Histochemical Study of the RNA-Effect on the Uteri of Castrated Mice (34502)

SHIH-FANG YANG AND CHIH-YUN HSÜ
(Introduced by M. C. Niu)
Department of Biomorphics, National Defense Medical Center, Taipei, Taiwan, Republic of China

Functional studies of uterine RNA have revealed that it is possible to bring the regressed uterus, caused by castration, back to the normal condition (1, 2). The effect was found to be localized primarily in the uterine epithelium and glands. While the epithelial cells were changing from the cuboidal to the columnar form, the number of the glands increased also. Associated with these alterations were the growth of the uterine size or volume and the augmentation of such enzyme activity as alkaline phosphatase (3, 4) and β-glucouronidase (5). Enzymatic analysis yielded data concerning the relative amount of enzyme activity, but no information regarding its distribution in various components of the uterine wall. As an outgrowth of the summer seminars (1967), sponsored jointly by Academia Sinica, National Taiwan University and Tsinghua University, M.C. Niu initiated a histochemical program studying the enzyme distribution in the mouse uterus. The aim of this paper, first of a series, is to present results showing that (1) the activity of alkaline phosphatase is primarily localized in the outer periphery of the uterine wall, *i.e.*, longitudinal muscle bundles and the connective tissue of the castrate uterus; (2) uterine RNA-induced enzyme activity is localized mainly in the epithelium and that of the associated glands; and (3) the functional activity of uterine RNA is destroyed by treatment with RNAse or boiling. Liver RNA was found ineffective.

Materials and Methods. Young mature female NIH mice weighing 20-30 g were bilaterally ovariectomized. Daily vaginal smears were followed. Two weeks after castration, 60 healthy mice with persistent diestrous stage were randomly divided into five groups. After anesthesia with ether, a cut through the back was made to expose the left horn of the regressed uterus. A volume of 0.05 ml of the following five preparations was injected respectively into the left uterine lumen using a 1-ml tuberculin syringe and a 27-gauge hypodermic needle: (1) endometrial RNA (E-RNA), (2) liver RNA (L-RNA), (3) RNAse-treated E-RNA, (4) boiled E-RNA and (5) physiological saline (0.9% NaCl, pH 7.0).

E-RNA was prepared from the endometrium of mature sow according to a modified method of Kirby (6, 7). L-RNA was prepared likewise from the liver of NIH mice. Both were used within 6 hr after isolation. The concentration was adjusted to $OD_{260}m\mu$ 200/ml. An aliquot of the E-RNA was hydrolyzed by pancreatic RNAse (Worthington) (20 μg enzyme per ml, 38° for 2 hr). Another aliquot was boiled at 100° for 30 min.

Three days after injection, the animals were killed. The left uterine horn was immediately excised and fixed in a dry-ice–acetone mixture (−78°) or in Bouin's solution. Acetone-fixed materials were sectioned at 8 μ according to the freeze-substitution microtechnique (8). The enzyme activity of alkaline phosphatase was shown by the histochemical procedure of Gomori (9). In order to check the specificity of the color reaction produced by the enzyme, sections from the five groups were separately incubated in the medium with and without the substrate, β-sodium glycerophosphate, or incubated with and without the inhibitor, L-phenylalanine (0.05 *M*) (10). Bouin-fixed uteri were embedded in paraffin, cut at 7 μ and stained with hematoxylin and eosin.

Results. Treatment of the left uterine horn with E-RNA resulted in an increase in size of approximately 3–4 times the diameter of any of the other four groups: saline, liver RNA, boiled, and RNAse-digested E-RNA-treated series. The details of the changes are described as follows:

Distribution of alkaline phosphatase:—Histochemical stained sections of the saline-treated uteri exhibited a mild reaction of the alkaline phosphatase activity. The enzymatic reaction occurred mainly in the outer border of the uterine wall. Some were found in circular muscle, but none in the endometrial stroma (Fig. 1).

Treatment with E-RNA increased the activity of alkaline phosphatase in a striking manner (Figs. 2 and 3). It can be seen that dark brown-colored cobalt sulfide was deposited copiously in the longitudinal muscle layer and in the columnar epithelial layer of the lumen and associated glands. Practically none was found in the endometrial stroma and only a slight amount in the circular muscle. That the dark-brown stain is due to the enzyme activity is supported by the fact that (a) the inclusion of an enzyme inhibitor in the incubation medium resulted in the lack of a color reaction in the epithelium and faint color in the myometrium (Fig. 4). Apparently this was due to the inhibitor of enzyme action by L-phenylalanine, a metal-chelating agent to make Mg^{2+} nonavailable; (b) the use of an incubation medium without substrate. The sections showed once again a lack of color reaction.

Under identical conditions, the uteri received injections of liver RNA, boiled and RNAse-digested E-RNA. These injections resulted in a very faint distribution of alkaline phosphatase in the epithelium and circular muscle. This enzyme activity was similar to that shown by the saline-treated specimens (Figs. 5–7). A summary of the uterine response to saline, liver RNA, and E-RNA is given in Table I. It can readily be seen that E-RNA is specifically responsible for the induction of alkaline phosphatase activity in the uterine epithelium. No reason could be given to the 4 of 18 mice that did not respond to E-RNA.

TABLE I. Response of Alkaline Phosphatase Activity in the Uteri of Ovariectomized Mice after Various Treatments.

Treatment	No. of animals	Response	
		Positive	Negative
Endometrial RNA	18	14	4
Liver RNA	12	0	12
Boiled E-RNA	12	0	12
RNAse-treated E-RNA	6	0	6
Saline	12	0	12

Histological changes:—In the saline-treated uteri, the epithelial cells were cuboidal (Fig. 8). Uterine glands were scarce. After treatment with E-RNA, the epithelium resumed the normal morphology of the columnar cells which contained elongated nuclei and became folded. A number of glands proliferated and their epithelium also became columnar. The endometrial stroma appeared edematous (Fig. 9). In addition to the endometrial changes, the muscle bundles were enlarged (Fig. 2). Both boiled and RNAse-digested E-RNA as well as liver RNA were incapable of elucidating an uterine response (Figs. 10–12).

Discussion. Histochemical study of the control and RNA-treated uteri of the castrated mice has confirmed the early findings (11, 12). The similarity of the uterine response to the RNA from pooled uteri of rats (1) and mice or calf (2) on the one hand and from sow's endometrium on the other suggests that the major components of the whole uterine RNA may actually be contributed by the endometrium. The effect of E-RNA on the distribution of alkaline phosphatase in the castrate uterus is comparable to that produced by the steriod sex hormone (13, 14). The question raised by this similarity is that the action of RNA may actually result from the contamination with the hormone. However, this is not supported by four lines of evidence: (1) the RNA prepared by the cold phenol procedure was washed three times with ether and three times with ethanol before using; and both ether and ethanol are good solvents of the hormone; (2) the

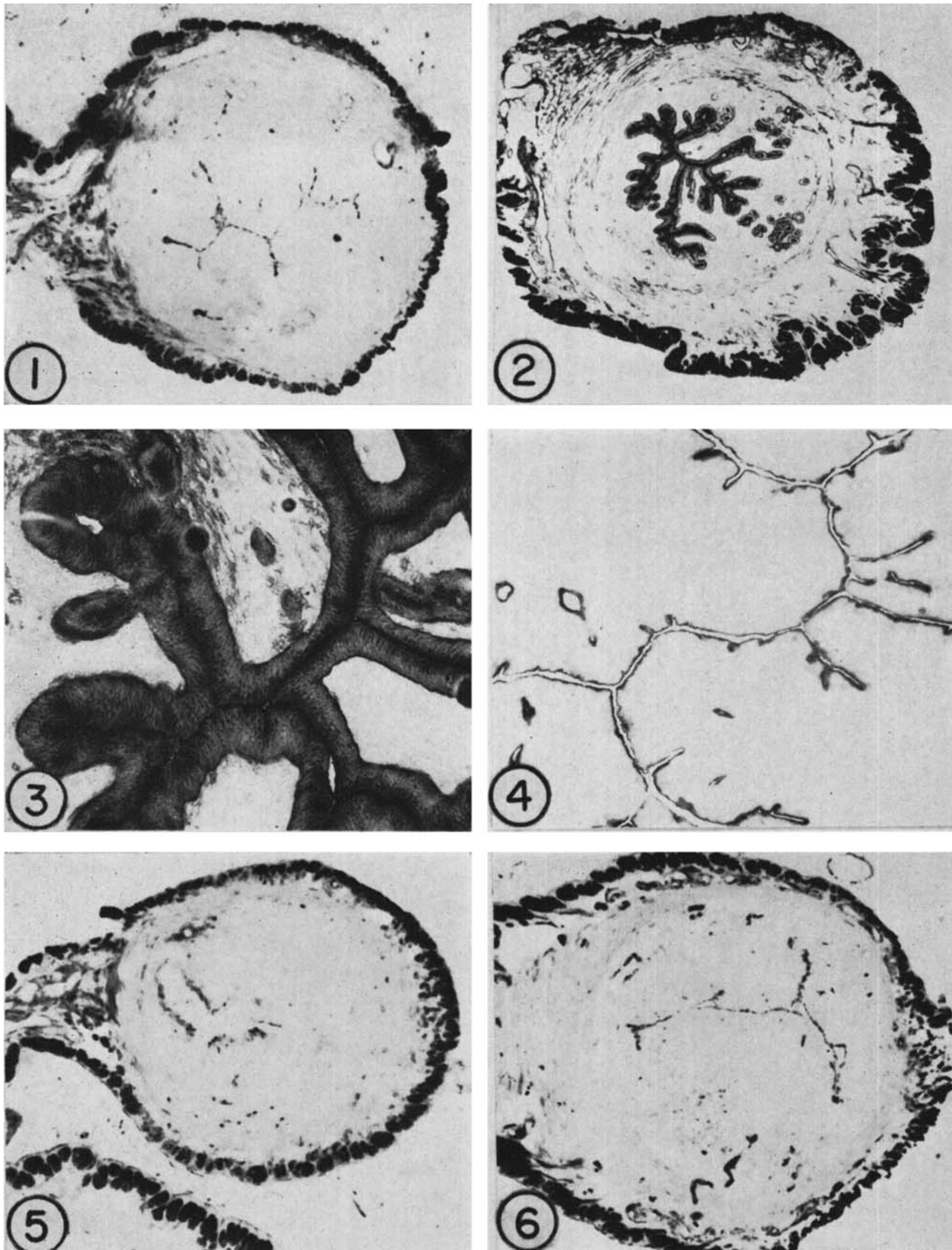

FIG. 1. Cross section of saline-treated uterus of the ovariectomized mouse showing mild distribution of alkaline phosphatase. Gomori's method; 45×.

FIG. 2. Cross section of E-RNA-treated uterus of the ovariectomized mouse showing positive distribution of alkaline phosphatase in the epithelial and muscular tissues and negative distribution in endometrial stroma. Gomori's method; 15×.

FIG. 3. Enlarged portion of Fig. 2. 150×.

FIG. 4. Cross section of E-RNA-treated uterus of the ovariectomized mouse showing inhibition of alkaline phosphatase activity by L-phenylalanine. Gomori's method; 150×.

FIG. 5. Cross section of L-RNA treated uterus of the ovariectomized mouse showing very mild distribution of alkaline phosphatase. Gomori's method; 45×.

FIG. 6. Cross section of boiled E-RNA-treated uterus of the ovariectomized mouse showing mild distribution of alkaline phosphatase. Gomori's method; 45×.

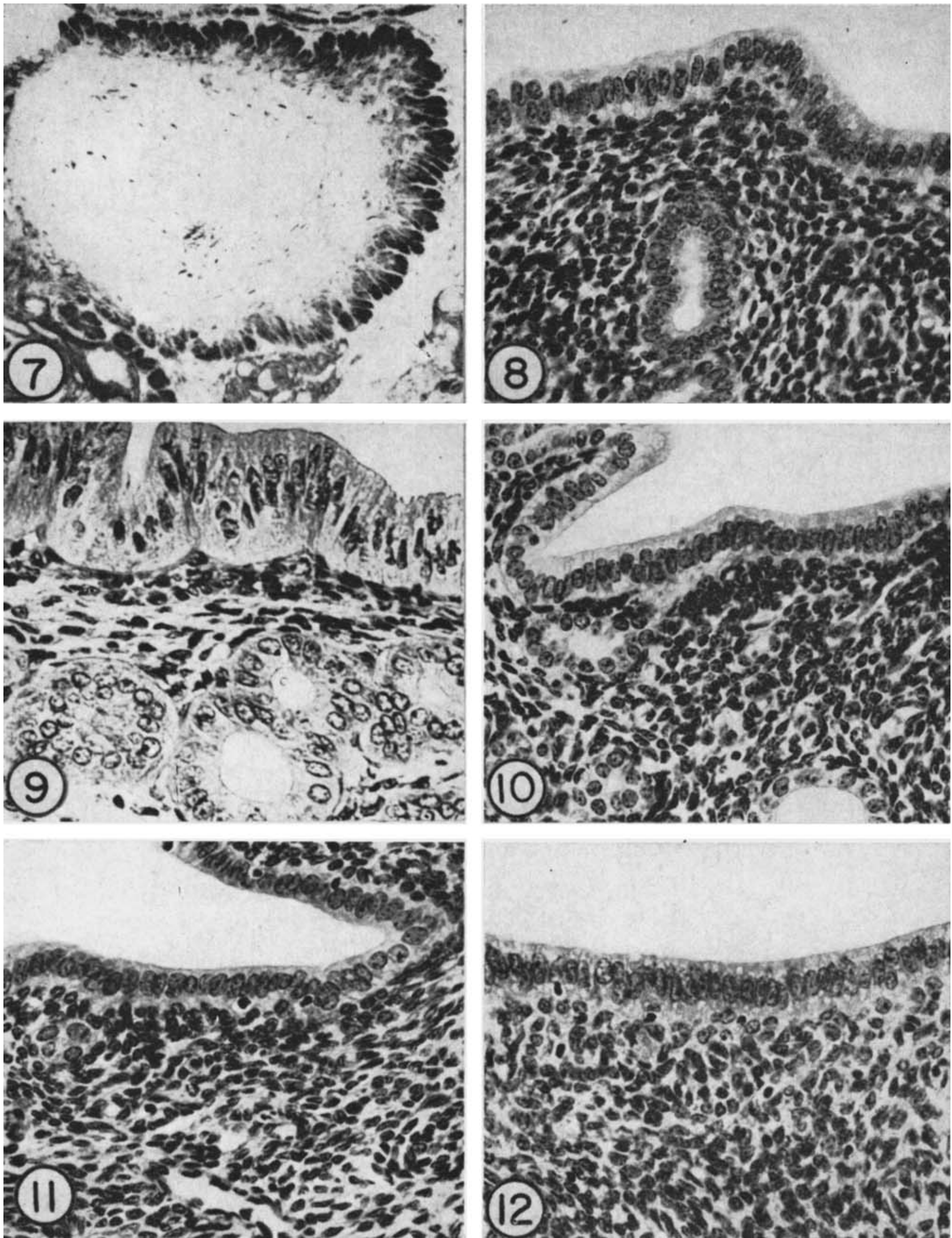

Fig. 7. Cross section of RNAse-treated E-RNA-injected uterus of the ovariectomized mouse showing mild distribution of alkaline phosphatase. Gomori's method; 45×.

Fig. 8. Cross section of saline-treated uterus of the ovariectomized mouse showing regressed endometrial structures. Hematoxylin and eosin; 400×.

Fig. 9. Cross section of E-RNA-treated uterus of the ovariectomized mouse showing regenerated endometrial structures. Hematoxylin and eosin; 400×.

Fig. 10. Cross section of L-RNA-treated uterus of the ovariectomized mouse showing regressed endometrial structures. Hematoxylin and eosin; 400×.

Fig. 11. Cross section of boiled E-RNA-treated uterus of the ovariectomized mouse showing regressed endometrial structures. Hematoxylin and eosin; 400×.

Fig. 12. Cross section of RNAse-treated E-RNA-injected uterus of the ovariectomized mouse showing regressed endometrial structures. Hematoxylin and eosin; 400×.

physiological function of E-RNA was abolished by the treatment of pancreatic RNAse or boiling at 100° for 30 min; (3) the RNA isolated from pooled uteri plus C-14 estrogen contained no radioactivity after two washings with fat solvent (1); and (4) the RNA isolated from liver plus excess estrogen was incapable of initiating the uterine response (15). The latter was interpreted as meaning that there is practically no estrogen present in the RNA sample and that the uterine response to E-RNA is specific.

The localization of alkaline phosphatase in the outer periphery of the castrate uterine wall, longitudinal muscle, and serosa, was unexpected and, therefore, subjected to further scrutiny. The fact that the histochemical reaction is sensitive to the enzyme inhibitor and requires the presence of a specific substrate rules out the possibility that the deposition of cobalt sulfide in the longitudinal muscle layer and serosa is an artifact. On the contrary, we are inclined to believe that this constitutes the material basis of the enzyme activity found in the homogenate of the castrate uterus (12). Two weeks after ovariectomy the presence of the enzyme activity in the outer uterine wall would suggest its independence of the hormone (sex) action. It seems, therefore, that the target tissue of the hormone in the uterus is primarily endometrium. This may well explain why E-RNA is equally, if not more, potent as the whole-uterus RNA in elucidating the uterine response. It may be relevant to add here that the RNA we employed is heterogeneous. No effort has yet been attempted to define the active component of the sample which contains less than 2% of protein according to Lowrys procedure (see 14).

Summary. RNA was extracted from the endometrium of the mature sow by the cold phenol procedure. Injection of this RNA into the lumen of the regressed uteri of castrated mice resulted in a significant increase in alkaline phosphatase activity, primarily in the epithelial layer but not in the endometrium stroma. In addition, structural restorations, such as the increase of epithelial height, proliferation of uterine glands, and enlargement of muscle bundles were observed. However, physiological saline, liver RNA, and endometrial RNA degraded by RNAse or boiling had no appreciable effect.

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