962), Proc. Nat. Acad. Sci. U.S. 48, 2179.

Peter, J. B. (1963), Ph.D. Thesis, University of Minnesota, Minneapolis, Minn.

Pitelka, D. R., Kerkof, P. P., Gagne, H. T., Smith, S., and Abraham, S. (1969), Expt. Cell. Res. 57, 43.

Pitot, H. C. (1969), Arch. Pathology 87, 212.

Plagemann, P. G. W., and Roth, M. F. (1969), Biochemistry 8, 4782.

Reel, J. R., and Kenny, F. T. (1968), Proc. Nat. Acad. Sci. U.S. 61, 200.

Rivera, E. M. (1964), Endocrinology 74, 853.

Rivera, E. M., and Bern, H. A. (1961), Endocrinology 69, 340.

Scherrer, K., and Darnell, Jr., J. E. (1962), Biochem. Biophys. Res. Commun. 9, 451.

Schingoethe, D. J., Hageman, E. C., and Larson, B. L. (1967), Biochem. Biophys. Acta 148, 469.

Schmid, W., Gallwitz, D., and Sekeris, C. E. (1967), Biochem. Biophys. Acta 134, 80.

Schneider, W. C. (1967), in Methods in Enzymology, Vol. 3, Colowick, S. P., and Kaplan, N. O., Ed., New York, N.Y., Academic Press, pp. 681.

Sirakov, L., and Rychlik, I. (1968), Collect. Czech. Chem. Commun. 33, 637.

Sirakov, L., Rychlik, I., and Sorm, F. (1968), Collect. Czech. Chem. Commun. 33, 951.

Slater, T. F., and Planterase, D. N. (1958), Biochem. J. 69, 417.

Soeiro, R., Vaughan, M. H., Warner, J. R., and Darnell, Jr., J. E. (1968), J. Cell. Biol. 39, 112.

Spirin, A. S. (1967), in Current Topics Devel. Biol., Vol. 2, Mascona, A. A., and Monroy, A., Ed. New York, N.Y., Academic Press, pp. 1.

Stellwagen, R. H., and Cole, R. D. (1968), J. Biol. Chem. 243, 4456.

Stellwagen, R. H., and Cole, R. D. (1969), J. Biol. Chem. 244, 4878.

Stockdale, F. E., Juergen, W. G., and Topper, Y. J. (1966), Devel. Biol. 13, 266.

Talwalker, P. K., Meites, J., and Nicoll, C. S. (1960), Am. J. Physiol. 199, 1070.

Talwalker, P. K., Nicoll, C. S., and Meites, J. (1961), J. Endocrin. 69, 802.

Tata, J. R. (1966), Devel. Biol. 13, 77.

Tata, J. R. (1968), Nature 219, 331.

Thibodeau, P. S., and Thayer, S. A. (1967), Endocrinology 80, 505.

Thompson, E. B., Tomkins, G. M., and Curran, J. F. (1966), Proc. Nat. Acad. Sci. U.S. 56, 296.

Tomkins, G. M., Garren, L. D., Howell, R. R., and Peterkofsky, B. (1965), J. Cellular Comp. Physiol. 66, 212.

Tonoue, T., and Frieden, E. (1970), J. Biol. Chem. 245, 2359.

Topper, Y. J. (1968), Ann. N.Y. Acad. Sci. 30, 869.

Traurig, H. H. (1967), Anat. Rec. 157, 489.

Tucker, H. A. (1966), Am. J. Physiol. 210, 1209.

Tucker, H. A., and Reece, R. P. (1963a), Proc. Soc. Exp. Biol. Med. 112, 370.

Tucker, H. A., and Reece, R. P. (1963b), Proc. Soc. Exp. Biol. Med. 112, 409.

Turkington, R. W. (1968), Endocrinology 82, 575.

Turkington, R. W. (1969), J. Biol. Chem. 244, 5140.

Turkington, R. W. (1970), Fed. Proceedings 29, 729.

Turkington, R. W., Brew, K., Vanaman, T. C., and Hill, R. L. (1968a), J. Biol. Chem. 243, 3382.

Turkington, R. W., Brew, K., Vanaman, T. C., and Hill, R. L. (1968b), J. Biol. Chem. 243, 3389.

Turkington, R. W., Juergens, W. G., and Topper, Y. J. (1965), Biochem. Biophys. Acta 111, 573.

Turkington, R. W., Lockwood, R. H., and Topper, Y. J. (1967), Biochem. Biophys. Acta 148, 475.

Turkington, R. W., and Topper, Y. J. (1967), Endocrinology 80, 329.

Turkington, R. W., and Ward, O. T. (1969), Biochem. Biophys. Acta 174, 291.

Wang, D. Y. (1962), Biochem. J. 83, 633.

Wang, D. Y., and Greenbaum, A. L. (1962), Biochem. J. 83, 626.

Warburg, O., and Christian, W. (1942), Biochem. Z. 310, 384.

Warner, J. R., and Soeiro, R. (1967), Proc. Nat. Acad. Sci. U.S. 58, 1984.

Warner, J. R., Soeiro, R., Birnboin, H. C., Girard, M., and Darnell, J. E. (1966), J. Mol. Biol. 19, 349.

Weinberg, R. A., Loening, U. E., Willems, M., and Penman, S. (1967), Proc. Nat. Acad. Sci. U.S. 58, 1088.

Weinberg, R. H., and Penman, S. (1970), J. Mol. Biol. 47, 169.

Weis, L. S., and Norahara, H. T. (1969), J. Biol. Chem. 244, 3084.

Wellings, S. R. (1970), in Lactogenesis, Reynolds, M., and Folley, S. J., Ed., Philadelphia, P., University of Penn. Press, pp. 5-25.

Willems, M., Musilova, H. A., and Malt, R. A. (1969), Proc. Nat. Acad. Sci. U.S. 62, 1189.

Williamson, J. R., Scholz, R., Browning, E. T., Thurman, R. G., and Fukami, M. H. (1969), J. Biol. Chem. 244, 5044.

Wool, I. G., and Cavicchi, P. (1967), Biochemistry 6, 1230.

Wool, I. G., and Manchester, K. L. (1962), Nature 193, 345.

Yokoyama, A., Shinde, Y., and Ôta, K. (1970), in Lactogenesis, Reynolds, M., and Folley, S. J., Ed., Philadelphia, Pa., University of Penn. Press, pp. 65-71.

A P P E N D I X

Appendix Table 1. Composition of Medium 199

Ingredients Per Liter		
1-Arginine HCl	70	mg
1-Histidine HCl H_2O	21.88	mg
1-Lysine HCl	70	mg
1-Tyrosine	40	mg
1-Tryptrophan	10	mg
1-Phenylalanine	25	mg
1-Cystine	20	mg
1-Methionine	15	mg
1-Serine	25	mg
1-Threonine	30	mg
1-Leucine	60	mg
1-Isoleucine	20	mg
1-Valine	25	mg
1-Glutamic Acid H_2O	75	mg
1-Aspartic Acid	30	mg
1-Alanine	25	mg
1-Proline	40	mg
1-Hydroxyproline	10	mg
Glycine	50	mg
Ca-D-Pantothenate	0.01	mg
Biotin	0.01	mg
Folic Acid	0.01	mg
Choline Chloride	0.5	mg
Inositol	0.05	mg
para-Aminibenzoic Acid	0.05	mg
Vitamin A Acetate	0.1147	mg
Calciferol	0.1	mg
Menadione	0.01	mg
Alpha Tocopherol Phosphate Na_2	0.01	mg
Ascorbic Acid	0.05	mg
Glutathione	0.05	mg
Cholesterol	0.2	mg
Sodium Acetate	50	mg
1-Glutamine	100	mg
Adenosinetriphosphate Na_2	10	mg
Adenylic Acid	0.2	mg
Ferric Nitrate $9H_2O$	0.1	mg
Ribose	0.5	mg
1-Cysteine HCl H_2O	0.1	mg
Adenine Sulfate	10	mg
Guanine HCl	0.3	mg
Xanthine	0.3	mg
Hypoxanthine	0.3	mg
Thymine	0.3	mg
Uracil	0.3	mg
Thiamine HCl	0.01	mg

Appendix Table 1. (Continued)

Ingredients Per Liter		
Riboflavin	0.01	mg
Pyridoxine HCl	0.025	mg
Pyridoxal HCl	0.025	mg
Niacin	0.025	mg
Niacinamide	0.025	mg
Deoxyribose	0.5	mg
Tween 80	5	mg

	Earle's Base		Hank's Base	
Sodium Chloride	6.8	g	8	g
Potassium Chloride	0.4	g	0.4	g
Calcium Chloride	0.2	g	0.14	g
Magnesium Sulfate	0.0977	g	0.108	g
Monosodium Phosphate	0.125	g	0.06	g
Dextrose	1	g	1	g
Sodium Bicarbonate	(2.2)	g	(0.35)	g

Appendix Table 2. Carnoy-Hematoxin Method for Preparing Samples for Histological Examination

1) sample placed in Carnoy's fixative overnight HOAC-ETOH-$CHCl_3$ (1:6:3)

2) sample stored in 70% ETOH

3) transferred to eosin for up to 12 hours

4) dehydrated for 1 hour each in 70% ETOH, 95% ETOH and 100% ETOH before transferring to toluene for 30 minutes

5) placed in Paraffin I (not over 65°) for 12 hours

6) placed in Paraffin II (not over 65°) for 2-12 hours

7) block and cut 8 μ thick

Staining:

1) Xylene I 5 min
   Xylene II 5 min

2) Alcohol - 100%, 95%, 70%, 50% - 3 min in each, rinse well after each step

3) Distilled H_2O - rinse well

4) Hematoxin - 4 min

5) Tap H_2O - rinse off blue color for few minutes and examine with microscope - look at nuclei

6) 0.17% HCl-70% ETOH - dip once and place immediately in tap H_2O to complete destaining blue for 15-20 minutes

   Note: Check microscopically to determine if slices have been adequately destained or if they have been overstained

7) Eosin - 3-5 min

8) Alcohol - 70%, 95%, 100%, 100%, - 3-5 min in each, rinse well after each step

9) Xylene I 3-5 min
   Xylene II 3-5 min

10) Mount

Appendix Table 3. Effect of Various Hormones Upon ^{32}P Incorporation Into Phenol Extracted RNA by Tissue Slices From Mammary Glands of Rats 3 Days Prepartum (Experiment I).

One g of tissue slices was incubated in 25 ml of Medium 199-E with 30 uc of ^{32}P for periods of time indicated. RNA was fractionated as outlined in Methods. The basal level of each hormone was 40 µg/100 mg of tissue.

	cpm ^{32}P Incorporated/10 µg RNA			
	28S	18S	15S	sRNA
1 hour	111	100	125	105
1 hour + 2x(HC-I)	96	124	145	182
1 hour + HC-I	210	209	215	217
3 hours	410	511	813	513
3 hours + 2x(HC-I)	421	403	383	546
3 hours + HC-I	841	830	1068	615
3 hours + P-I	448	490	543	365
3 hours + HC-P-I	487	465	770	459
5 hours	769	760	608	1044
5 hours + HC-P-I	790	730	789	1055
10 hours	1695	1666	1681	2161
10 hours + 2x(HC-I)	2420	2633	3597	3357
10 hours + HC-I	2437	2041	1738	2331
10 hours + P-I	4013	3571	4231	2903
10 hours + P	3384	2936	3225	2925
10 hours + HC-P-I	2055	2042	1477	2360

Appendix Table 4. Effect of Various Hormones Upon ^{32}P Incorporation Into Phenol Extracted RNA by Tissue Slices From Mammary Glands of Rats 2 Days Prepartum (Experiment II).

One g of tissue slices was incubated in 25 ml of Medium 199-E with 50 uc of ^{32}P and hormones for periods of time indicated. RNA was fractionated as outlined in Methods. Actinomycin D and ^{32}P were added at beginning of incubation to 12 hour sample. The basal level of hormone was 40 ug/100 mg of tissue. Seven ug of actinomycin/100 mg tissue was present.

	^{32}P Incorporation cpm/10 ug RNA			
	28S	18S	15S	sRNA
7 hours + HC-P-I	1845	1905	1507	2802
12 hours	3172	3091	3450	4877
12 hours + HC-I	2972	3331	3819	4688
12 hours + HC-P-I	3876	4100	4596	5577
12 hours + normal-199[1]	1973	2080	1433	2712
12 hours + normal-199 + HC-P-I	2799	2319	2806	2942
3 hours + actinomycin D[2]	304	305	359	660
12 hours + actinomycin D	26	20	26	220

[1]Normal-199 was Medium 199-Earles salts without added glucose and lactalbumin hydrolyzate.

[2]The 3 hours sample was preincubated for 1 hour with actinomycin D before ^{32}P was added, thus total incubation time was 4 hours.

Appendix Table 5. Effect of Various Hormones Upon ^{32}P Incorporation Into Phenol Extracted RNA by Tissue Slices From Mammary Tissue of Rats 6-18 Hours Postpartum

One g of tissue slices was incubated in 25 ml of Medium 199-E with 50 uc of ^{32}P and hormones for periods of time indicated. RNA was fractionated as outlined in Methods. The basal level of each hormone was 40 ug/100 mg of tissue.

	^{32}P Incorporation cpm/10 ug RNA			
	28S	18S	15S	sRNA
Experiment I				
3 hours	159	236	138	200
7 hours	1501	1903	1703	661
7 hours + HC-P-I	1870	2552	2603	1109
12 hours	2247	2722	2093	1978
12 hours + HC-P-I	2637	3336	2077	1965
Experiment II				
5 hours	509	453	470	331
12 hours	2532	2516	780	2140
12 hours + HC-P-I	3178	3629	3119	2897
12 hours + I[1]	555	565	288	317
24 hours[1]	326	335	351	301
24 hours + HC-I[1]	222	152	213	236
24 hours + HC-P-I[1]	211	209	291	238

[1]The ^{32}P was present only for the last 5 hours of incubation.

Appendix Table 6. Effect of Various Hormones on ^{32}P Incorporation Into Phenol Extracted RNA by Tissue Slices From Mammary Glands of Rats 72-84 Hours Postpartum (Experiment I)

One g of tissue slices was incubated in 25 ml of Medium 199-E with 50 µc of ^{32}P and hormones (40 ug/100 mg of tissue). RNA was fractionated as outlined in Methods. Ten µg of actinomycin D/100 mg tissue was present as indicated.

	^{32}P Incorporation cpm/10 ug RNA			
	28S	18S	15S	sRNA
1 hour	---	---	---	---
1 hour + HC-I	5	10	16	16
1 hour + HC-P-I	11	18	44	32
3 hours	103	99	187	192
3 hours + I	103	118	87	101
3 hours + P-I	158	174	145	238
3 hours + HC-P-I	113	118	103	114
5 hours	205	205	173	184
5 hours + HC-P-I	338	380	228	298
10 hours	883	860	1050	901
10 hours + I	1083	1227	860	964
10 hours + HC-I	1357	1157	1185	1158
3 hours + actinomycin D[1]	---	17	5	85
10 hours + actinomycin D	5	7	9	50

[1]The 3 hour sample was preincubated in the presence of actinomycin D for 1 hour before the ^{32}P was added.

Appendix Table 7. Effect of Various Hormones Upon ^{32}P Incorporation Into Phenol Extracted RNA by Tissue Slices From Mammary Glands of Rats 48-60 Hours Postpartum (Experiment II).

One g of tissue was incubated in 25 ml of Medium 199-E with 50 μc of ^{32}P and hormones (40 μg/100 mg tissue). RNA was fractionated as outlined in Methods.

	^{32}P Incorporation cpm/10 μg RNA			
	28S	18S	15S	sRNA
3 hours	48	40	91	121
7 hours	217	155	194	417
7 hours + HC-P-I	419	304	315	911
12 hours	679	825	744	996
12 hours + HC-I	1105	975	1031	1435
12 hours + P	655	604	677	1198
12 hours + HC-P-I	1137	595	527	1401
12 hours + normal-199[1]	474	524	606	648
12 hours - normal-199[1] + HC-P-I	683	558	512	759
12 hours[2]	87	85	221	211
12 hours + HC-P-I[2]	98	119	189	222

[1]Normal-199 was Medium 199-Earles salts without added glucose and lactalbumin hydrolyzate.

[2]The sample was preincubated for 9 hours and the isotope was only present for the last 3 hours.