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Role of RNA Synthesis in the Estrogen Induction of a Specific Uterine Protein*

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Abstract. The rate of amino acid incorporation into a specific uterine protein (induced protein band) isolated by gel electrophoresis has been shown to be markedly stimulated within an hour after estrogen administration. Injection of actinomycin D (8 mg/kg) prior to estrogen blocks the synthesis of induced protein. The accumulation of the product of the actinomycin D-sensitive step (induced protein band RNA) is significant 15 minutes after estrogen, and its synthesis would appear to be initiated as soon as the estrogen-receptor complex reaches the nucleus. Blocking protein synthesis with puromycin or cycloheximide did not affect the accumulation of induced protein band RNA, indicating that this is one of the earliest macromolecular synthetic events to occur after estrogen administration.

Introduction. Protein and RNA synthesis have been implicated in the mechanism of action of a number of hormones, including the estrogens.[1] Which one of these two steps is the primary response to estrogen is not clear on the basis of previous data. Studies involving the inhibitors puromycin and cycloheximide indicate that the expression of the estrogenic response by uterine cells (including increased RNA polymerase activity and increased incorporation of nucleosides into RNA) is dependent upon protein synthesis.[2,3] Since general protein synthesis was found not to be increased until 2–4 hr after hormone treatment, whereas a number of other metabolic activities were increased earlier,[2] it was concluded that the synthesis of a few specific uterine proteins probably occurs during early estrogen action. Notides and Gorski[4] demonstrated an increased incorporation of labeled amino acid into a specific protein band when soluble proteins of the uterus were separated by starch gel electrophoresis. Attempts to discern whether this induction was dependent upon RNA synthesis were inconclusive.[4] Levels of actinomycin D reported to block uterine RNA synthesis caused only a 50% reduction in the incorporation of labeled amino acid into the induced protein band (IP).

In this paper data are presented which demonstrate that the induced protein is not synthesized when RNA synthesis is completely inhibited and that a large dose of actinomycin D alone is sufficient to block this induction. It is further shown that this actinomycin D-sensitive step is not in turn dependent upon protein synthesis. Kinetic studies on the appearance of the product of the actinomycin D-sensitive step (presumably synthesis of a new RNA) are also presented.

Methods. Holtzman rats 21–26 days old (immature) or 3.5-month-old animals (mature) ovariectomized for a period of at least 1 week were used in this study. Experimental animals (3–10/group) were injected intraperitoneally with estradiol-17β (5 μg/immature and 10 μg/mature) in 0.154 M saline. Control animals received saline injections alone. In the experiments in which RNA or protein synthesis inhibitors were used, actinomycin D was injected alone (4 mg/kg body wt or 8 mg/kg body wt) or in combination with nogalamycin (Upjohn Company, 8 mg/kg each) 15–30 min prior to estrogen administration. Puromycin (100 and 200 mg/kg, Nutritional Biochemical) or cycloheximide (4 mg/kg) was administered 30 min prior to the hormone. All inhibitors were dissolved in 0.15 M NaCl. At designated time intervals after estradiol injection, the animals were decapitated and their uteri excised, stripped of all adhering fatty tissue, and incubated in 1–3 ml Eagle's HeLa medium (Difco) at 37°C for 1–2 hr under an atmosphere of 95% O_2 and 5% CO_2. Uterine proteins were labeled with 20 μCi/ml ^{3}H-L-leucine (2.0 Ci/mmol, Schwarz) or 5 μCi/ml ^{14}C-L-leucine (175–240 mCi/mmol, Schwarz). At the end of the incubation period, the control and estrogen-treated uteri were rinsed thoroughly with cold 0.05% disodium ethylenediaminetetraacetate (Na_2EDTA) and homogenized separately or together in 1.0 ml of the Na_2EDTA solution. The homogenates were centrifuged for 30 min at 15,000 $\times$ g and the resulting supernatant fraction was frozen until use.

Both starch gel and polyacrylamide gel electrophoresis were employed. Starch gel electrophoresis was performed as described previously.[4] Acrylamide gels were prepared in 0.066 M tris(hydroxymethyl)aminomethane (Tris, Sigma), 0.02 M boric acid, and 0.003 M Na_2EDTA buffer (TBE) at pH 8.6. Twenty milliliters of 6% Cyanogum-40 (Fisher) were mixed with 0.4 ml of 10% ammonium persulfate (prepared fresh) and 50 μl of *N,N,N',N'*-tetramethylenediamine (Eastman). Aliquots (4 ml) were placed in glass tubes (10 $\times$ 0.7 cm), layered with buffer, and allowed to polymerize. Supernatant fractions (100–200 μl) from control and/or estrogen-treated uteri were mixed with 25 μl of 13% Ficoll (Pharmacia) containing electrophoretic tracking dye (Canalco). Electrophoresis was run in TBE buffer at room temperature at 1 mA/tube for 1 hr and raised to 2 mA/tube for 4–5 hr. At the termination of a run the gels were removed from the tubes, stained with 1% amido Schwarz in 7% acetic acid for at least 1 hr, and electrophoretically destained. The distance of the protein bands from the origin was measured, and the gels were frozen over solid CO_2 and sectioned into 1-mm slices. Each slice was placed in a scintillation vial, dried, and dissolved in 0.2 ml of 30% hydrogen peroxide at 40°C. One milliliter NCS solubilizer (Nuclear-Chicago) was added to the dissolved gel and the mixture incubated for an additional hour at the same temperature. Ten milliliters of scintillation fluid (0.5% PPO (New England Nuclear); 0.03% dimethyl POPOP (Nuclear Equipment) in toluene) were added. Radioactivity was measured in a Packard Tri-Carb scintillation spectrophotometer. Counting efficiency was 20% for ^{3}H and 55% for ^{14}C.

Results. Figure 1 shows an electrophoretic separation of labeled uterine proteins on starch gel. Estrogen increased the rate of incorporation of labeled amino acid into only one protein band above that of the control group. Pretreatment with the RNA synthesis inhibitors actinomycin D and nogalamycin blocked cytidine incorporation into RNA by 85–90% and effectively blocked increased synthesis of induced protein. Data from this study were pooled and summarized and the estrogen-plus-inhibitor groups did not differ significantly from the controls. The RNA synthesis inhibitors did not significantly affect the rate of incorporation of labeled amino acid into total soluble protein, although a trend to lower incorporation (approximately 20% in nine trials) could be seen.

Since actinomycin D alone was able to suppress the induction of the specific uterine protein, this inhibition was studied in greater detail using a double isotope technique and acrylamide gel electrophoresis (Fig. 2). Briefly, uterine proteins

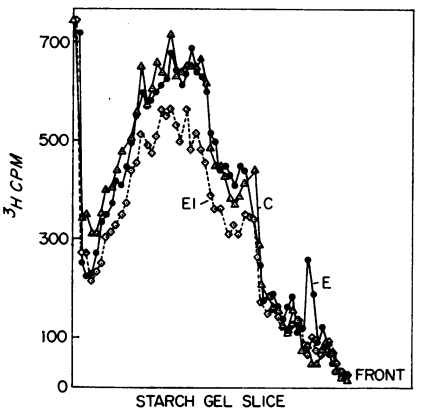

FIG. 1.—Electrophoretic separation of uterine soluble proteins on starch gels after 30 min *in vivo* estrogen stimulation (Group *E*). Group *EI* received actinomycin D and nogalamycin (8 mg/kg each) 15 min prior to the hormone. Group *C* received saline alone. Uteri (8–10/group) were incubated with 60 μCi ^{3}H-leucine in 3 ml Eagle's HeLa medium for 1 hr. Uterine soluble proteins were prepared and electrophoresed as described in *Methods*.

from estrogen-treated animals were labeled with ^{3}H and those from control uteri with ^{14}C. The supernatant fraction from the combined uteri was electrophoretically separated, the gel sectioned, and the radioactivity in each slice as a result of ^{3}H and ^{14}C counted. The ratio of these two isotopes was then calculated. Barnea and Gorski[5] have shown that only in the area on the gel corresponding to the induced protein band is an increase in the ^{3}H/^{14}C ratio noted. Reversing the isotopes had no effect on the estrogen response, and no difference could be seen between *in vivo* and *in vitro* labeling patterns. Figure 3 shows the labeling patterns and ^{3}H/^{14}C ratios of uterine proteins from estrogen-treated animals with or without actinomycin D pretreatment 30 min prior to estrogen. The lower inhibitor dose (4 mg/kg) was reported to block uterine RNA synthesis

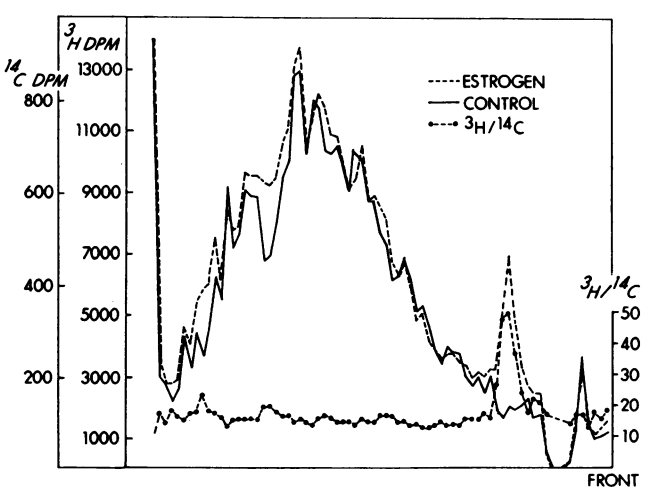

FIG. 2.—Separation of uterine soluble proteins by acrylamide gel electrophoresis. Uterine soluble proteins from mature ovariectomized animals (1/group) were incubated in 1 ml HeLa medium containing either 0.015 μmole ^{3}H-leucine (estrogen) or 0.015 μmole ^{14}C-leucine (control) for 1 hr at 37°C. The soluble proteins were separated on a 6% acrylamide gel, stained, and cut. The radioactivity and ^{3}H/^{14}C ratio in each slice were determined.

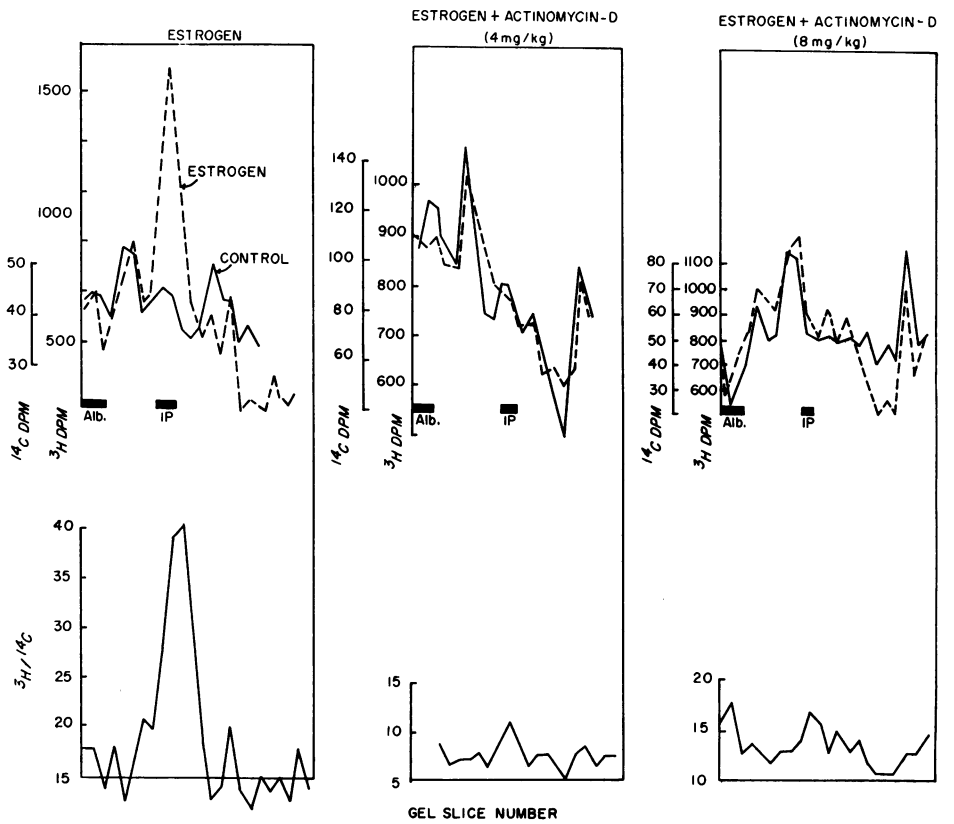

FIG. 3.—Acrylamide gel electrophoresis of uterine soluble proteins from rats (5/group) stimulated 30 min *in vivo* with estradiol-17β after a 15-min pretreatment with actinomycin D at two dose levels. Uteri from estrogen-treated animals were incubated with 30 μCi ^{3}H-leucine (0.015 μmole) and those from control animals with 5 μCi ^{14}C-leucine (0.015 μmole) in 1.5 ml Eagle's HeLa medium for 1 hr. The upper figure shows the radioactivity profile of proteins migrating from the albumin band to dye front. $^{3}H/^{14}C$ in each gel slice appears in the lower portion of the figure. dpm, disintegrations per minute.

85–90%.[4] Both levels of the inhibitor suppressed the synthesis of the induced protein as shown by the lowered incorporation of labeled amino acid into induced protein and the decreased $^{3}H/^{14}C$ ratio in the induced protein peak relative to other proteins on the gel. By fixing the increase in induced protein ratio due to estrogen at 100%, we found that pretreatment with 4 mg/kg actinomycin D reduced this increase to 23% ± 2%. The higher dose (8 mg/kg) of inhibitor further reduced it to 10% ± 4%. It is concluded that the increase in the rate of synthesis of the induced protein by estrogen is dependent upon a prior actinomycin D-sensitive step, presumably the synthesis of a new RNA.

In order to determine if this actinomycin D-sensitive step was in turn dependent upon protein synthesis, the following experiment was performed. Estrogen was administered to animals in which uterine protein synthesis was inhibited by puromycin or cycloheximide (injected 30 min prior to estrogen).

The uteri were removed 30 min after estrogen injection, washed extensively, and incubated with labeled amino acid for 2 hr. Actinomycin D was present in the incubation medium (50 μg/ml) to prevent any RNA synthesis from occurring at this time. Blocking protein synthesis during the time the hormone was present *in vivo* failed to inhibit the induction of the induced protein (Fig. 4), demonstrating that prior protein synthesis is not necessary for the appearance of the actinomycin D-sensitive step. When the estrogen response was set at 100%, the increases in the induced protein $^3H/^{14}C$ ratio were 90 and 84% for 100 mg/kg puromycin (general protein synthesis was inhibited 65%), 98% for 200 mg/kg puromycin (95% inhibition), and 121% for cycloheximide (97% inhibition) (Fig. 5).

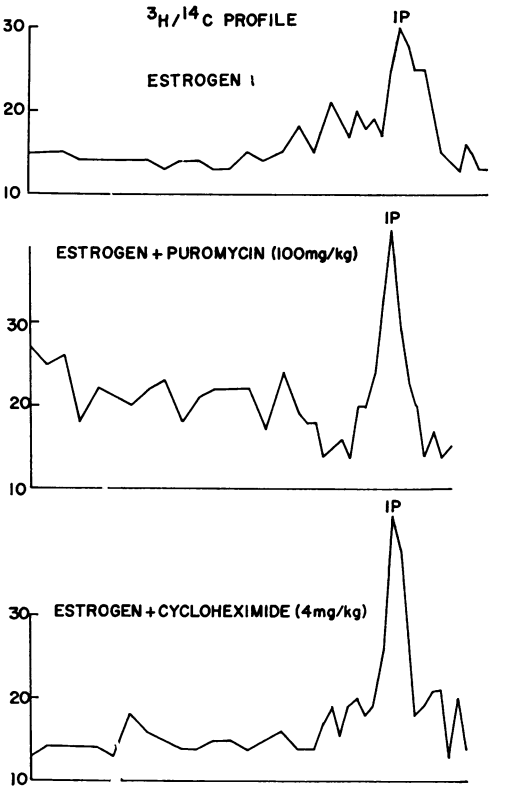

FIG. 4.—The effect of protein synthesis inhibition on the actinomycin D-sensitive step. Puromycin (100 or 200 mg/kg) or cycloheximide (4 mg/kg) was administered 30 min prior to a 30-min estrogen stimulation. Uteri were excised, washed thoroughly, incubated in 1.0 ml Eagle's HeLa medium containing 0.015 μmole of labeled amino acid (^{3}H-leucine for estrogen-treated uteri and ^{14}C-leucine for controls), and 50 μg actinomycin D for 2 hr. Uteri were pooled, extracted, and the soluble proteins electrophoresed on acrylamide gels. The $^3H/^{14}C$ ratios were calculated and are plotted for uterine proteins from estrogen-stimulated rats with and without concomitant protein synthesis inhibition.

The time course for the appearance of the product of the actinomycin D-sensitive step after estrogen treatment is shown in Figure 6. Estrogen was administered for time intervals up to 90 min. At the end of each interval, the uteri were allowed to incorporate labeled amino acid into protein in the presence of actinomycin D, which inhibited further RNA synthesis. It may be seen in both immature and ovariectomized animals that this step occurs with little or no lag period after estrogen and reaches a maximum 30–60 min after administration of the hormone. In contrast, the rate of induced protein synthesis measured *in vivo* is increased only after a lag period of 45–60 min after estrogen treatment.

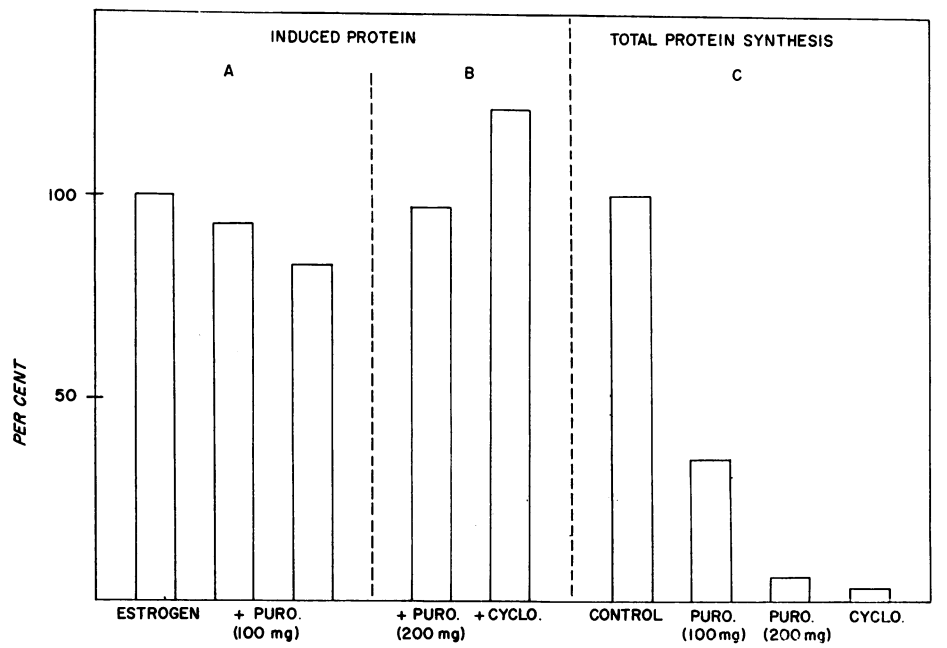

FIG. 5.—The effect of protein synthesis inhibition on the actinomycin D-sensitive step Puromycin (100 or 200 mg/kg) or cycloheximide (4 mg/kg) was administered 30 min prior to a 30-min estrogen stimulation. Uteri were excised, washed thoroughly, incubated in 1.0 ml Eagle's HeLa medium containing 0.015 μmole of labeled amino acid (^{3}H-leucine for estrogen-treated uteri and ^{14}C-leucine for controls) and 50 μg actinomycin D for 2 hr. Uteri were pooled, extracted, and the soluble proteins electrophoresed on acrylamide gels. The increase in the induced protein ^{3}H/^{14}C ratio is plotted with the estrogen response set at 100%. General protein synthesis was determined by incorporation of injected labeled leucine into total uterine proteins during *in vivo* exposure to estrogen.

Discussion. As far as we can determine, this is the first report of an estrogen response that is not blocked by the protein-synthesis inhibitors puromycin and cycloheximide. The estrogen-stimulated increases in incorporation of nucleoside into uterine RNA as well as RNA polymerase activity in uterine nuclei are decreased to control levels by protein-synthesis inhibitors.[6] This strongly suggests that the actinomycin D-sensitive step that precedes induced protein synthesis is a primary event in the action of estrogen. This step, if it truly represents RNA synthesis, is different in two aspects from the increased RNA synthesis one measures with nucleoside incorporation into RNA. First, as mentioned above, it is not dependent on prior protein synthesis. Secondly, it occurs earlier in time than after estrogen. The data presented above indicate that accumulation of the actinomycin D-sensitive step is detectable within minutes after estrogen injection. As synthesis must have started earlier, it is apparent that about as soon as the estrogen molecules reach the uterine nuclei, new RNA synthesis is initiated. It is also apparent that the accumulation of the actinomycin D-sensitive step starts slowing down by 30 min after estrogen, implying that the rate of synthesis is decreasing by this time. This is of interest because estrogen is still accumulating in the uterine nuclei at this time and remains there for

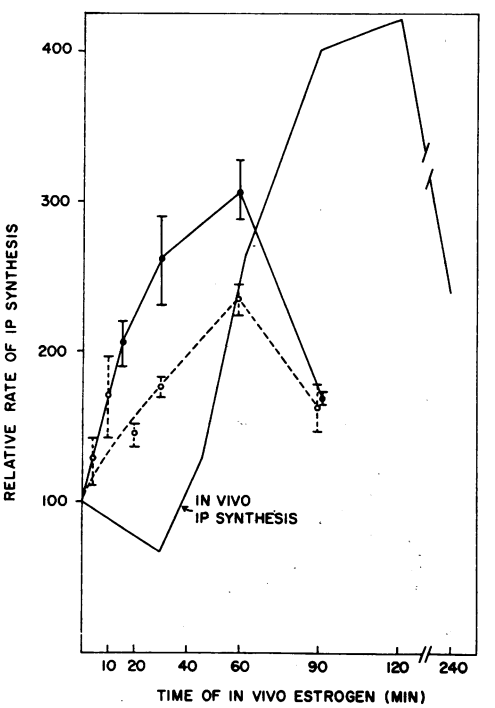

FIG. 6.—Time course for the accumulation of IP-mRNA (product of actinomycin D-sensitive step) in immature and ovariectomized rats. After estradiol-17β administration (5μg), the immature animals (3/group) were killed at 5, 10, 20, 30, 60, and 90 min intervals. The ovariectomized animals (2/group and 10 μg/rat) were sacrificed at 15, 30, 60, and 90 min. The uteri were incubated in 1.0 ml Eagle's HeLa medium containing 5 μCi of labeled amino acid (^{3}H-leucine for estrogen-treated animals and ^{14}C-leucine for controls) and 20 μg actinomycin D for 2 hr. Control and estrogen-treated uteri were combined, homogenized in 1.0 ml of EDTA (0.05%), and the resulting supernatant fraction separated by acrylamide gel electrophoresis. The ratio of dpm in the induced protein band as compared to the dpm in the gel slices above the induced protein band were calculated for both ^{3}H and ^{14}C. The ratio for ^{3}H (estrogen) was then compared to the ratio for ^{14}C (control) within a gel and the data, expressed as percentage of control, used as an estimate of relative rate of induced protein synthesis. (●), ovariectomized rats; (○), immature rats. Each point represents the average ±SE for three groups. Rate of induced protein synthesis in ovariectomized rats (—) was performed *in vivo* and is from data reported by Barnea and Gorski (1970).

several hours. This actinomycin D-sensitive step (RNA synthesis) occurs at a time when we are unable to detect any changes in nucleoside incorporation into RNA. Hamilton and his colleagues[7] have reported increased incorporation of uridine into RNA within minutes, but extensive investigations in our laboratory have failed to confirm their results.[8] We conclude, therefore, that one of the earliest responses to estrogen is the selective and specific stimulation of a limited number of RNA's. This is probably the result of the initial interaction of estrogen with uterine cytoplasmic receptor and the subsequent movement of the estrogen-receptor complex into the nucleus.[9] The simplest model is one in which a few new messenger RNA's are being synthesized in response to the estrogen-receptor complex. However, transfer RNA or even some type of ribosomal RNA cannot be ruled out.

Similar results have been reported for the induction of chromosomal puffs by ecdysone,[10] the induction of tyrosine aminotransferase in tissue culture cells,[11] and for glutamine synthetase in chick embryo retina by hydrocortisone.[12] The failure of puromycin to block this estrogen response in the uterus is also interesting in relation to reports that this inhibitor causes nonspecific side effects on the uterine vascular bed.[13] Our data, therefore, can be interpreted as indicating that neither protein synthesis nor vascular changes affect this specific RNA synthesis.

In vivo studies on the rate of synthesis of the induced protein show an initial lag phase of about 40 min followed by a marked increase in the rate of synthesis starting 45–60 min after administration of the hormone.[5] The present data imply that the synthesis of induced protein band mRNA begins without a measurable lag phase after estrogen treatment and that its accumulation is complete by 60 min after the hormone treatment. The lag phase between the accumulation of the induced protein band mRNA and its translation probably corresponds to the time necessary for translocation to the cytoplasm or other translational events. It should be pointed out that in all these studies RNA involvement is based on use of inhibitors, and no direct evidence is yet available to prove that specific RNA's are synthesized in response to estrogen.

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