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ROLE OF RNA IN THE REGULATORY ACTION OF ESTROGEN

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Estrogens, as well as other steroid hormones, may exert their stimulatory effect on target tissues by initial activation of RNA synthesis.[1, 2] This concept is supported by the observation that estrogen stimulation of the uterus leads quickly to an increase in the rate of incorporation of precursors into RNA,[3, 4] the initial increase being noted in the nuclear fraction of RNA.[5] The uterotrophic action of estradiol-17β is prevented by the metabolic inhibitor actinomycin D either administered systemically[6] or by injection into the lumen of the uterus.[7] The action of estrogen on the vagina can be prevented also by topical application of actinomycin.[8] A mechanism for the effect of estradiol on RNA synthesis has been suggested by the identification of a macromolecular fraction of the cell which possesses receptors for the hormone. This fraction, when not combined with the hormone, inhibits the activity of RNA polymerase. When the hormone is present, the inhibition is released.[9] The biochemical evidence that the biosynthesis of RNA by rat uteri is immediately accelerated by estrogen suggests that the stimulating hormone may have no further role in causing the transition from the atrophic to the stimulated state. To test this postulate, RNA was extracted from the uteri of estrogen-stimulated rats and administered by local, intraluminal application to estrogen-deprived ovariectomized rats. The resultant morphologic changes in treated uteri are indistinguishable from those that occur following the administration of estrogen.[10]

Materials and Methods.—Preparation of total RNA extract: Method A: Female rats of the Sprague-Dawley strain weighing 180–200 gm were ovariectomized, and the certainty of anestrus was established by following the vaginal smears for at least two weeks. Each batch of RNA was prepared from the uteri of at least ten ovariectomized females but frequently as many as 100 were used. Each animal was injected intravenously (tail vein) with 10 μg estradiol-17β per 1 ml 1% ethyl alcohol in saline. After 4 hr, the animals were sacrificed by decapitation; the uteri were removed and quickly frozen. In the 4°C cold room, the uteri were homogenized with a Virtis homogenizer in 10 times volume of saline, with or without 0.001 *M* $MgCl_2$. The tissue brei was filtered through one layer of cheesecloth and mixed with an equal volume of water-saturated phenol. The mixture was stirred vigorously on a magnetic stirrer for 30 min at room temperature, and the stirring was repeated for 30 min in the 4°C cold room. It was centrifuged at 2,000 gravities for 30–60 min at 2–4°C, and the top aqueous layer was saved. The other layers were washed once with an equal volume of saline, centrifugation was repeated, and the saline was combined with the original supernatant with extreme care to avoid carrying over any saline-

phenol interphase. Potassium acetate was added to a final concentration of 2% and 2 vol of chilled 95% ethyl alcohol were mixed. The mixture was agitated well by hand shaking and placed at −20°C for 2 hr. It was then centrifuged at 1,750 gravities for 15 min at 2–4°C. The supernatant fluid was discarded and the precipitate dissolved in cold physiological saline and centrifuged. Reprecipitation with 2 vol of 95% ethyl alcohol and 2% potassium acetate was followed by centrifugation as before. The precipitate was dissolved in physiological saline and the optical density determined spectrophotometrically at 260 mμ. Concentration was estimated on the basis of 1 optical density unit equivalent to 45 μg.[11] For all preparations, extinction ratios at 280/260 mμ were determined and in most instances lay between 0.47 and 0.49. Maximal absorption is at 258–259 mμ, and minimal at 230 mμ.

Most RNA extracts were used in experiments within a few days of preparation. One preparation was stored frozen in saline at −20°C at a concentration of 750 μg/ml, and after three months retained biologic activity.

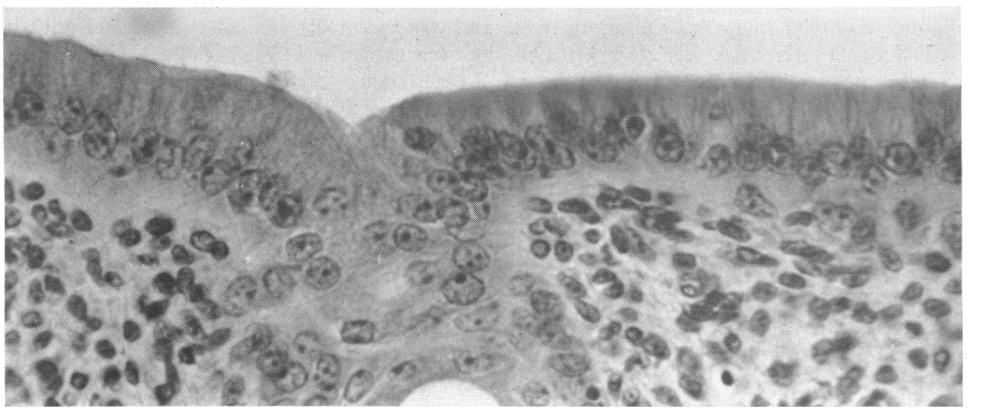
Method B: Two batches of uterine RNA were made by a different procedure intended to preserve nuclear RNA. The uteri were homogenized in 0.01 *M* acetate buffer pH 5.0 containing 0.1 *M* NaCl, 0.001 *M* $MgCl_2$, and 0.5% sodium lauryl sulfate. After an equal volume of phenol was added, the mixture was agitated for 30 min at room temperature and then placed at 60°C for 5 min; it was centrifuged for 60 min at 2,000 gravities at 4°C. In the cold room, the phenol phase was removed by suction and discarded. The interphase was suspended in 0.01 *M* Tris (hydroxymethylaminomethane) buffer pH 8.5 and was repeatedly treated with phenol until it formed a thin interphase. An alcohol precipitation was carried out as in *Method A*, and the precipitate was dissolved in 0.01 *M* tris buffer pH 7.4 containing 0.1 *M* NaCl and 0.001 *M* $MgCl_2$. This solution was treated with DNase (Worthington Biochemical Corp.) at a final concentration of 10 μg/ml for 30 min at 40°C. A second alcohol precipitation was then performed and the final precipitate was dissolved in saline. Following this procedure, the 280/260 mμ extinction ratio was 0.50, maximal absorption was 258–259 mμ, and minimal absorption was 230 mμ. Material prepared by *Methods A and B* was indistinguishable in the biological tests used, so that it is not separately identified in the report of results. Total RNA obtained by either extraction procedure from the uteri of estrogen-treated rats is designated U-RNA^e, from liver or vagina of the same animals L-RNA^e and V-RNA^e, respectively, and from uteri of ovariectomized rats *not* treated with estrogen, U-RNA^n. Regardless of tissue source, concentration of extract was always adjusted to 750 μg/ml at 260 mμ.

Uterine catheterization and infusion: Female rats (Sprague-Dawley) weighing 150–180 gm were ovariectomized, and daily vaginal smears recorded for two weeks. Only totally anestrous animals were used. With the animal under ether anesthesia, the tubal end of the uterus was exposed by a dorso-lateral incision. The uterine wall was nicked with iris scissors. A fine polyethylene tubing (Clay-Adams Intramedic PE-10) with a small lip made by mild heat was introduced into the uterine lumen and was fixed with two sutures of silk thread (000). Abdominal muscles were sutured, and the catheter was tunneled through the dorsal fascia and brought to the surface at the nape of the neck. Here, the catheter from each uterine horn was affixed to the skin by means of wound clips and then protected with a gauze pad and adhesive covering. Each uterine horn of the rat opens separately into the vagina, with a complete septum separating the cervices through their entire length. To ascertain if retrograde flow from the vagina can result in the flow of fluid from one uterine horn to the other, several tests were performed with Evans blue dye perfused through the right or left uterine horn. In no case did dye appear in the contralateral horn. Thus, it was possible to compare an experimental horn with a control horn in a single animal. Each received a test substance in one uterine horn (either right or left) and a control substance in the opposite horn. The total dose of any substance was applied in 12 infusions of 0.2 ml at 4-hr intervals. Each dose of 0.2 μg contained 150 μg RNA. The appearance of a drop of fluid at the vaginal orifice was taken as proof that the catheter was intraluminal and not displaced. Only animals which retained the catheters in place throughout the infusion schedule were evaluated. The animals were sacrificed with ether 4 hr after the last infusion, and the uteri and vagina were dissected and preserved in Bouin's fixative. The experimental and control horns were embedded side by side in paraffin and the two horns compared microscopically.

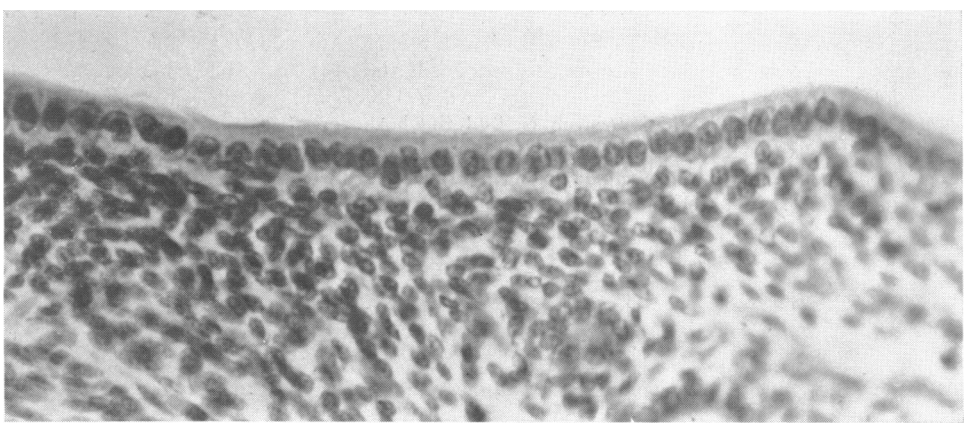
Results.—Intraluminal administration of estradiol-17β: When administered in divided doses every 4 hr for 48 hr (12 administrations), estradiol-17β in a total dose

as low as 6×10^{-4} μg causes stimulation of the endometrium (Fig. 1*a*). The opposite uterine horn of the same animal, treated in the same manner with normal saline, has the nonstimulated cuboidal epithelium and atrophic uterine glands characteristic of the castrate rat's uterus (Fig. 1*b*).

Intraluminal stimulation by RNA: Histological changes characteristic of estrogen stimulation occur when ovariectomized rat uteri are treated with U-RNA^e, at a total dose of 1.8 mg administered over 48 hr (Fig. 2*a*). A stimulatory effect, measured by the height of the uterine epithelium, is found in 14 out of 20 rat uterine horns treated with U-RNA^e (Table 1). All rats which failed to respond received U-RNA^e of a single batch, which had no stimulatory action on any animal in which it was used. The responding animals were treated with U-RNA^e of five batches prepared at different times. That the activity of the extract is due to the RNA and not an unidentified contaminant is suggested by the virtual loss of activity when the samples of U-RNA^e, shown by previous tests to be active, are preincubated with RNase (Fig. 2*b*). The specificity of U-RNA^e in causing stimula-

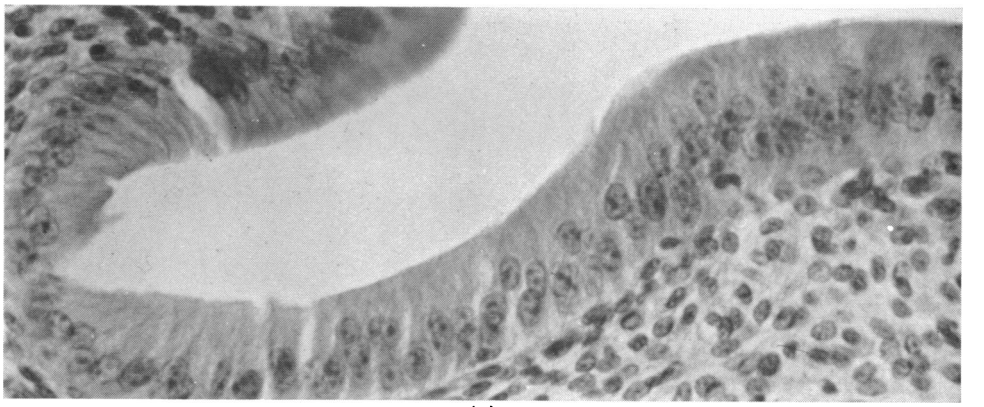


(*a*)

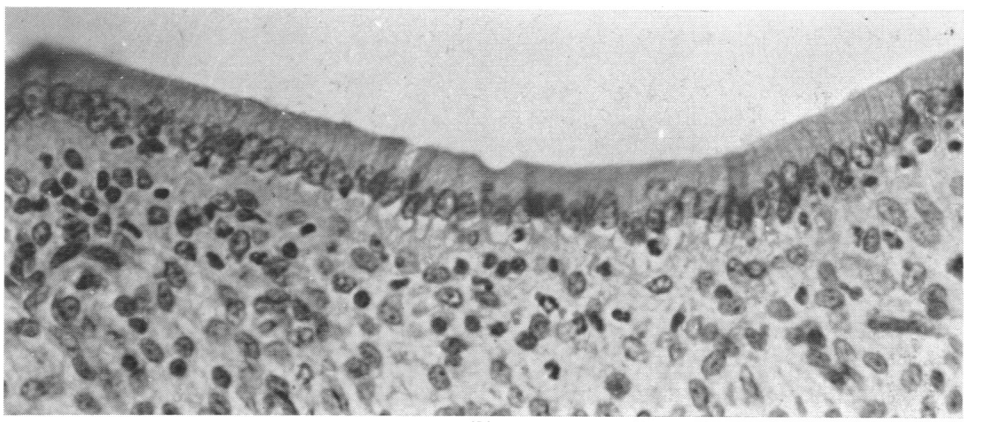


(*b*)

FIG. 1.—(*a*) Cross-section of endometrium of ovariectomized rat which received intrauterine application of estradiol-17β. A total dose of 6×10^{-4} μg was administered in divided doses every 4 hr for 48 hr and the animal sacrificed 4 hr after the twelfth intraluminal application. ×900. (*b*) Opposite uterine horn of animal shown in (*a*). This horn treated with saline on same schedule. ×900.



(a)



(b)

FIG. 2.—(a) Cross-section of endometrium of ovariectomized rat which received intrauterine application of U-RNA^e. A dose of 150 μg was administered every 4 hr for 48 hr and the animal sacrificed 4 hr after the twelfth intraluminal application. ×900. (b) Opposite uterine horn of animal shown in (a). This horn treated with U-RNA^e incubated with 5 μg/ml RNase (pancreatic) for 60 min at 37°C. ×900.

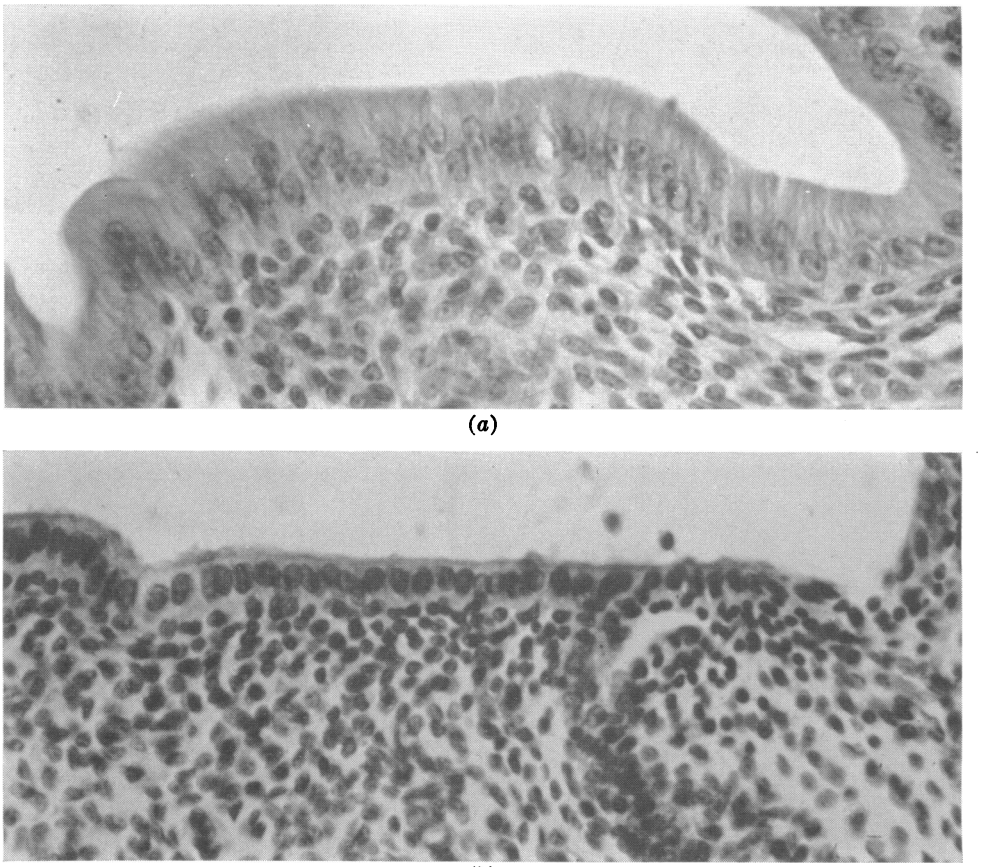
tion of the endometrial epithelium is shown in Table 1. In an animal that receives U-RNA^e in one uterine horn (Fig. 3a) and liver-RNA at the same concentration in the opposite uterine horn (Fig. 3b), only the former causes transformation of the endometrial surface to high columnar cells.

TABLE 1

EFFECT OF RNA EXTRACTS ON ENDOMETRIUM OF CASTRATE RATS

Substance*	Cases	Response†			
		−	+	++	+++
Saline	20	18	2		
U-RNA^e	20	5	1	5	9
U-RNA^n	10	6	4		
L-RNA^e	20	14	6		
(U-RNA^e) RNase‡	10	8	2		
V-RNA^e	20	15	5		

* All RNA preparations adjusted to equivalent concentration based on O.D. at 260 mμ.
† Response represents average height of uterine epithelial cells based on cell measurement of 1000 cells per endometrium: − = 10–12 μ; + = 15–19 μ; ++ = 20–24 μ; +++ = 25 μ or greater.
‡ U-RNA^e incubated with 5 μg/ml RNase (pancreatic) for 60 min at 37°C.



(a)

(b)

FIG. 3.—(a) Cross-section of endometrium of ovariectomized rat which received intrauterine application of U-RNA^e (as described for animal shown in Fig. 2a). ×900. (b) Opposite uterine horn of animal shown in (a). This horn treated with L-RNA^e on identical schedule and concentration used for contralateral horn. ×900.

Discussion.—By preparing ovariectomized rats with indwelling polyethylene catheters, it has been possible to have access to the uterine horns for local application of materials which could be tested for stimulatory activity characteristic of estrogenic hormones.

The observations indicate that the total RNA fraction of estrogen-stimulated rat uteri is capable of initiating the morphologic changes in the endometrium characteristic of estrogen stimulation. Precise identification of the active constituents of the RNA extract remains to be established. That the activity is not caused by a contamination of the uterine RNA extract with estradiol-17β is indicated by several facts. First, the activity of the RNA extract is virtually eliminated by preincubation with RNase although the enzyme has no effect on the chemical integrity or biological activity of estradiol-17β. Second, a recovery of RNA was carried out with uteri obtained from animals which had received radioactive estradiol-17β-6, 7-H^3. At each step in the RNA recovery procedure, the radioactivity of the product was tested. The final RNA extract was not radioactive. Finally, in experi-

ments to be reported elsewhere, it was found that although the application of actinomycin D prevents the local action of estradiol-17β, the same dose of actinomycin D does not prevent the stimulatory effect of the RNA extract.[7]

The source of the tissue RNA able to initiate estrogen-like morphologic events appears to be highly specific. Neither liver, which inactivates circulating estrogen, nor vagina, an estrogen target organ, contains RNA capable of initiating the biological activity characteristic of hormone stimulation of the uterus. The activity is limited to RNA synthesized by uterine cells under the stimulus of estrogen. The quiescent uterine cells of the ovariectomized rat uterus possess RNA unable to produce the effect. This suggests that hormone stimulation causes not only a quantitative change in uterine RNA biosynthesis,[12] but a qualitative change as well.

Previously, the theories relating estrogen action with alteration in biosynthesis of RNA were based on evidence derived from biochemical studies describing acceleration of RNA synthesis as a result of estrogen treatment, and from the observations that inhibition of DNA-dependent RNA biosynthesis by treatment with actinomycin D prevents target organs from responding to the hormone. Now may be added the evidence that biologically active RNA can be extracted from estrogen-treated uteri and that this extract is capable of initiating the changes in protein synthesis required to transform a nonstimulated uterine endometrium to the stimulated state. Thus, it may be concluded that after the hormone initiates RNA biosynthesis, it is not necessarily involved in subsequent steps leading to the secondary morphologic changes in the uterus. The response we have measured, cell growth, may represent a special estrogen effect totally and exclusively dependent on protein synthesis. It remains to be established whether the principle observed here applies to estrogen effects in general, and in particular to physiological, as well as morphological, manifestations of the action of the hormone.

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