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Early Estrogen Action: Stimulation of the Metabolism of High Molecular Weight and Ribosomal RNAs

(estradiol-17β/RNA isolation/uterus/ribosomal RNA precursors/actinomycin D)

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ABSTRACT **Samples of RNA, isolated from uteri of ovariectomized adult rats treated with estrogen, have been analyzed on sucrose gradients. Treatment with estrogen either for 20 min or 2 hr increased the specific activity of all classes of uterine RNA, but produced no significant alteration in the distribution of radioactivity in the gradients, when animals received [^{3}H]uridine intraperitoneally 15 min before they were killed. After labeling periods of 30 min, 1 hr, or 2 hr, however, the RNAs isolated from animals treated with estrogen had a smaller percentage of rapidly sedimenting (faster than 28S) species of RNA than did RNA from animals not treated with the hormone. The decreased percentage of high molecular weight RNA correlated with increases in both the specific activity of 28S and 18S RNA and the concentration of RNA in the whole organ. The labeled RNA of high molecular weight was also demonstrated, by the use of actinomycin D *in vivo*, to have a more rapid turnover rate in the estrogen-stimulated uterus. Our results indicate that estrogen increases not only the rate of synthesis of ribosomal RNA in the uterus of the ovariectomized adult rat, but also the rate or efficiency of processing of precursor RNA species of high molecular weight.**

One of the earliest effects of estrogen on the metabolism of the uterus of the immature or ovariectomized adult rat is an acceleration of the rate of RNA synthesis (for review, see ref. 1). Furthermore, the ability of estrogen to elicit pronounced physiological responses in the uterus appears to be mediated, at least in part, by this modification of RNA metabolism (2). Attempts have been made to isolate rat uterine RNA and to compare the rapidly labeled RNA formed in the organ stimulated by estrogen with that formed in the absence of the hormone, but the results obtained by different investigators (3-5) have been difficult to interpret. One reason for this is that the methods used in the past to extract total, and particularly nuclear, RNA from the uterus have generally resulted in the isolation of partially degraded and/or contaminated RNA samples, as indicated either by an examination of the procedures used or by the sucrose gradient analyses reported. In investigations designed to measure the effect of estrogen on the metabolism of RNA in the uterus of the ovariectomized adult rat, we have developed a procedure for the isolation of RNA from this organ that routinely yields an undegraded and uncontaminated product. The procedure is similar to that described by Joel and Hagerman (5). We now report the results of sucrose gradient analyses of isolated uterine RNA labeled *in vivo*. We conclude that estrogen very early in its action in the uterus stimulates the synthesis of all major classes of RNA. In addition, RNA samples isolated from hormone-treated animals showed a smaller percentage of labeled, rapidly sedimenting RNA species (faster than 28 S) than RNA from rats not given estrogen. This observation indicates that the rate or efficiency of processing of precursor RNA species of high molecular weight is also increased during the early action of the hormone.

MATERIALS AND METHODS

Adult Sprague-Dawley rats of uniform weight (160–180 g) and age, ovariectomized 3–4 weeks before experimentation, were used.

RNA was routinely extracted from the combined uteri of four animals by a modification of the sodium dodecyl sulfate–hot phenol procedure (6, 7). The rats were killed by cervical dislocation and their uteri were rapidly removed and dropped directly into ethanol–dry ice. The frozen tissue was then broken into small pieces, blotted, and homogenized in a mixture of 4 ml of aqueous phase (0.05 M sodium acetate, 0.05 M NaCl, 0.5% bentonite, and 0.001% polyvinyl sulfate, pH 5.1) and 4 ml of water-saturated phenol containing 0.1% 8-hydroxyquinoline at 2°C. Homogenization was with a Polytron (PT 10) tissue disintegrator (Kinematica Gmbh., Lucerne, Switzerland) run at 60 V for 60 sec. To the homogenate was added a hot (75°C) mixture of 4 ml of aqueous phase made to 1% with sodium dodecyl sulfate, and 12 ml of phenol solution. Homogenization was continued at 60 V for 30 sec. The homogenate was then stirred for 10 min at 40°C, cooled to 2°C, and centrifuged at 20,000 × *g* for 10 min. Material not homogenized sedimented to the bottom of the tube, and was designated the residue. The clear aqueous layer was carefully withdrawn, leaving the interfacial material undisturbed, and then stirred for 20 min at 5°C with an equal volume of phenol solution containing *m*-cresol (1 ml/10 ml of phenol) and 1 ml of the aqueous phase. The mixture was then centrifuged, and the resulting aqueous layer, designated the extract, was separated. Ethanol containing 2% potassium acetate (2.5 volume) was added to the extract. After the suspension stood overnight at minus 20°C, the nucleic acid precipitate was collected by centrifugation, rinsed twice with ethanol, dissolved in 1 ml of 0.05 M Tris·HCl (pH 7.4) containing 0.05 M NaCl, 1 mM $MgCl_2$, and 4 μg/ml dextran sulfate, and treated with 0.15 mg of ribonuclease-free deoxyribonuclease (Worthington Corp.) for 30 min at room temperature. The solution was then made 1% with respect to sodium dodecyl sulfate, and extracted with an equal volume of the phenol-cresol solution for 20 min at 5°C. After centrifugation the aqueous layer was separated, and the nucleic acids were precipitated again. This precipitate was dissolved in 0.01 M sodium acetate (pH 5.1) containing 1 mM EDTA and 10 μg/

TABLE 1. *Recovery of RNA, DNA, and acid-insoluble radioactivity throughout the isolation procedure*

	RNA (mg)	DNA (mg)	RNA:DNA ratio	RNA recovered (%)	Acid-insoluble radiactivity recovered (%)
Homogenate	1.48 ± 0.05	4.19 ± 0.35	0.35	100	100
Residue	0.24 ± 0.05	0.72 ± 0.08	0.33	16.2	17.0
Interfacial material	0.09 ± 0.03	1.16 ± 0.11	0.16	12.8	30.0
Extract	1.04 ± 0.05	2.10 ± 0.24	0.50	70.3	52.2
Final sample	0.77 ± 0.04	0.0	—	52.0	39.0

RNA was isolated from the combined uteri of four ovariectomized adult rats that received 200 μCi of [^{3}H]uridine (12.5 nmol in 0.2 ml of water, intraperitoneally) 15 min before killing. The total acid-insoluble radioactivity of the homogenate was 36,400 ± 6,500 cpm. The results are the means (±SE) of four replicate experiments.

ml of polyvinyl sulfate, and RNA was separated from oligodeoxyribonucleotides by four precipitations with 25% ethanol containing 2 M potassium acetate (8). The final sample of isolated RNA was washed thoroughly with 70% ethanol to remove salts.

For measurements of nucleic acid and radioactivity of the uterine homogenates, duplicate 1-ml portions were removed and the nucleic acids were precipitated by the addition of 2.5 ml of ethanol containing 2% potassium acetate. After the precipitate was washed, the nucleic acids were hydrolyzed with 0.5 N perchloric acid for 30 min at 75°C, and the hydrolyzates were extracted with ether to remove traces of phenol. Portions of these extracts were used to determine DNA (9), with calf-thymus DNA as a standard, and RNA by the orcinol reaction (10), with yeast RNA as a standard. Other portions of the hydrolyzates were counted to determine their acid-insoluble radioactivity. Total-tissue radioactivity was computed by summation of acid-insoluble and acid-soluble counts.

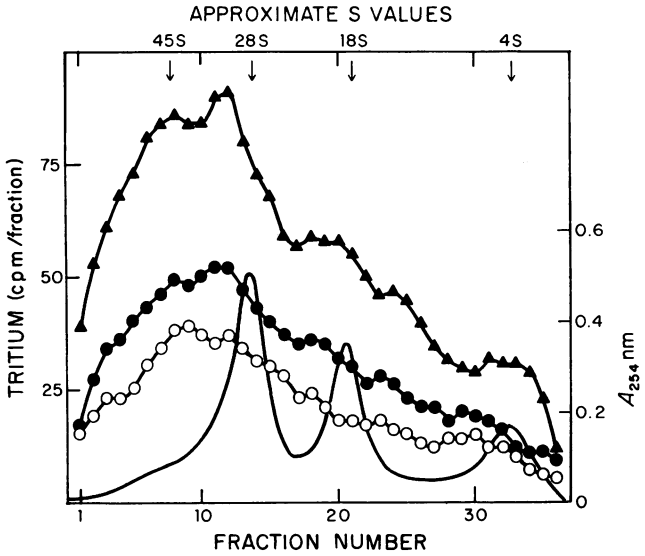

FIG. 1. Effect of estrogen on the sucrose gradient profile of rapidly labeled RNA from the uterus of the ovariectomized adult rat, as a function of time after hormone treatment. Four animals received (intraperitoneally) 20 μg of estradiol-17β in 0.2 ml of 1,2-propanediol 20 min before they were killed. Four other animals received the same dose of the hormone 2 hr before death, and four control animals received the solvent alone 20 min before death. All animals received [^{3}H]uridine, as described in Table 1, 15 min before death. Uteri of each group were combined before RNA isolation. The specific activity of the RNA from the animals treated with estrogen for 20 min was 17,400 cpm/mg, and from those treated with the hormone for 2 hr was 28,400 cpm/mg. In the absence of hormone, the specific activity was 12,400 cpm/mg. O——O, Radioactivity in the absence of estrogen; ●——●, radioactivity in the presence of the hormone for 20 min; ▲——▲, radioactivity in the presence of the hormone for 2 hr; ——, A_{254}.

Samples of labeled RNA of exactly 1 A_{260} unit, dissolved in about 0.1 ml of 0.01 M sodium acetate (pH 5.1) containing 1 mM EDTA and 10 μg/ml of polyvinyl sulfate, were layered on 5-50% (w/v) sucrose gradients (4.6 ml) prepared in 0.01 M sodium acetate (pH 5.1) containing 0.1 M NaCl and 1 mM EDTA (7), and centrifuged at 50,000 rpm for 4 hr at 4°C in the SW 65 rotor of a Beckman L2-65B ultracentrifuge. The gradients were separated into fractions of either five or six drops each (to give 36 or 30 fractions, respectively) by an ISCO (Instrument Specialties Co.) density gradient fractionator with an automatic absorbance recorder. Tritium in each fraction was counted to 3% error in a Beckman liquid scintillation spectrometer with an efficiency of 40%.

RESULTS

Table 1 shows the recovery of RNA from whole organs. With different procedures, including the one finally adopted, DNA was always extracted with the RNA and had to be removed subsequently. Although about 70% of the RNA was extracted from the tissue, the final recovery of RNA, after DNA was eliminated, averaged 52%. No DNA or protein could be detected in the isolated RNA samples by sensitive colorimetric (9, 11) and spectrophotometric (12) methods. The final recovery of rapidly labeled RNA, formed *in vivo* during a 15-min pulse of [^{3}H]uridine, averaged 39%. Attempts to improve the yield of RNA, either by means of more complete homogenization of the uterine tissue or by an increased temperature of extraction, led to the recovery of partially degraded products.

Fig. 1 shows the early and later effects of estrogen administered *in vivo* on the radioactivity and absorbancy profiles of sucrose gradient analyses of labeled RNA isolated from the uteri of ovariectomized rats that received [^{3}H]uridine 15 min before they were killed. The RNA with the highest specific activity formed during this short labeling period had a high molecular weight, which indicated that minimal degradation of the isolated RNA had occurred. The absorbancy profiles of

TABLE 2. *Time course for the effect of estrogen on the ratio of RNA to DNA and the uptake and incorporation of [^{3}H]uridine into acid-insoluble material and RNA in the uterus of the ovariectomized rat*

Time after treatment (hr)	RNA:DNA ratio	Total-tissue radioactivity (cpm/mg DNA)	Acid-insoluble radioactivity (%)	Specific activity of isolated RNA (cpm/mg)
		Animals given [^{3}H]uridine without estrogen:		
0.5	0.34	80,900	14	17,500
1	0.36	95,700	18	43,100
2	0.34	128,000	23	52,700
8	0.35	180,500	28	120,000
		Animals given estrogen and [^{3}H]uridine:		
0.5	0.35	100,400	19	27,800
1	0.38	115,900	24	68,300
2	0.43	166,200	30	81,100
8	0.62	225,700	36	94,800

The details of experimentation are described in Fig. 2.

the RNA samples were identical. Estrogen administered 20 min before rats were killed, followed by [^{3}H]uridine 5 min later, caused a 40% stimulation of the specific activity of the RNA. In animals given estrogen 2 hr before they were killed, followed by [^{3}H]uridine 1.75 hr later, the specific activity of the RNA was increased 130%. Apart from the increased specific activity, however, no differences in the shapes of the radioactivity profiles shown in Fig. 1 could be attributed to the hormone. In other similar experiments, rapidly labeled RNA from uteri stimulated by the hormone was also found to give the same pattern of distribution on sucrose gradients as those described in Fig. 1.

We next examined the effect of estrogen, as a function of time, on the radioactivity and absorbancy profiles of RNA isolated from uteri of ovariectomized rats that received the hormone and [^{3}H]uridine together. Table 2 shows the ratios of RNA to DNA in the various uterine homogenates, as well as the specific activities of the RNA isolated. As expected, estrogen increased the concentration of RNA in the uterus, and also increased the specific activity of the RNA isolated 30 min, 1 hr, or 2 hr after hormone treatment (13). The higher specific activity of the RNA from animals that received [^{3}H]uridine for 8 hr, compared to that from animals that received both estrogen and the labeled precursor for 8 hr, is a consequence of the much larger amount of unlabeled RNA synthesized at later times in the hormone-stimulated uterus. This RNA dilutes the labeled RNA formed initially. In 8 hr, the concentration of RNA in the uterus increased about 77% in response to the hormone *in vivo*, and more than half of this increase occurred between 2 and 8 hr (Table 2).

The profiles shown in Fig. 2 are those obtained from sucrose gradient analyses of the same RNA samples described in Table 2. The absorbancy profiles of all of the RNA samples analyzed were the same. The radioactivity profiles, however, reflected differences in the relative amounts of the various classes of labeled RNA present in the uterus at various times after the injection of [^{3}H]uridine. After only 30 min of exposure to [^{3}H]uridine (Fig. 2*A*), most of the labeled RNA formed had a high molecular weight (faster than 28S). But, as the time of exposure to [^{3}H]uridine increased, the amounts of labeled 28S, 18S, and 4S RNA increased and the amount of labeled RNA of high molecular weight decreased (Fig. 2*B–D*). These observations, made in the absence of estrogen, are in accord with the current concept of the formation of 28S and 18S ribosomal RNAs from a precursor RNA of high molecular weight (45S) (6, 14, 15). The radioactivity profiles shown in Fig. 2 further reveal that the rate at which the concentration of labeled RNA of high molecular weight decreased, in parallel with the increased labeling of 28S and 18S RNA, was accelerated by the hormone. The ratio of the radioactivity in fraction 12 (about 28 S) to that in fraction 6 (about 45 S) in Fig. 2*A–D* increased, respectively, 1.2, 1.4, 1.7, and 4.8 in the absence of the hormone, whereas the corresponding ratios are 1.3, 1.6, 2.8, and 4.7 in the presence of the hormone. These experiments have been repeated several times, and in each case the characteristic differences in the labeling patterns of the RNAs shown in Fig. 2*A–C* were observed.

We also performed experiments in which RNA was synthesized normally in either the control or the estrogen-stimulated uterus for 30 min in the presence of [^{3}H]uridine, and then further synthesis was blocked by treatment with actinomycin D. Maturation of the labeled RNA initially formed was then allowed to proceed for about 90 min before rats were killed and their uterine RNA isolated. Table 3 shows that treatment with estrogen alone for 3 hr increased the specific activity of the RNA about 400%. Actinomycin D given 30 min after [^{3}H]uridine did not affect the uptake of [^{3}H]uridine by the uterus, but decreased its incorporation into acid-insoluble material by 50%, whether or not the organ was stimulated by the hormone. However, when actinomycin D was administered at the same time as the hormone or its solvent (1,2-propanediol) alone, the specific activities of RNA from the control and hormone-treated animals were depressed 97 and 93%, respectively.

The sucrose gradient profiles of RNA samples isolated from animals given [^{3}H]uridine and then actinomycin D (see Table 3) are shown in Fig. 3. In the absence of actinomycin D, it was again observed that the relative amount of labeled RNA of high molecular weight (faster than 28 S) was greater in the control uterus than in the hormone-stimulated organ. The ratio of the radioactivity in fraction 12 (28 S) to that in fraction 6 (45 S) was 2.3 for the control and 3.4 as a result of estrogen action (Fig. 3*A*). Fig. 3*B* also shows that the ratio

of the radioactivity in fraction 12 to that of fraction 6 was 1.6 in the absence, and 3.7 in the presence, of estrogen. This result demonstrates that during the incubation for 90 min with actinomycin D after [^{3}H]uridine had been administered, more of the rapidly labeled RNA of high molecular weight formed in the hormone-stimulated uterus was processed than was the RNA in the control organ, which indicates that these precursor RNA species have an accelerated rate of turnover during early estrogen action.

DISCUSSION

Many investigations have demonstrated that a major feature of several growth-promoting and developmental hormones acting in their target organs is an acceleration of ribosome formation (1, 16). For example, Moore and Hamilton (17) have found that estrogen increases both the rate of synthesis and the cytoplasmic concentration of monomeric ribosomes in the uterus of the ovariectomized rat. The capacity of the uterus to make more ribosomes in the presence of the hormone than in its absence must derive from an increase in the rate of one or more of the several steps known to be involved in the overall process of the formation of a mammalian ribosome (18). Among these are the synthesis of 45S RNA and of ribosomal proteins, the methylation and processing of the RNAs and their combination with proteins, and the passage of the subribosomal particles into the cytoplasm. The results described in this paper, and especially those presented in Figs. 1 and 2, suggest that the rise in the rate of ribosome synthesis observed (17) in the estrogen-stimulated uterus results from an acceleration of the rate of formation of 45S RNA.

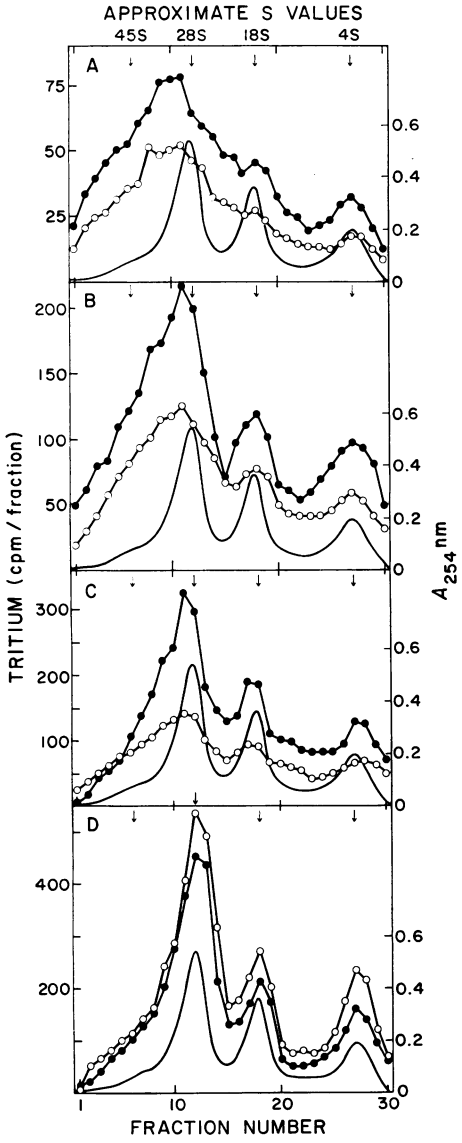

FIG. 2. Radioactivity and absorbancy profiles of RNA isolated from the uterus of the ovariectomized rat, as a function of time after administration of estrogen and [^{3}H]uridine together. The experimental details were the same as described in Fig. 1, except that the experimental animals received hormone and the precursor at time zero, and were killed 30 min (*A*), 1 hr (*B*), 2 hr (*C*), or 8 hr (*D*) later. O——O, Radioactivity in the absence of estrogen; ●——●, radioactivity in the presence of the hormone; ——, A_{254}.

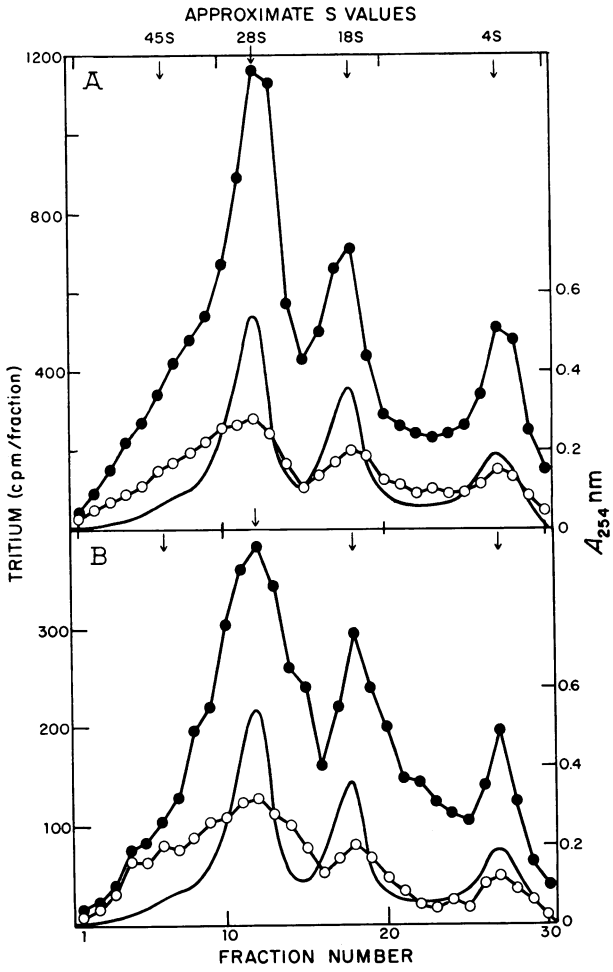

FIG. 3. Combined effect of estrogen and actinomycin D on the radioactivity and absorbancy of RNA isolated from the uterus of the ovariectomized rat. Two groups (*A*) of animals received estrogen or solvent alone for 3 hr and the labeled precursor for 2 hr before they were killed. Two other groups (*B*) were given the hormone or solvent and the precursor, but received actinomycin D 90 min before they were killed. O——O, Radioactivity in the absence of estrogen; ●——●, radioactivity in the presence of the hormone; ——, A_{254}.

TABLE 3. *Effect of actinomycin D on the ratio of RNA to DNA and the uptake and incorporation of [^3H]uridine into acid-insoluble material and RNA in the estrogen-stimulated uterus of the ovariectomized rat*

Treatment	RNA:DNA ratio	Total-tissue radioactivity (cpm/mg DNA)	Acid-insoluble radioactivity (%)	Specific activity of isolated RNA (cpm/mg)
		Without actinomycin D:		
Controls	0.35	83,700	27	40,300
Estrogen-treated	0.48	163,500	35	201,600
		Actinomycin D given 30 min after [^3H]uridine:		
Controls	0.34	93,500	13	24,800
Estrogen-treated	0.41	163,600	17	78,800
		Actinomycin D given 60 min before [^3H]uridine:		
Controls	0.35	54,800	6	2,600
Estrogen-treated	0.36	81,600	8	4,400

12 rats received estradiol-17β and 12 solvent only 3 hr before they were killed, followed by [^{3}H]uridine (200 μCi; 25 nmol in 0.4 ml of water) 1 hr later. Four of the hormone-treated animals and four of the controls also received 0.5 mg of actinomycin D in 0.2 ml of isotonic saline, 90 min before they were killed. Four hormone-treated and four control rats also received the same dose of actinomycin D, together with an injection of the hormone or the solvent alone 60 min before they received [^{3}H]uridine.

The observation of a larger amount of labeled RNA of high molecular weight in the uterus in the absence of estrogen, compared to that in the organ stimulated by the hormone, may be consistent with the very slow turnover of ribosomes known to occur in the atrophied organ in the absence of the hormone (16, 17). Although our data do not distinguish between effects of the hormone on ribosomal precursor RNAs versus heterogeneous, rapidly sedimenting RNAs, precedents for our observation have been reported. Cooper (19) has shown that in the resting human lymphocyte, a large amount of labeled 45S RNA is formed that is subsequently degraded without ever entering the cytoplasm. Furthermore, stimulation of RNA synthesis in the lymphocyte by phytohemagglutinin results from an increase in the rate of 45S RNA synthesis, coupled with a reduction in the wastage of ribosomal RNA. Chaudhuri and Lieberman (20) have also indicated that in regenerating rat liver a more efficient use of 45S RNA appears to facilitate a substantial increase in the rate of ribosome formation, as compared to normal liver. Green, Bunting, and Peacock (21), working with explants of mouse mammary glands, observed an increase in the relative amount of labeled high molecular weight RNA as a result of hydrocortisone stimulation. Petri, Fristrom, Stewart, and Hanly (22) have noted that the addition of ecdysone to imaginal discs of *Drosophila* cultured *in vitro* stimulated the net amount of ribosomal RNA synthesized, and increased the rate of processing of 38S RNA into 28S and 18S ribosomal RNAs.

Two interpretations of the results of our experiments seem most reasonable. First, estrogen stimulates the rate of conversion of ribosomal precursor RNAs into 28S and 18S RNA. Second, estrogen increases the efficiency of utilization (in other words, decreases the wastage) of the RNA of high molecular weight. Whatever the mechanism responsible for producing the altered labeling pattern of uterine RNA during early estrogen action, we conclude that it is most probably concerned with the regulation of ribosome formation.

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