# Progesterone-Mediated Inhibition of Casein mRNA and Polysomal Casein Synthesis in the Rat Mammary Gland during Pregnancy†

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ABSTRACT: The levels of casein mRNA, polysomal casein synthesis, and intracellular casein have been determined during mid-pregnancy in the rat mammary gland. Ovariectomy of the mid-pregnant rat and hormone replacement were used as a model for studying the regulation of casein synthesis. Following ovariectomy a twofold increase in both casein mRNA activity, determined in a wheat germ translation assay, and casein mRNA sequence concentration, measured using a selective cDNA hybridization probe, was observed. This effect on mRNA accumulation was first observed within 16 h following ovariectomy and was maximal between 24 and 48 h. Progesterone, but not estradiol or hydrocortisone, administered at the time of ovariectomy prevented the increase in casein mRNA. These changes in casein mRNA were compared with the mRNA levels normally observed during development of the rat mammary gland from virginity until lactation. A comparison of polysomes isolated from mid-pregnant and lactating mammary glands revealed a shift from small polysomes, containing predominantly monosomes, disomes, and trisomes to larger polysomal aggregates, containing 7 to 11 ribosomes per mRNA. A similar shift was observed within 48 h following ovariectomy. Using specific immunoprecipitation, casein synthesis on isolated polysomes was measured in a cell-free translation assay. Casein comprised more than 40% of the protein synthesized in polysomes isolated from either lactating or ovariectomized mid-pregnant animals. This represented about twice the level of casein synthesis observed in a 15 day pregnant animal. A direct comparison of polysomal casein synthesis and casein mRNA activity demonstrated a coordinate increase in both responses following ovariectomy, that was prevented by the administration of progesterone. The levels of intracellular casein during mid-pregnancy and following ovariectomy were also shown to correlate with the changes observed in casein mRNA and casein synthesis. Thus, progesterone inhibits casein mRNA accumulation, and casein synthesis during pregnancy. The regulation of casein synthesis is, therefore, a complex process involving potentially both transcriptional and translational mechanisms.

Previous results from our laboratory have demonstrated that appreciable amounts of casein mRNA are present in the rat mammary gland during early and mid-pregnancy (Rosen et al., 1975). Although secretion of casein does not occur at these times we found that at mid-pregnancy the level of casein mRNA per alveolar cell was 25 to 50% of the maximal levels reached during lactation. This increase in casein mRNA sequences, measured with a selective cDNA-hybridization probe, was coincident with an increase in casein mRNA activity, as measured by cell-free translation (Rosen and Barker, 1976). Thus the relationship between the levels of casein mRNA and the synthesis and secretion of casein are not understood, and the precise hormonal mechanisms regulating these processes remain unclear.

The levels of casein mRNA observed during mid-pregnancy may reflect both the positive and negative effects of several peptide and steroid hormones. Thus, the increase in casein mRNA activity seen during early and mid-pregnancy may be related to changes in serum prolactin and, especially in the rat, placental lactogen. These two polypeptide hormones have been shown to induce casein synthesis (Lockwood et al., 1966; Turkington, 1968) and casein mRNA (Houdebine and Gaye, 1975; Houdebine, 1976) both in vivo and in mammary gland organ culture. Furthermore, high levels of progesterone during pregnancy are thought to antagonize the lactogenic effects of these hormones (Davis et al., 1972; Assairi et al., 1974). The present studies were therefore designed to investigate the relationship among casein mRNA levels, polysomal casein synthesis, and the intracellular concentrations of casein during mid-pregnancy in the rat.

It has been previously demonstrated that ovariectomy of a mid-pregnant rat induces the appearance of a milk-like secretion with the immunological properties of casein (Liu and Davis, 1967). This response could be blocked by the administration of progesterone at the time of ovariectomy (Davis et al., 1972). Therefore, in the present studies ovariectomy of the midpregnant rat and hormone replacement have been used as a model for studying the regulation of both casein mRNA levels and polysomal casein synthesis in vivo. These experiments were a necessary prerequisite for current experiments in our laboratory in which the effects of progesterone in mammary gland organ culture are being studied (Matusik and Rosen, submitted for publication). A preliminary report of these findings has been published previously (Rosen and Comstock, 1976).

## Experimental Procedures

*Animals and Hormonal Treatment.* The breeding of animals and the isolation of mammary tissue have been described previously (Rosen et al., 1975). Day zero of pregnancy is defined as the day on which copulation plugs were observed. Bilateral ovariectomy was performed under ether anesthesia. Hormones were administered at the time of ovariectomy and continued for the total length of time indicated in the figures. Dosage of hormone and routes of administration were: (1) progesterone, 3 mg every 8 h in sesame oil, sc[1]; (2) estradiol

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[1] Abbreviations used: sc, subcutaneously; ip, intraperitoneally; NaDodSO$_4$, sodium dodecyl sulfate; $R_0t$, the concentration of RNA in mol $\times$ L$^{-1}$ times the time in seconds; cDNA, complementary DNA; AMV, avian myeloblastosis virus; Na$_2$EDTA, disodium ethylenediaminetetraacetic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Tris,tris(hydroxymethyl)aminomethane; $A_{260}$, absorbance at 260 nm; PhCH$_2$SO$_2$F, phenylmethanesulfonyl fluoride; DEAE, diethylaminoethyl-cellulose.

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benzoate, 2 μg every 8 h in sesame oil, sc; (3) prolactin, ovine, 35 IU/mg, NIH-P512, 0.5 mg every 12 h, ip; (4) hydrocortisone succinate, 5 mg every 8 h, ip.

*Isolation of RNA.* The isolation of total cellular RNA by direct homogenization of pulverized, frozen tissue in a phenol–$NaDodSO_4$ solution at pH 8.0 has been previously described (Rosen et al., 1975). Prior to hybridization all RNA extracts were treated with Proteinase K (EM Laboratories, 25 μg/mL at 37 °C for 10 min) followed by a final phenol–$CHCl_3$ extraction at pH 8.0. The amount of DNA in the total nucleic acid extracts was determined by a standard diphenylamine assay (Burton, 1968) and the rate of hybridization adjusted accordingly. DNA did not contribute to the extent of hybridization observed in any of the samples at the $R_0t$ values assayed (Rosen and Barker, 1976). When cell-free translation assays were performed, DNA was removed from the total nucleic acid by three extractions with 3 M sodium acetate, pH 6.0 (Palmiter, 1974).

*Quantitation of Casein mRNA Levels.* Casein mRNA was measured by both molecular hybridization using a full-length selective cDNA probe (Rosen and Barker, 1976) or by specific immunoprecipitation of the released peptides following translation in a wheat germ cell-free translation assay (Rosen, 1976). Equivalent results were obtained using either assay during mid-pregnancy and lactation. However, the increased sensitivity of the cDNA probe was required to quantitate casein mRNA levels in virgin tissue and during early pregnancy.

The synthesis of cDNA using a purified 15S rat casein mRNA template was performed as previously described with the following modifications: The cDNA was synthesized using [$^3$H]dCTP (80 μM, 4.7 Ci/mmol) instead of [$^3$H]dGTP to minimize tritium exchange during the hybridization; the remaining deoxynucleotide triphosphates were adjusted to 400 μM. Approximately 50 μg/mL of a purified 15S casein mRNA fraction and 125 units/mL of AMV RNA-directed DNA polymerase (provided through the courtesy of Dr. J. Beard) were added in a 1.0-mL final reaction volume. Following a 20-min incubation at 46 °C a full-length casein cDNA probe was isolated by chromatography on Sephadex G-50 followed by alkaline sucrose gradient centrifugation as described previously (Rosen and Barker, 1976). RNA excess hybridizations were performed in 0.6 M NaCl, 0.01 M Hepes, pH 7.0, 0.002 M $Na_2EDTA$ at 68 °C and the extent of hybridization was determined with $S_1$ nuclease (Miles Laboratories, Monahan et al., 1976). The data are expressed as the percent hybridization vs. the log equivalent $R_0t$. Rates of hybridization have been adjusted to the standard conditions of 0.18 M $Na^+$ at 62 °C (Britten et al., 1974).

Casein mRNA activity was determined by translation in a wheat germ cell-free system using L-[5-$^3$H]proline (43 Ci/mmol, Schwarz/Mann) as previously described (Rosen, 1976). Between 0.25 and 2.0 μg of the total RNA extracts were assayed and the casein mRNA and total mRNA activities were determined from the linear portions of the activity curves.

*Measurement of Polysomal Casein Synthesis.* Polysomes were isolated by the method of Morton et al. (1975) using yeast RNA as an RNase inhibitor instead of heparin, which may inhibit protein synthesis. Freshly excised rat mammary tissue was rinsed in ice-cold saline and minced thoroughly at 0–4 °C. Two volumes (v/w) of polysome homogenization buffer (0.25 M sucrose, RNase-free, Schwarz/Mann; 50 mM Tris-HCl, pH 7.5; 25 mM KCl; 50 mM $MgCl_2$; 2 mg/mL purified yeast total RNA) were added to yield a 33% homogenate. Commercial yeast total RNA (Schwarz/Mann) was purified by treatment with 50 μg/mL self-digested nuclease-free Pronase (Calbiochem) for 1 h at 37 °C, followed by phenol–$CHCl_3$ extraction and alcohol precipitation as previously described for the total nucleic acid extraction. The tissue was gently homogenized in a Teflon–glass homogenizer, pestle clearance 0.01 in., with five strokes at 100–200 rpm. The homogenate was then filtered through one layer of cheesecloth and 0.13 mL of a freshly prepared 20% deoxycholate solution (DOC) was added per mL of homogenate. The detergent treated sample was thoroughly mixed with a glass rod and 2 volumes of 2.3 M sucrose (RNase-free) containing 50 mM Tris, pH 7.4, 25 mM KCl, 5 mM $MgCl_2$, and 1 mg/mL yeast RNA (TKMR) were added. Following mixing, this solution was layered over 4 mL of 2.0 M sucrose (RNase-free) also containing TKMR in polyallomer tubes. The tubes were centrifuged at 65 000 rpm (278 000$g$) in a type 75 Ti rotor for 16 h at 4 °C. To avoid contamination of the polysome pellet, the tube was first clamped with a large hemostat below the interface of the stepwise gradient. The tube was then inverted and the bottom of the tube, containing the polysome pellet, rapidly cut off and allowed to drain. The polysome pellet was rinsed with TKM buffer (50 mM Tris, pH 7.4, 25 mM KCl, 5 mM $MgCl_2$). Polysomes were finally resuspended in TKM buffer at a concentration of 100 $A_{260}$ units/mL and stored in 100-μL aliquots in liquid nitrogen. Yields of polysomes ranged from 4.0 $A_{260}$/g tissue for mid-pregnant samples to 10 $A_{260}$/g tissue for lactating tissue with $A_{260}/A_{280}$ ratios of 1.6 to 1.8. This procedure has been applied to both pregnant and lactating tissue and yields polysomes that actively incorporate amino acids into casein when incubated in a cell-free translation assay. Polysomes were analyzed by centrifugation of 2 $A_{260}$ units on 0.3 to 1.0 M linear sucrose gradients for 90 min at 35 000 rpm at 4 °C in TKM buffer using an SW40 rotor. Gradients were fractionated using an ISCO density gradient fractionator (Model 640) and an ISCO UA-5 monitor at 254 nm.

The total protein synthetic activity of polysome preparations and the determination of polysomal casein synthesis were performed as follows: polysomes, 0 to 2 $A_{260}$, were added to a reaction mixture containing 10 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 98 mM KCl, 1 mM ATP, 0.25 mM GTP, 1 mM dithiothreitol, pyruvate kinase (0.15 IU), L-[5-$^3$H]proline (43 Ci/mmol, 2 μCi), 19 amino acids (20 μM, each), uncharged oviduct tRNA, 81 μg/mL (Anderson, 1969), 12 μL of reticulocyte polysomal salt wash (Gilbert and Anderson, 1970), and 8 μL of reticulocyte supernatant fraction (Moldave et al., 1971) in a final volume of 100 μL. Following a 20-min incubation at 37 °C, ribosomes were removed by centrifugation in a type 65 Ti rotor (1 h at 4 °C at 48 000 rpm). The incorporation of radioactive proline into total protein and into specifically immunoprecipitable casein was determined as previously described (Rosen, 1976).

*Radioimmunoassay of Casein.* Fresh tissue was homogenized at 4 °C in 2 volumes (v/w) of a buffer containing 0.25 M sucrose, 50 mM Tris-HCl, pH 7.6, 25 mM KCl, 5 mM $MgCl_2$, 2% Triton X-100 and 25 μg/mL $PhCH_2SO_2F$ (Sigma) using a Polytron homogenizer (Brinkman) at a setting of 6 for 30 s to 1 min. The homogenate was centrifuged at 18 000$g$ for 15 min at 4 °C and the supernatant removed from the pellet and lipid layer. The supernatant was recentrifuged at 105 000$g$ for 1 h at 4 °C in a type 65 Ti rotor and aliquots of the supernatant stored in liquid $N_2$ until assayed.

Radioactive casein was synthesized in the wheat germ translation assay using a twice dT-bound casein mRNA fraction as previously described (Rosen, 1976). Ribosomes were removed by centrifugation at 105 000$g$ for 1 h and aliquots of the [$^3$H]casein stored in liquid $N_2$. A rabbit anti-rat casein IgG fraction (Rosen et al., 1975), sufficient to precipitate 50% of the added [$^3$H]casein, was diluted 75-fold with

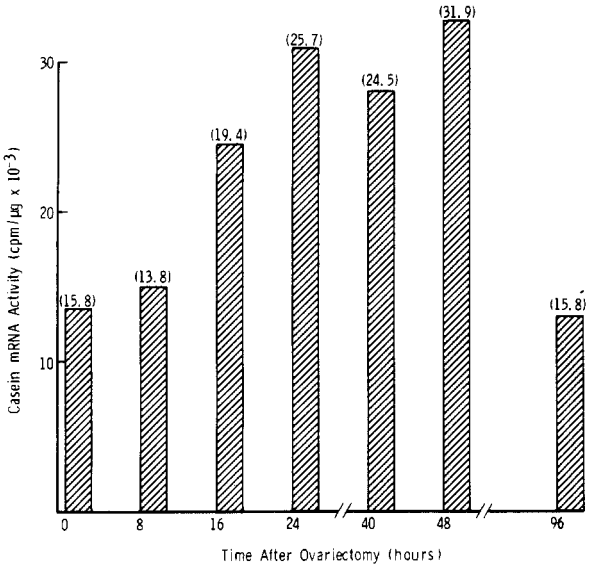


FIGURE 1: Effect of ovariectomy of 12 day pregnant rats on casein mRNA activity as determined with the wheat germ translation assay. Tissue was removed at the times designated in the figure and rapidly frozen, and RNA was extracted as described in Experimental Procedures. The numbers in parentheses express the specific casein mRNA activity as a percentage of the total mRNA activity.

rabbit IgG (10 mg/mL, Miles Laboratories) and used under the same conditions previously described for the indirect immunoassay of rat casein (Rosen, 1976). Approximately 50 000 cpm of [$^3$H]casein was added per point with increasing amounts of the 105 000$g$ supernatants added. The amount of protein in the supernatants was determined after $Cl_3CCOOH$ precipitation to remove the Triton X-100 (Lowry et al., 1951). The data are expressed as a percentage of the maximum amount of [$^3$H]casein immunoprecipitated when no competitor was added.

## Results

Previous studies by Liu and Davis (1967) have demonstrated that ovariectomy of rats approximately midway through pregnancy induced a lactation-like response, characterized by an increase in casein-like protein 24 h postovariectomy. The effect of ovariectomy of a 12 day pregnant rat on the level of casein mRNA activity is shown in Figure 1. The initial levels of casein mRNA prior to ovariectomy ranged from 14 to 16% of the total mRNA activity. This is in reasonable agreement with our previous data (Rosen et al., 1975). Within 16 h following ovariectomy an increase in casein mRNA activity was observed. Between 24 and 48 h casein mRNA activity remained approximately twofold higher than the control and comprised 25 to 32% of the total mRNA activity. By 96 h following ovariectomy, casein mRNA activity had returned to the level observed in the 12 day pregnant mammary gland. These increases in casein mRNA activity (cpm incorporated into casein/$\mu$g of total nucleic acid extract) were due in part to increases in the mRNA activity of the total nucleic acid extract (18.6% increase at 48 h) but were primarily the result of a selective increase in the specific casein mRNA activity.

The specific cDNA hybridization assay was employed to determine if this increase in casein mRNA activity represented an activation of preexisting mRNA or an actual increase in casein mRNA sequences. The effect of ovariectomy at either 5 days or 12 days of pregnancy is compared with the normal levels of casein mRNA observed throughout pregnancy (Figure 2). The hybridization of a total RNA preparation isolated from virgin mammary tissue and a pure 15S rat casein mRNA preparation with the casein cDNA are also shown. This permits the direct calculation of the percentage of the RNA present as casein mRNA in each RNA preparation (100 $\times$ $R_0t_{1/2}$ pure mRNA $\div$ $R_0t_{1/2}$ given RNA). A tenfold increase in casein mRNA was observed between the virgin (0.0018% casein mRNA/total cellular RNA) and 5 day pregnant (0.019% casein mRNA) RNA extracts. Ovariectomy of the 5 day pregnant animal resulted in an additional fivefold increase reaching a level of casein mRNA slightly greater than that observed in a 10 day pregnant animal. Ovariectomy of the 12 day pregnant animal produced approximately a twofold increase within 48 h, equivalent to the level of casein mRNA normally observed in a 20 day pregnant rat (Rosen and Barker, 1976). This is in agreement with the increase in casein mRNA activity observed in the wheat germ assay. Thus, the increase in casein mRNA activity represents an actual increase in the number of casein mRNA molecules. It should be noted that the effect of ovariectomy observed after 48 h was considerably greater than the expected increase in casein mRNA 2 days later in normal mammary development (Rosen et al., 1975; Rosen and Barker, 1976, Figure 2). Furthermore, the 5-fold increase in casein mRNA observed 48 h after ovariectomy of a 5 day pregnant rat actually represents a smaller absolute

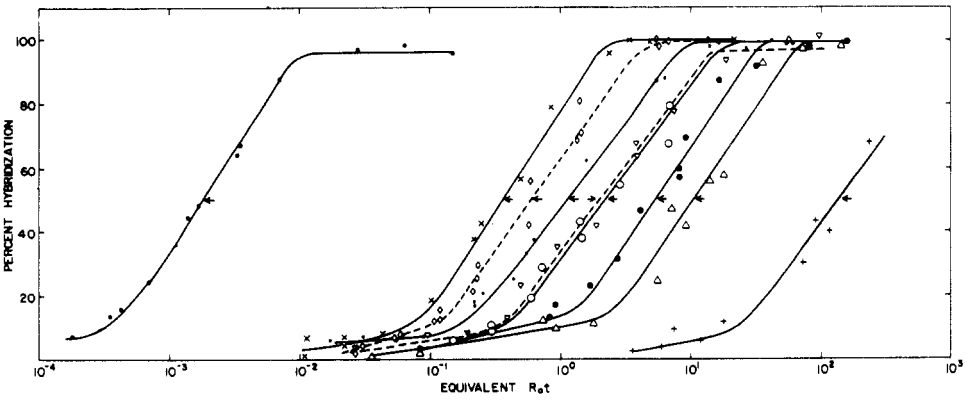


FIGURE 2: Effect of ovariectomy on casein mRNA sequence concentration determined using cDNA hybridization. A comparison with casein mRNA levels during normal mammary development. The following RNA samples were assayed: (solid lines) virgin (+); 5 day pregnant (△); 8 day pregnant (●); 10 day pregnant (○); 12 day pregnant (·); 8 day lactating (x); and on the far left, the purified 15S casein mRNA back hybrid (●); dashed lines, 48 h following ovariectomy of a 5 day pregnant animal (▽); 48 h following ovariectomy of a 12 day pregnant rate (◇). The arrows designate the estimated $R_0t_{1/2}$ values. Since the hybridization with RNA extracted from virgin tissue did not go to completion due to the low concentration of casein mRNA, the $R_0t_{1/2}$ value at 50% hybridization was employed.

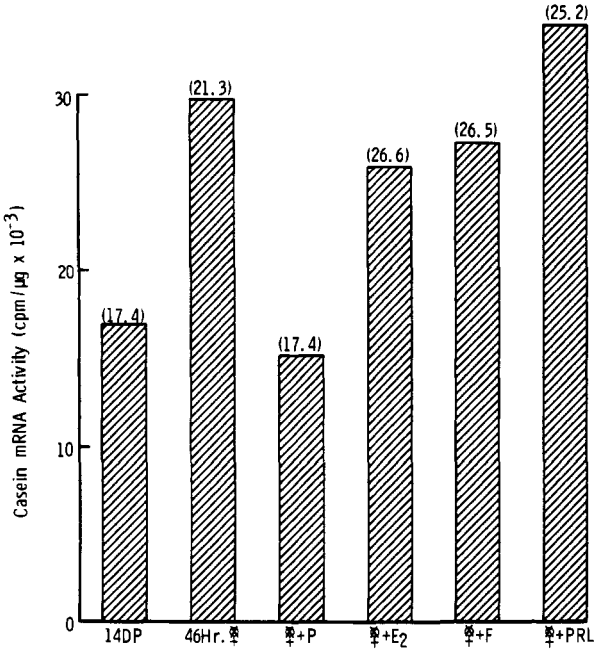


FIGURE 3: Effect of hormone administration at the time of ovariectomy on casein mRNA activity. Hormones were administered as described in Experimental Procedures for 46 h following ovariectomy of a 14 day pregnant rat. The casein mRNA activity determined in the wheat germ assay is shown on the ordinate and the percentage of total mRNA activity is shown in parentheses. The following abbreviations are used: 14DP (14 day pregnant); 46 H ♀ (46 h following ovariectomy); ♀ + P (ovariectomized rat given progesterone); ♀ + $E_2$ (ovariectomized rat given estradiol benzoate); ♀ + F (ovariectomized rat given hydrocortisone succinate); ♀ + PRL (ovariectomized rat given prolactin).

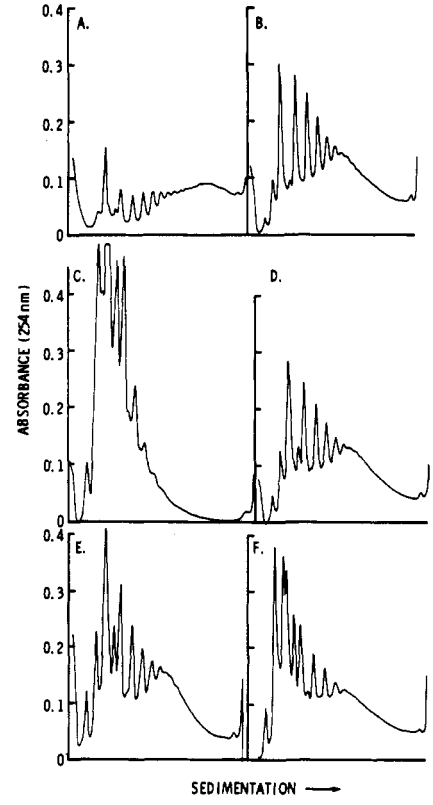


FIGURE 4: Effect of ovariectomy and progesterone administration on mammary gland polysome distribution. Details are described in Experimental Procedures. (Panel A) Liver polysomes; (B) polysomes isolated form 8 day lactating mammary tissue; (C) polysomes from 12 day pregnant mammary glands; (D) mammary polysomes isolated 46 h following ovariectomy of 12 day pregnant animals; (E) mammary polysomes isolated 46 h following ovariectomy of a 14 day pregnant animal; (F) mammary polysomes isolated from progesterone-treated rats 46 h following ovariectomy of a 14 day pregnant rat.

increase in casein mRNA sequences than the 2-fold increase observed at 12 days of pregnancy (0.076% of the total RNA vs. 0.124%). An overall 300-fold increase in the concentration of casein mRNA in an RNA extract is seen between the virgin gland and 8 day lactating mammary tissue. Since the RNA content of the lactating gland is considerably greater than the virgin this results in almost a 4000-fold increase in total casein mRNA molecules per g of tissue during this developmental period. In agreement with previous results (Rosen and Barker, 1976) the mid-pregnant mammary gland (10 to 14 days pregnant) contained between one-quarter to one-third the concentration of casein mRNA and approximately 8 to 16% the total amount of casein mRNA present at 8 days at lactation.

In order to investigate the precise hormonal mechanisms responsible for these changes the abilities of various hormones administered at the time of ovariectomy to modulate the induction of casein mRNA were also studied (Figure 3). Ovariectomy of a 14 day pregnant animal resulted in a 1.75-fold increase in casein mRNA activity, although the percentage of casein mRNA activity was slightly reduced in this experiment. Administration of progesterone at the time of ovariectomy and every 8 h for the next 46 h was able to prevent this increase. This response was not, however, prevented by the administration of either estradiol or hydrocortisone and, therefore, appeared to be selective for progesterone. Administration of prolactin only increased minimally the level of casein mRNA observed after ovariectomy alone.

In order to study further the role of progesterone in the regulation of casein synthesis, a protocol was developed for the isolation of functional rat mammary polysomes. Procedures which have been routinely utilized for the isolation of rat liver (Blobel and Potter, 1967), hen oviduct (Palacios et al., 1972), or rabbit mammary gland polysomes (Houdebine and Gaye, 1975) could not be successfully applied to the isolation of undegraded rat mammary polysomes. This appeared to result from the difficulty in homogenization of rat mammary tissue which contains large amounts of adipose and connective tissue, rather than to nuclease susceptibility of rat mammary polysomes. Using a modification of the procedure of Morton et al. (1975), which employs yeast RNA as a RNase inhibitor and an elevated $Mg^{2+}$ concentration, we were able to obtain polysomes containing 7 to 11 ribosomes from lactating mammary tissue (Figure 4B). A liver polysome profile isolated by the same procedure is shown in Figure 4A. Liver polysomes were used as a control for nuclease activity in mixing experiments and as a standard in the cell-free protein synthesis assays. Polysomes isolated during mid-pregnancy by this procedure were predominantly monosomes, disomes, and trisomes (Figure 4C). Forty eight hours following ovariectomy of 12 day pregnant animals, however, large polysomes characteristic of the lactating tissue appeared (Figure 4D). Mixing experiments between lactating, liver, and pregnant tissue suggested that these polysome profiles were not a reflection of intracellular RNase levels, but might instead represent a difference in the efficiency of initiation of protein synthesis in these tissues (data not shown). The effect of progesterone administration at the time of ovariectomy is also shown (Figure 4F). Progesterone partially blocked the appearance of large polysomes and resulted in the appearance of quantitatively more monosomes and small polysomes (Figure 4F compared with 4E). However, because of the limited magnitude of this shift its significance

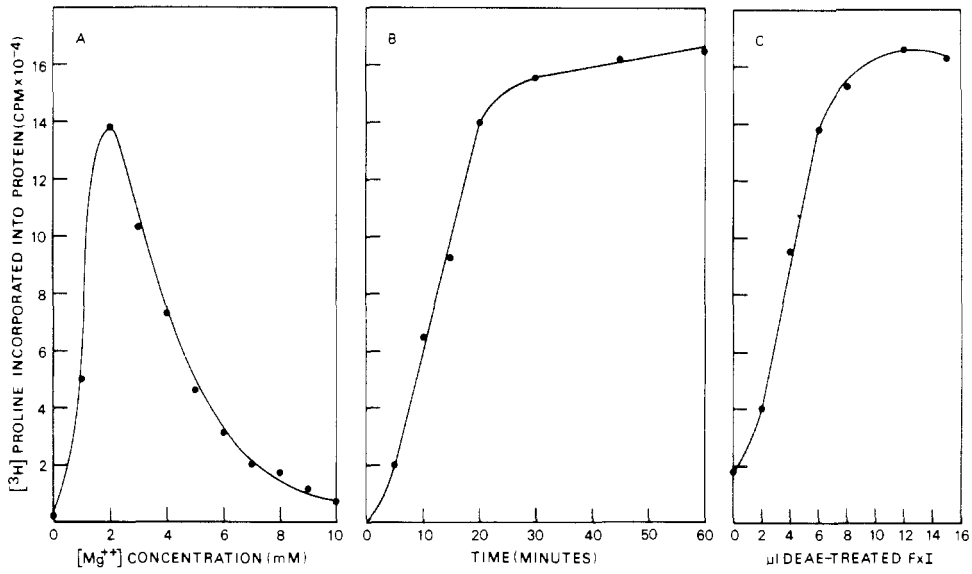


FIGURE 5: Characteristics of polysomal cell-free translation assay. (Panel A) Effect of $Mg^{2+}$ on total protein synthetic activity; (B) kinetics of protein synthesis; (C) requirement for the addition of DEAE-cellulose treated fraction I (FxI). In each of the experiments approximately 1 $A_{260}$ of polysomes was assayed as described in Experimental Procedures.

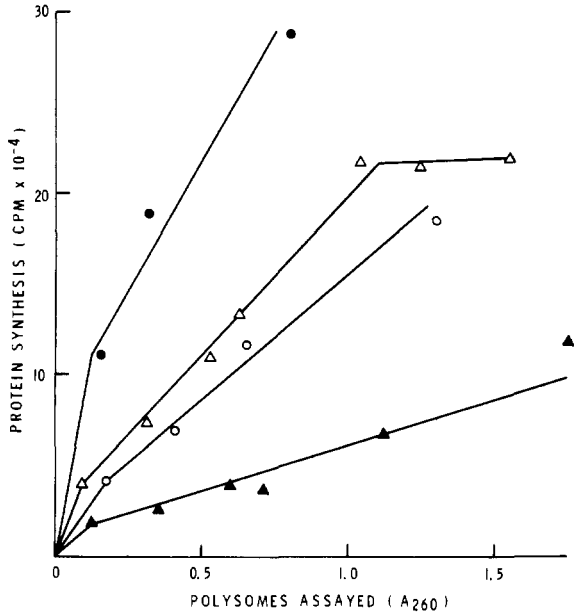


FIGURE 6: Effect of ovariectomy on polysomal protein synthesis. Increasing amounts of polysomes were assayed using the optimal $Mg^{2+}$ and FxI concentrations determined in Figure 5 for 20 min as described in Experimental Procedures. Polysomes were isolated from 12 day pregnant mammary glands (▲); 46 h following ovariectomy of 12 day pregnant rats (○); from 8 day lactating animals (△); and from rat liver (●).

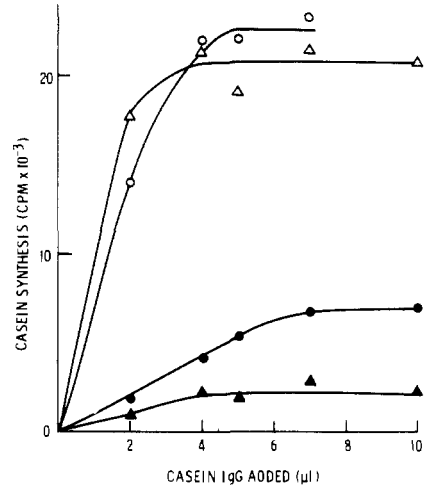


FIGURE 7: Effect of ovariectomy on polysomal casein synthesis. Increasing amounts of a primary anti-casein IgG fraction were added to precipitate all the radioactive casein synthesized using a constant secondary to primary antibody ratio of 15:1 (Rosen, 1976). A trapping control was employed using liver polysomes (▲), which represented about 3% of the total protein synthetic activity. Approximately 1 $A_{260}$ of polysomes isolated from 15 day pregnant mammary glands (●), from 14 day pregnant animals 48 h following ovariectomy (○), and from 3 day lactating rats (△) were assayed as described in Experimental Procedures. After subtracting the trapping background the levels of casein synthesis were 21, 42, and 40% of the total protein synthesis observed in the 15 day pregnant, the 48 h ovariectomized sample, and the lactating mammary polysomes, respectively.

is more difficult to assess (see Discussion). However, the marked changes in polysome profiles observed within 48 h following ovariectomy and between polysomes isolated during mid-pregnancy and lactation, all obtained using the identical isolation procedure, clearly suggested that significant differences existed in polysomal protein synthesis at these respective stages.

The ability of polysomes to synthesize protein, and, specifically, casein was determined by incubation in a cell-free translation assay containing a salt wash of rabbit reticulocyte polysomes. Some of the characteristics of this assay are depicted in Figure 5. The reaction was linear for 20 min at 37 °C (Figure 5B) and optimal total protein synthesis occurred at 2 mM $Mg^{2+}$. In addition, the reaction was markedly dependent upon the presence of a polysomal salt wash from which all exogenous mRNA had been removed by DEAE-cellulose chromatography (Figure 5C). The protein-synthetic capacity of polysomes isolated from lactating, mid-pregnant, and ovariectomized mid-pregnant animals was next determined and is shown in Figure 6 in comparison with the control liver polysomes. Ovariectomy of a mid-pregnant animal resulted in a 2.5-fold increase in polysomal protein synthesis compared with the mid-pregnant control. Within 48 h after ovariectomy the total protein-synthetic activity approached that of the

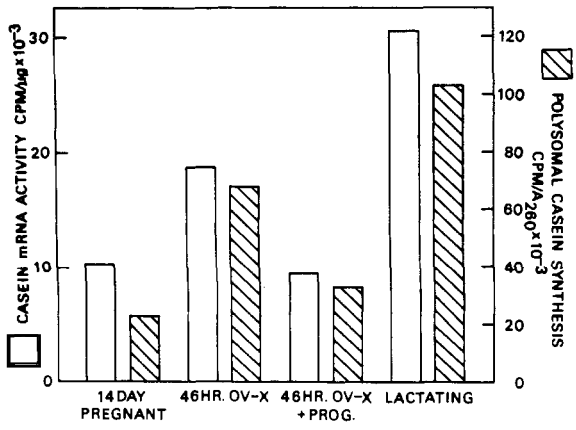


FIGURE 8: A comparison of casein mRNA activity and polysomal casein synthesis following ovariectomy and progesterone treatment. Mammary tissue was removed from 14 day pregnant animals, either 46 h following ovariectomy (OV-X), or following progesterone administration at the time of ovariectomy for 46 h (OV-X + Prog) and from 8 day lactating rats. Polysomes were prepared from half of the tissue and polysomal casein synthesis assayed as described in Experimental Procedures. RNA was extracted from the other half of the tissue and casein mRNA activity determined in the wheat germ translation assay.

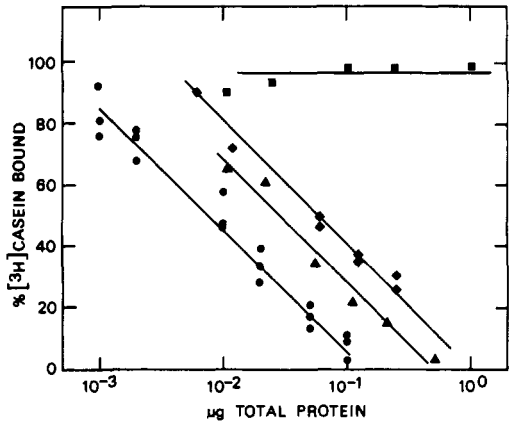


FIGURE 9: Radioimmunoassay of intracellular casein concentration during mid-pregnancy and following ovariectomy. The details of the radioimmunoassay are described in Experimental Procedures. The data are expressed as the percentage of the [$^3$H]casein immunoprecipitated with no competitor added vs. the amount of protein competitor assayed. The displacement curves obtained with purified rat casein (●), and 105 000-*g* supernatants from 14 day pregnant mammary glands (◆), 46 h following ovariectomy of a 14 day pregnant animal (▲) or rat liver (■) are shown.

polysomes isolated from 8 day lactating tissue. This is consistent with the appearance of the large polysomes (Figures 4D and 4F).

Polysomal casein synthesis was determined by the specific immunoprecipitation assay as shown in Figure 7. The amount of polysomes assayed, usually 1 $A_{260}$, was determined from the linear portion of the total protein synthesis assay shown in Figure 6. A liver polysome control was included to correct for nonspecific trapping. Casein synthesis comprised 21% of the total synthetic capacity of polysomes isolated from 15 day pregnant mammary tissue. Following ovariectomy a twofold increase in polysomal casein synthesis was observed resulting in a level comparable to that observed in a lactating polysome preparation of greater than 40%. Therefore, casein mRNA is associated with mammary gland polysomes in the pregnant rat and these polysomes have the capacity to synthesize casein in vitro.

Changes in polysomal casein synthesis displayed an excellent correlation with the levels of total cellular casein mRNA when compared in the same experiment (Figure 8). Thus, following ovariectomy a twofold increase in both mRNA activity and polysomal casein sythesis was observed. The levels of both casein mRNA and polysomal casein synthesis found after ovariectomy of a 14 day pregnant animal were 50 to 60% of the maximal levels found at 8 days of lactation. Finally, progesterone administration at the time of ovariectomy blocked both of these responses.

Although mammary polysomes isolated from mid-pregnant animals had the capability of synthesizing casein in vitro when supplemented with heterologous protein synthesis factors and tRNA, this was not proof that casein was being synthesized in vivo. Furthermore, if casein synthesis was occurring in vivo during pregnancy, the fate of the protein in the absence of secretion was unknown, i.e., was casein being stored in anticipation of subsequent lactation, or was the protein turning over prior to lactation and the initiation of secretion? In order to answer these questions the levels of casein present in the 105 000*g* supernatant of extracts of pregnant mammary tissue were measured using a radioimmunoassay procedure. The [$^3$H]casein was generated in the wheat germ translation assay using a partially purified rat casein mRNA fraction, rather than using the more commonly employed radioiodinated protein. The assay was capable of detecting casein in amounts as low as 1 ng and was sensitive to 100 ng (Figure 9). Triton X-100 was added to the homogenization buffer in order to solubilize any stored casein in the tissue and $PhCH_2SO_2F$ was utilized in order to minimize proteolysis. With this method casein was detected in the soluble protein of a 14 day pregnant mammary gland homogenate. Following ovariectomy, the level of intracellular casein increased two- to threefold (Figure 9) and a white milky fluid could be seen in the minced mammary tissue prior to homogenization (Rosen, unpublished observations). This appearance was blocked by progesterone administration at the time of ovariectomy (Davis et al., 1972). Thus, it appears that casein synthesis is occurring in vivo during pregnancy in the rat and that at least some of the newly synthesized casein is stored in the pregnant gland prior to lactation.

## Discussion

The regulation of casein synthesis and secretion is a complex process which is regulated by the multiple interactions of several peptide and steroid hormones (Topper, 1970). Development of the mammary gland during pregnancy is characterized by an increased synthesis of rRNA, polysomes, and tRNA (Gaye et al., 1973; Banerjee and Banerjee, 1973) including changes in the ratios of specific isoaccepting species of tRNA (Elska et al., 1971). This increase in RNA synthesis is accompanied by the extensive development of endoplasmic reticulum and the appearance of membrane-bound polysomes (Oka and Topper, 1971; Turkington and Riddle, 1970). Furthermore, an increased DNA content (Griffith and Turner, 1961) and the proliferation of alveolar cells occurs (Munford, 1963) resulting in a highly sophisticated protein-synthetic machinery capable of secreting several grams of casein per day during lactation (Jenness, 1974). Developmental changes in both mRNA levels and the components of the protein synthetic apparatus have been observed in other hormonally inducible systems, notably during estrogen-induced egg white protein synthesis in the immature chick oviduct (Rosen and O'Malley, 1975) and estrogen-induced vitellogenin synthesis in the male *Xenopus* liver (Tata, 1976). In both of these systems increases

in specific mRNA levels are accompanied by changes in mRNA and tRNA synthesis, endoplasmic reticulum biosynthesis, levels of protein synthesis initiation factors and membrane-bound polysomes, eventually resulting in the synthesis and secretion of large quantities of a specific protein.

The regulation of casein synthesis and secretion is, therefore, not an "all or none" phenomenon dependent solely upon the induction of casein mRNA. Casein mRNA is present during early and mid-pregnancy, and to a limited extent even in the virgin mammary gland (Figure 2). A similar observation has recently been reported during pregnancy in the rabbit, where casein mRNA was detected well before the onset of lactation (Shuster et al., 1976). A low level of casein mRNA was also detected in the virgin mammary gland in these studies using a 200 nucleotide long rabbit casein mRNA-cDNA probe. However, in the rabbit a 900-fold increase in total casein mRNA content was detected between mid-pregnancy and lactation, whereas in the rat mammary gland this increase was approximately 10-fold (Figure 2). This 90-fold greater relative difference in the amount of casein mRNA observed in the rat during mid-pregnancy may reflect the high concentrations of placental lactogen in the rat, e.g., 1200 ng/mL at day 12 of pregnancy (Kelly et al., 1976) compared with very low levels of placental lactogen activity in the rabbit, e.g. a maximal level of 25 ng/mL at day 30 of pregnancy was observed (Kelly et al., 1976).

The exact relationship between the levels of casein mRNA and the rates of casein synthesis in the developing rat mammary gland remains to be established. Experiments measuring the rate of casein mRNA transcription, the half-life of casein mRNA, the transit time of casein mRNA on mammary gland polysomes and the rates of casein synthesis and turnover will be required before the stoichiometry of casein mRNA and casein synthesis can be determined. These experiments will require an in vitro system in which the above processes can be measured directly following the addition of peptide and steroid hormones. However, the presence of casein synthesis during mid-pregnancy in the rat has been established both by the measurement of polysomal casein synthesis (Figures 7 and 8) and by direct radioimmunoassay of mid-pregnant mammary tissue homogenates (Figure 9). Similar results have been observed in both the rabbit (Shuster et al., 1976) and mouse mammary (Terry et al., 1975) glands during mid-pregnancy. These results suggest that casein synthesis and storage occur in a variety of mammals prior to the onset of lactation.

A comparison of polysome profiles between mid-pregnancy and lactation indicates a shift from monosomes, disomes, and trisomes to larger polysomes containing 7 to 11 ribosomes (Figure 4). A similar shift was observed within 48 h following ovariectomy of a mid-pregnant rat. In both cases the concentration of casein mRNA only increased two- to threefold during these periods. These results suggest that the polysome size distribution during mid-pregnancy may not reflect merely the level of casein mRNA, but instead the efficiency of initiation of protein synthesis. The efficiency of initiation of casein synthesis may be influenced by such variables as the specific isoaccepting tRNA population (Le Meur et al., 1976), the availability of endoplasmic reticulum and an increase in protein synthesis initiation factors (Comstock et al., 1972). For example, in the rabbit a shift in the total polysome profile from monomeric forms to polymeric forms is also observed during pregnancy (Shuster et al., 1976) and this shift is accompanied by a progressive increase in the proportion of polysomes bound to membranes (Gaye and Denamur, 1969). It has been suggested that the actual secretion of casein may be influenced by the proportion of free and bound polysomes (Houdebine, 1977). In addition, during prolactin-stimulated lactogenesis in the pseudo-pregnant rabbit, the capacity of the tissue to synthesize casein mRNA and casein may actually increase more rapidly than the capacity of the tissue to secrete casein (Houdebine, 1977). A similar phenomenon is probably occurring during mid-pregnancy in the rat. However, difficulties in the isolation and quantitation of free and membrane-bound polysomes from pregnant rat mammary tissue preclude the direct assessment of this hypothesis at this time. These studies will require improved homogenization and polysome isolation procedures.

The role of progesterone as the principal hormone suppressing lactation during pregnancy in the rat is given additional support by the experiments reported in this manuscript. Removal of progesterone by ovariectomy resulted in an increased level of casein mRNA, increased polysomal casein synthesis, an increased concentration of intracellular casein, and finally the appearance of a white, milk-like secretion. These results extend the previous observations of Kuhn (1969), Davis et al. (1972), and Liu and Davis (1967) concerning the mechanism of action of progesterone during pregnancy in the rat. Studies using the pseudo-pregnant rabbit mammary gland have also demonstrated that progesterone administered either prior to or simultaneously with prolactin will inhibit the induction of casein mRNA, casein synthesis, and subsequent lactogenesis (Houdebine and Gaye, 1975; Houdebine, 1976). This effect appears to be selective for progestational steroids. In the rat the plasma level of progesterone increases as early as day 4 of pregnancy to levels of 80 ng/mL and reaches levels of 120 ng/mL during mid-pregnancy (Morishige et al., 1973). The inhibitory effect of progesterone observed in these experiments requires the administration of several daily injections of the steroid. This was necessary to maintain continuous high serum levels of progesterone in the presence of a high metabolic clearance rate for the steroid (Pepe and Rothchild, 1973), although a comparable inhibitory effect by continuous infusion of low levels of progesterone (12–48 $\mu$g/h) has also been reported (Davis et al., 1972). Administration of progesterone after maximal induction of casein mRNA was obtained, either following ovariectomy or during lactation, was unable to reduce the levels of casein mRNA (Rosen, unpublished data). This further suggests that the continuous presence of progesterone is necessary during pregnancy in order to exert its inhibitory effect on lactogenesis (Chatterton, 1975). In addition to the previously mentioned data, the lack of an inhibitory effect of an even larger dose of another steroid, hydrocortisone, also argues for the specificity of the action of progesterone during pregnancy. Thus, the response of casein mRNA and casein synthesis to progesterone does not appear to represent a nonspecific toxic effect of the hormone, but rather is a selective effect of this steroid hormone, which may be important for the regulation of casein synthesis during pregnancy.

Progesterone appears to regulate casein synthesis and secretion in a pleiotropic fashion. At least three different potential mechanisms of action have been suggested: (1) progesterone has been reported to counteract the self-regulated increase in prolactin receptors observed in the mammary gland (Djiane and Durand, 1977). This may account for the relatively low levels of prolactin receptors detected in the rabbit mammary gland during pregnancy (Djiane et al., 1977) and may result in a reduced ability of prolactin or placental lactogen to induce casein mRNA. (2) Progesterone is also an effective competitor for glucocorticoid receptor binding sites in the mammary gland (Shyamala, 1973) and, therefore, may prevent the glucocorticoid-induced development of the rough endoplasmic reticulum necessary for lactogenesis (Wynn et

al., 1976). Plasma corticosteroid levels during mid-pregnancy in the rat have been reported to be comparable to serum progesterone levels approaching levels of 200 ng/mL (Simpson et al., 1973). Thus, the development of secretory capacity may depend on the relationship between the stimulatory effects of corticosteroids and the inhibitory effect of progesterone during pregnancy. Finally, a specific, unique progesterone receptor has been demonstrated in mammary carcinomas (Horwitz et al., 1975). However, until the characterization and function of a progesterone receptor in normal mammary tissue are demonstrated, the possibility that a unique progesterone receptor may mediate direct effects of progesterone distinct from those previously discussed is purely speculative. These potential multiple sites of progesterone action may explain the coordinated responses to ovariectomy observed during mid-pregnancy, i.e., an increase in casein mRNA levels, an increase in large polysomes and casein synthesis, and finally increased levels of intracellular casein and the initiation of secretion.

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References

Anderson, W. F. (1969), *Biochemistry 8,* 87.

Assairi, L., Delouis, C., Gaye, P., Houdebine, L. M., Alliver-Bousquet, M., and Denamur, R. (1974), *Biochem. J. 144,* 245.

Banerjee, D. N., and Banerjee, M. R. (1973), *J. Endocrinol. 56,* 145.

Blobel, G., and Potter, V. R. (1967), *J. Mol. Biol. 28,* 539.

Britten, R. J., Graham, D. E., and Neufeld, B. R. (1974), *Methods Enzymol. 29,* 363.

Burton, K. (1968), *Methods Enzymol. 128,* 163.

Chatterton, R. T., Jr., King, W. T., Ward, D. A., and Chien, J. L. (1975), *Endocrinology 96,* 861.

Comstock, J. P., Rosenfeld, G. C., O'Malley, B. W., and Means, A. R. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 2377.

Davis, J. W., Wikman-Coffelt, J., and Eddington, C. L. (1972), *Endocrinology 91,* 1101.

Djiane, J., and Durand, P. (1977), *Nature (London) 266,* 641.

Djiane, J., Durand, P., and Kelly, P. A. (1977), *Endocrinology 100,* 1348.

Elska, A., Matsuka, G., Matiash, U., Nasarenko, I., and Semenova, N. (1971), *Biochim. Biophys. Acta 247,* 430.

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

Gaye, P., Houdebine, L. M., Petrissant, G., and Denamur, R. (1973), *Acta Endocrinol. (Copenhagen), Suppl. No. 180,* 426.

Gilbert, J. M., and Anderson, W. F. (1970), *J. Biol. Chem. 245,* 2342.

Griffith, D. R., and Turner, C. W. (1961), *Proc. Soc. Exp. Biol. Med. 106,* 448.

Horwitz, K. B., McGuire, W. L., Pearson, O. H., and Segaloff, A. (1975), *Science 189,* 726.

Houdebine, L. M. (1976), *Eur. J. Biochem. 68,* 219.

Houdebine, L. M. (1977), *Mol. Cell Endocrinol. 7,* 125.

Houdebine, L. M., and Gaye, P. (1975), *Mol. Cell Endocrinol. 3,* 37.

Jenness, R. (1974), *J. Invest. Dermatol. 63,* 109.

Kelly, P. A., Tsushima, T., Shiu, R. P. C., and Friesen, H. G. (1976), *Endocrinology 99,* 765.

Kuhn, N. J. (1969), *J. Endocrinol. 44,* 39.

Le Meur, M.-A., Gerlinger, P., and Ebel, J.-P. (1976), *Eur. J. Biochem. 67,* 519.

Liu, T. M., and Davis, J. W. (1967), *Endocrinology 80,* 1043.

Lockwood, D. H., Turkington, R. W., and Topper, Y. J. (1966), *Biochim. Biophys. Acta 130,* 493.

Lowry, O. H., Rosebrough, N. J., Darr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.

Moldave, K., Galunsink, W., and Rao, P. (1971), *Methods Enzymol. 20C,* 337.

Monahan, J. J., Harris, S. E., Woo, S. L. C., Robberson, D. L., and O'Malley, B. W. (1976), *Biochemistry 15,* 223.

Morishige, W. K., Pepe, G. J., and Rothchild, I. (1973), *Endocrinology 92,* 1527.

Morton, B., Nwizu, C., Henshaw, E. C., Hirsch, C. A., and Hiatt, H. H. (1975), *Biochim. Biophys. Acta 395,* 28.

Munford, R. E. (1963), *J. Endocrinol. 28,* 1.

Oka, T., and Topper, Y. J. (1971), *J. Biol. Chem. 246,* 7701.

Palacios, R., Palmiter, R. D., and Schimke, R. T. (1972), *J. Biol. Chem. 247,* 2316.

Palmiter, R. D. (1974), *Biochemistry 13,* 3606.

Pepe, G. J., and Rothchild, I. (1973), *Endocrinology 93,* 1200.

Rosen, J. M. (1976), *Biochemistry 15,* 5263.

Rosen, J. M., and Barker, S. W. (1976), *Biochemistry 15,* 5272.

Rosen, J. M., and Comstock, J. P. (1976), *Proc. 58th Endocrin. Soc. Meeting,* 123.

Rosen, J. R., and O'Malley, B. W. (1975), *Biochem. Action. Horm. 3,* 271.

Rosen, J. M., Woo, S. L. C., and Comstock, J. P. (1975), *Biochemistry 14,* 2895.

Shuster, R. C., Houdebine, L.-M., and Gaye, P. (1976), *Eur. J. Biochem. 71,* 193.

Shyamala, G. (1973), *Biochemistry 12,* 3085.

Simpson, A. A., Simpson, M. H. W., Sinha, Y. N., and Schmidt, G. H. (1973), *J. Endocrinol. 58,* 675.

Tata, J. R. (1976), *Cell 9,* 1.

Terry, P. M., Ball, E. M., Ganguly, R., and Banerjee, M. R. (1975), *J. Immunol. Methods 9,* 123.

Topper, Y. J. (1970), *Recent Prog. Horm. Res. 26,* 287.

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

Turkington, R. W., and Riddle, M. (1970), *J. Biol. Chem. 245,* 5145.

Wynn, R. M., Harris, J. A., and Chatterton, R. T., Jr. (1976), *Am. J. Obstet. Gynecol. 126,* 920.