²² Suhrland, L. G., and A. S. Weisberger, *J. Lab. reg Clin. Med.*, **60**, 1022 (1962).

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THE NATURE OF THE RNA RESPONSE TO ESTRADIOL ADMINISTRATION BY THE UTERUS OF THE RAT*

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Over the past several years abundant evidence has accrued to indicate that the administration of sex hormones results in an increase both in the content²⁻⁶ and in the rate of synthesis⁷ of ribonucleic acid (RNA) in the accessory sex tissues. Furthermore, although evidence has been presented to the contrary,⁸ several kinds of studies have indicated that the enhancement of protein synthesis by these hormones is secondary to the increased rate of RNA synthesis. First, the biochemical site of action of testosterone on protein synthesis in rat seminal vesicle and of estradiol on protein synthesis in hen oviduct has been localized to the RNA-rich microsomes.⁹⁻¹¹ Second, inhibition of protein synthesis does not interfere with the estrogen-induced acceleration of RNA synthesis.¹² Finally, the addition of synthetic polyribonucleotides has been shown to accelerate the synthesis of protein in homogenates of control but not of testosterone-treated prostate.¹³

The implication that the acceleration of protein synthesis following hormone administration is secondary to enhanced RNA synthesis does not of itself, however, explain the mechanism by which protein synthesis is accelerated. At least three different types of RNA are involved in protein synthesis—soluble or transfer RNA, ribosomal structural RNA, and messenger or template RNA. That transfer RNA is not rate-limiting for protein synthesis in these hormone-responsive tissues has been suggested by two types of experiments—measurements of transfer C¹⁴-RNA-amino acids in slices of rat seminal vesicle,⁹ and the demonstration that no soluble factors appear to be rate-limiting in protein synthesis by homogenates of hen oviduct.^{10, 11} Furthermore, the studies of Liao and Williams-Ashman indicate that template RNA is rate-limiting for protein synthesis in the prostate of the castrated rat.¹³

The present communication describes studies on the sequence and nature of the RNA response to estradiol administration by the immature rat uterus. Evidence is presented that the administration of estradiol initially enhances the synthesis of transfer RNA and of template RNA, and that enhanced synthesis of ribosomal structural RNA is a late effect.

Materials and Methods.—Female Sprague-Dawley rats, weighing 100–150 gm, were used for the experiments. Oophorectomies were performed under ether anesthesia; 10–20 days following the operation the animals were given by injection into the tail vein either 10 μ g of estradiol-17 β dissolved in 1 ml of 0.9% saline or the saline alone. The animals were killed by decapitation, and the uteri were excised and immediately chilled. For each experiment the tissues from 20 to 50 animals were pooled. The subscripts E and C refer to the source—either estradiol-treated or control uterus—of the fractions studied.

Incubation and extraction techniques: The chilled tissues were sliced with a McIlwain tissue slicer, blotted, weighed, and transferred to incubation tubes containing 4×10^{-4} M adenine-8-C¹⁴ (2.1×10^6 cpm), 6×10^{-3} M glucose, and Krebs-Ringer-bicarbonate buffer, pH 7.4, to make a final volume of one ml. After gassing with 95% oxygen–5% carbon dioxide, the tubes were capped and incubated in one of two ways. In the pulse experiments the incubations were stopped after 30 min; in the chase experiments one μ mole of adenine was added to the tubes after 30 min, and the incubation was continued for three hr. Following incubation 10 ml of the cold Krebs-Ringer-bicarbonate buffer was added, and the tubes were centrifuged for 30 seconds.

An RNA extraction procedure similar to that described by Perry¹⁴ was used. The pellets were suspended at 0° in 2 ml of 0.01 M acetate buffer, pH 5.0, containing 0.5% recrystallized sodium dodecyl sulfate, 0.1 M NaCl, and 10^{-3} M MgCl₂. The extracts were shaken for 3 min at 60°C with 3 ml of freshly distilled, water-saturated phenol, quickly chilled, and centrifuged. The aqueous phase was removed and extracted twice more with phenol at 60°C. The final extract was shaken five times with ether to remove the phenol, and the RNA was precipitated with 2 volumes of ethanol at –20°C. The RNA precipitate was dissolved in 2 ml of acetate-NaCl-MgCl₂ buffer and passed through a Sephadex G25 column (1×20 cm). The RNA was eluted with 4–9 ml of the buffer and again precipitated at –20°C with 2 volumes of ethanol. In experiments in which the messenger activity was assayed, the RNA was dialyzed in the cold for 2–6 hr against 100 volumes of acetate-NaCl-MgCl₂ buffer containing 0.1% mercaptoethanol and again precipitated at –20°C with ethanol. The phenol extraction procedure of Kirby,¹⁵ as described by Hoagland *et al.*,¹⁶ was used in some experiments; no difference was noted between the two procedures either in the centrifugation pattern or in the messenger activity of the final RNA preparation.

The RNA was then dissolved in 0.2 ml of the acetate-NaCl-MgCl₂ buffer and applied to a 5–20% sucrose gradient according to the method of Britten and Roberts.¹⁷ The preparations were centrifuged at 27,000 rpm for 8–11 hr at 5°C in a Spinco SW 39 swinging bucket rotor. After completion of the centrifugation a hole was punched in the bottom of each tube, and 10 or 20 drop fractions were collected as described by Szybalski.¹⁸ Absorbances at 260 m μ were determined, and portions of each sample were added to 10 ml of the scintillation liquid described by Bray¹⁹ and assayed for C¹⁴ in a Packard liquid scintillation counter. In experiments in which individual peaks of RNA were assayed for messenger activity several fractions were combined, precipitated with ethanol as before, dried under nitrogen, dissolved in 0.1 ml of buffer, and assayed in the amino acid incorporating system.

Preparation of amino acid incorporation system: The system employed was essentially that of Keller and Zamecnik²⁰ as described by Maxwell.²¹ Fresh liver microsomes and pH 5 enzymes were prepared daily from two female Sprague-Dawley rats weighing 100–150 gm each. All quantities of fractions are expressed as gram equivalents (gm eq), i.e., the weight of liver from which the particular fraction was derived. Incubation mixtures routinely contained 0.15 M sucrose, 0.07 M KCl, 0.1 M tris buffer pH 7.3, 5×10^{-3} M MgCl₂, 5×10^{-3} M mercaptoethanol, 5×10^{-4} M ATP, 5×10^{-5} M GTP, 1×10^{-2} M potassium phosphoenolpyruvate, 100 μ g crystalline pyruvate kinase (California Corp. for Biochemical Research), 5×10^{-5} M C¹⁴-amino acid (1×10^6 cpm), 1×10^{-4} M each of 19 L-amino acids minus the C¹⁴-amino acid, 0.2 gm eq microsome, 1.0 gm eq pH 5 fraction, and RNA as indicated. The final volume was 1.0 ml, and incubation was performed at 37° for 30 min. Protein was precipitated by the addition of 1 ml of 1 N perchloric acid, and the precipitate was washed according to the method of Hoagland *et al.*¹⁶ The final precipitate was dissolved in 0.5 ml of 0.5 N NaOH, added to 10 ml of the scintillation solution described by Bray,¹⁹ and assayed for C¹⁴ in a Packard liquid scintillation counter.

Results and Discussion.—In an attempt to characterize the pattern of response of RNA synthesis to steroid hormone administration, the influence of a single in-

FIG. 1.—Sedimentation diagrams of uterine RNA from castrated (control) rats and from rats administered estradiol either 4 or 24 hr previously. The left hand part of each diagram represents the bottom of the tube and consequently the faster sedimenting RNA components. Each diagram represents the pooled tissue from seven rats.

travenous injection of 10 μ g of estradiol-17 β on the synthesis of the various fractions of RNA was studied in slices of uterus from castrated rats (Fig. 1). After a 30-min pulse with adenine-8-C¹⁴ (upper half) or a 30-min pulse with adenine-8-C¹⁴ followed by a three-hr chase with adenine (lower half), the RNA was separated by the phenol extraction technique of Perry¹⁴ and centrifuged for 10 hr in a sucrose gradient density. Fractions were collected and analyzed both for radioactivity (the solid line) and for RNA concentration (the dotted line). In these diagrams, fraction 1 represents the bottom of the tube.

In the pulse experiment of the four-hr treated uteri (upper middle panel) there was a striking increase in the incorporation of adenine-C¹⁴ into the phenol extractable RNA; the radioactivity was localized in both the control and treated samples to a peak in the 4–6 S region corresponding to transfer RNA.¹⁴ No radioactivity and only traces of RNA were detected in the regions which, according to Perry¹⁴ correspond to ribosomal structural RNA (approximately 18 and 32 S). However, 24 hr after estradiol treatment (upper right panel) a major portion of the isolated RNA consists of these two peaks of ribosomal structural RNA.

The results of the studies of pulse labeling followed by a three-hr chase (lower half, Fig. 1) are similar to the pulse experiments; in each instance, the major peak of RNA-C¹⁴ is in the 4–6 S region. However, at the end of the 3 $\frac{1}{2}$ -hr incubation, small amounts of radioactivity were found in the regions of structural RNA in the slices from the animals treated four hr previously (lower middle panel), and larger quantities of radioactivity were detectable in the regions in the uterine slices from

animals injected 24 hr previously with estradiol (lower right panel). Thus, it was concluded that the estradiol-induced increase in the synthesis of ribosomal structural RNA occurs as a relatively late event, several hr after demonstrable increases in the synthesis of 4-6 sRNA.

The probable explanation for this relatively late increase in the synthesis of ribosomal structural RNA can be derived from study of the histological changes of the uterine mucosa during estradiol treatment. From studies of RNA synthesis in tissue cultures of L strain fibroblasts, Perry has demonstrated that soluble RNA and a rapidly labeled labile RNA are synthesized in the chromatin of the nucleus; ribosomal structural RNA, on the other hand, appears to be synthesized in the nucleolus of the nucleus.¹⁴ Moreover, it has previously been reported that, following testosterone administration to castrated rats, the shrunken nucleoli of the prostate undergo hypertrophy²² and that the nucleolar volume of the uterus varies during the estrous cycle.² The histological studies demonstrated in Figure 2 were undertaken in an attempt to correlate the temporal sequence of the appearance of ribosomal structural RNA and the enlargement of the nucleoli of the uterus following estradiol administration. In both the hematoxylin and eosin and the azure-blue stained sections, it is quite evident that the nucleoli of uterine endometrium from the immature castrated rat are small and dense; the epithelium is cuboidal in nature, and the cytoplasm contains only trivial quantities of basophilic material. It is noteworthy that four hr following estradiol administration the nucleoli remain dense and pyknotic, and there is no evidence of transformation from the cuboidal to the columnar type of epithelium. By the end of the 24 hr period, however, striking changes have occurred. The nucleoli are large and pale, the epithelium has completed the transition to the columnar state, and the cytoplasm is laden with dense basophilic material. Thus, the enlargement of the endometrial nucleoli, the appearance of an RNA-rich cytoplasm, and the increase in the content of the ribosomal structural RNA all occur after marked increases in the synthesis of other RNA components. This demonstration is entirely consistent with the view that the synthesis of ribosomal structural RNA in uterus is a relatively late event following estradiol treatment. It should be noted that these results might be construed to be at variance with the study of Liao and Williams-Ashman,¹⁸ who demonstrated that ribosomes (unsaturated with template RNA) continued to be present in rat prostate following castration. However, these investigators studied rats at a much shorter interval after castration (2-3 days), and it is possible that the cytoplasmic elements with slow turnover times had not had time to atrophy.

Next, an attempt was made to determine whether the RNA, synthesized within a four-hr period following estradiol treatment, included RNA with template activity (Table 1). Phenol-extractable uterine RNA from castrated and four hr estradiol-treated rats was assayed for template activity in the mammalian messenger RNA assay system as described by E. S. Maxwell;²¹ this system consists of rat liver microsomes and pH 5 enzyme, ATP, GTP, an ATP generating system, and a mixture of amino acids. As shown in Table 1 the addition of RNA from estradiol-treated uterus enhanced amino acid incorporation into protein about 50 per cent when lysine-C¹⁴ was used as the tracer and about 75 per cent using histidine-C¹⁴, whereas equal amounts of uterine RNA from the castrated control had no effect on amino acid incorporation.

Fig. 2.—Photomicrographs of the uterine mucosa from castrated (control) rats and from rats administered estradiol either 4 or 24 hr previously. In each instance the magnification is 800 X.

The influence of four additional preparations of phenol-extracted RNA from control and four-hr estradiol-treated uterus on amino acid-C¹⁴ incorporation by rat liver enzymes is illustrated in Table 2. Using three different C¹⁴-labeled amino

TABLE 1		
INFLUENCE OF UTERINE RNA ON AMINO ACID INCORPORATION BY RAT LIVER ENZYMES		
System	C ¹⁴ incorporation into protein from;	
	L-Lysine-U-C ¹⁴ (cpm)	L-Histidine-U-C ¹⁴ (cpm)
Complete system	92	76
Complete system plus RNA _E	136	130
Complete system plus RNA _C	96	66
Complete system minus microsomes	12	0
Complete system minus pH 5 enzyme	34	6
Complete system minus ATP, GTP, and pyruvate kinase	4	22

Reaction mixture is described in *Methods*. The quantities of RNA_E and RNA_C were 1.1 and 1.4 OD₂₆₀ units, respectively.

TABLE 2
INFLUENCE OF VARIOUS PREPARATIONS OF UTERINE RNA ON AMINO ACID INCORPORATION BY
RAT LIVER ENZYMES

Exp. no.	C ¹⁴ amino acid	Per cent enhancement by added RNA	
		RNA _E	RNA _C
1	Lysine	20	11
	Histidine	18	1
2	Leucine	37	19
3	Leucine	20	0
4	Lysine	31	17

Reaction mixture is described in *Methods*. The quantities of added RNA_E and RNA_C varied from 0.9 to 1.3 OD₂₆₀ units; in each instance, the results have been expressed as per cent enhancement per 1.0 OD₂₆₀ unit.

acids in the assay system, the enhancement by the RNA_E varied from 18–37 per cent, whereas the addition of the control RNA produced 0–19 per cent enhancement. Although the ability to enhance protein synthesis did vary among preparations, it should be emphasized that in every instance the enhancement was at least twice as great in the four-hr estradiol-treated tissues as in the control preparation. While these results are consistent, the degree of enhancement is not nearly so large as has been reported by Nirenberg and Matthaei for synthetic polyribonucleotides²³ or by Barondes, Dingman, and Sporn for mammalian messenger RNA²⁴ in bacterial assay systems; the results reported here (18–73 per cent enhancement/OD₂₆₀ RNA_E) are, however, comparable to those reported for rat liver template RNA in rat liver enzymes by Maxwell.²¹ The difference between the bacterial assay system and the mammalian assay system is probably twofold; the mammalian assay system is partially saturated with endogenous template RNA, and the naturally occurring template RNA may be either altered or partially damaged during the extraction and purification. That the latter is a likely possibility is suggested by the recent demonstration by Singer, Jones, and Nirenberg that the template activity depends, in part, on the ordered secondary structure of the polyribonucleotide.²⁵ Thus, the assay of the template activity of these RNA preparations cannot be regarded as absolute measurements of the template RNA content of the intact tissue in question, and it is possible that the magnitude of the increased template activity of uterine RNA within four hr following estradiol treatment is much greater than has been demonstrated in these experiments.

Studies which further characterize the template RNA activity of rat uterus are shown in Figure 3. When the various RNA fractions obtained following pulse labeling with adenine-C¹⁴ and gradient density centrifugation were assayed for their ability to enhance amino acid incorporation (the dotted lines), all the template activity was found plastered to the bottom of the tube (here indicated as tube 0), indicating that in this tissue, at least, messenger RNA must be greater than 32 S in size. This observation confirms the report by Barondes, Dingman, and Sporn that the template RNA of rat liver nuclei is at least 40 S in size.²⁴

As the result of these studies, three general conclusions may be drawn. First, a direct demonstration has been made that uterine RNA with template activity, as assayed in an amino acid incorporating system from rat liver, is increased within a four-hr period following estradiol administration to castrated rats. Second, although the synthesis of all RNA components does increase following estradiol administration, the synthesis of RNA in the nuclear chromatin appears to increase first, resulting in an increase both in template RNA and in transfer RNA; the

FIG. 3.—Sedimentation diagrams of template activity of uterine RNA from castrated (control) rats and from rats administered estradiol either 3 or 4 hr previously. Each diagram represents the pooled tissue from 10 rats. The extractions and centrifugations were performed following a 30 min pulse labeling of uterine slices with adenine-8- C^{14} . In each instance radioactivity was obtained in tube 0; however, due to the method of collection it is impossible to prevent contamination of the bottom of the tube with radioactivity from the last samples collected. As a consequence, no radioactive data were reported for these tubes.

enhancement of the synthesis of ribosomal structural RNA, on the other hand, appears to be a somewhat later event. Finally, since transfer RNA is not rate-limiting for protein synthesis in accessory sex tissue,⁹⁻¹¹ it seems justifiable to conclude that the early acceleration of protein synthesis in this tissue by estradiol administration is secondary to an increase in template RNA.

Summary.—On the basis of histological and chemical studies it is concluded that following estradiol administration chromosomal RNA synthesis is enhanced, as the result of which transfer RNA and template RNA activities are increased. The subsequent enhancement of the nucleolar RNA—the structural RNA of the ribosomes—appears to be a relatively late event. Finally, since the enhancement of RNA synthesis in the nuclear chromatin is an early result of estradiol action, it is likely that the syntheses of other RNA components and of protein are subsequent responses.

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COUPLED PHOTOREDUCTION OF UBIQUINONE AND
PHOTOOXIDATION OF FERROCYTOCHROME C CATALYZED BY
CHROMATOPHORES OF RHODOSPIRILLUM RUBRUM*. †

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A light-induced reaction between bacteriochlorophyll and a cytochrome of type *c* in *Chromatium* cells has been reported by Chance and Nishimura,¹ who showed a temperature-independent oxidation (down to 80°K) of the cytochrome under anaerobic conditions at a rate reported to be 0.39 μ M Fe⁺⁺/sec. Duysens² had earlier described a light-induced oxidation of cytochrome in *Rhodospirillum rubrum* under anaerobic conditions. In the experiments of Duysens, as well as in those of Chance and Nishimura, cytochrome was again reduced in the dark. Clayton³ showed that the light-induced oxidation of cytochrome in *Chromatium* chromatophores is closely connected to the absorption changes of a special component of bacteriochlorophyll. Evidence to date therefore favors a cytochrome as the electron donor reacting directly with bacteriochlorophyll upon illumination.

Ubiquinone (UQ)† has been implicated as a primary electron acceptor in bacterial photosynthesis by Clayton,⁴ who related the light-induced absorption changes in the 240–350 $m\mu$ range to the reduction of UQ in chromatophores from *Chromatium*, *R. rubrum*, and *Rps. spheroides*. He also showed there was almost a 1:1 relation-