

# ENDOGENOUS PEROXIDASE: SPECIFIC MARKER ENZYME FOR TISSUES DISPLAYING GROWTH DEPENDENCY ON ESTROGEN

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## ABSTRACT

Data derived from a correlated morphological and biochemical study suggest the following: (a) estradiol-17 $\beta$ , diethylstilbestrol, the estrogen antagonists nafoxidine (Upjohn 11,100), and Parke Davis CI628 induce synthesis of an endogenous peroxidase in the epithelium of target tissues like the vagina, the cervix, the uterus, and in the acinar cells of the estrogen-dependent rat mammary tumor; (b) peroxidase is a "specific" secretory protein of the estrogen-sensitized uterine endometrium; (c) peroxidase synthesis is not a nonspecific response to steroid hormone action, since progesterone and testosterone do not induce its synthesis; (d) endogenous peroxidase is a possible diagnostic protein for the detection of estrogen-dependent growing tissues, including breast cancer; (e) movement of exogenous horseradish peroxidase from the interstitium to the uterine lumina is restricted by tight junctions located at the apices of epithelial cells. Estrogen and antagonists do not appear to influence the transepithelial movement of exogenous peroxidase into the lumen.

The uterus, cervix, vagina, and mammary gland of mammals require estrogen for their postpubertal trophic development and for the maintenance of their differentiated state. Indeed, the presence of estrogen-binding proteins (estrogen receptors or estrophiles, Jensen et al., 1966 *a*) in these tissues and of estrogen dependency of rat and human mammary carcinoma are well documented (Huggins et al., 1959; Jensen et al., 1966 *b*; Jensen and DeSombre, 1972; Jensen et al., 1972; Heuson et al., 1972).

In the course of our investigation it was discovered that tissues displaying growth dependency on estrogen synthesize and secrete "specific marker proteins" into their lumina. These results show (a)

that synthesis of peroxidase<sup>1</sup> (or a hemoprotein with peroxidatic activity) is a consistent marker for estrogen-dependent tissues (carcinogen-induced rat mammary carcinoma, cervix, vagina, and uterus), (b) estrogen antagonists (Upjohn 11,100;

<sup>1</sup> Throughout this paper, the DAB-positive reaction product will be referred to as peroxidase, since uterine peroxidase is a well-documented enzyme (see text) and since the histochemical medium employed is designed to demonstrate peroxidase activity. It is of course possible that what is being visualized in either a hemoprotein, which may possess peroxidase activity, or several substances with peroxidatic activity. The biochemical characterization of this enzyme as a peroxidase is still very limited.

Parke-Davis CI628) that competitively bind estradiol-17 $\beta$  receptors, and which have pronounced antiuterotrophic and growth-inhibitory effects on estrogen-dependent rat mammary carcinoma, also activate genes for the synthesis of peroxidase in the uterus, cervix, and vagina, and (c) the cycle of peroxidase synthesis and secretion in the cervix, vagina, and uterus coincides with the estrous cycle of mature rats, and of that of immature rats and mature ovariectomized rats after injection of estradiol-17 $\beta$  and diethylstilbestrol.

## MATERIALS AND METHODS

### *Preparation of the Mammary Tumor Specimens*

Mammary carcinomas were induced in 50-day old female Sprague-Dawley rats by a single feeding of 20 mg of 7,12-dimethylbenz(a)anthracene (DMBA) in 2 ml sesame oil according to Huggins et al. (1959). In bilaterally ovariectomized animals, estradiol-17 $\beta$  was given at a dose of 2.5  $\mu$ g per day for 6 days per wk. Biopsy samples of tumor were excised from ether-anesthetized intact and ovariectomized rats, and from ovariectomized rats receiving estrogen. The tissue was immediately immersed in cold Formalin-glutaraldehyde fixative (Karnovsky, 1965). After 30–45 min, tissue samples were rinsed in several changes of 0.1 M cacodylate buffer, pH 7.2, for 8 h, and prepared for the demonstration of endogenous peroxidase activity by techniques described below.

### *Administration of Estrogen Antagonists, Estrogen, and Other Steroids*

23-day old female rats were given subcutaneous injections of varying amounts (30–500  $\mu$ g) of nafoxidine (Upjohn 11,100, Duncan et al., 1963) or Parke-Davis CI628, originally called CN 55,945-27 (Callantine et al., 1966), in 0.1 ml glycerol. Some animals were also given injections of the antagonist 30 min before injections with estrogen. In other experiments, the antagonists were administered at 6-h intervals from the 23rd through the 28th day of life.

Diethylstilbestrol (Sigma Chemical Co., St. Louis, Mo.), and estradiol-17 $\beta$  (Sigma) at concentrations of 0.1–0.4  $\mu$ g dissolved in 0.1 ml glycerol were injected into 23-day old rats. Higher doses of estrogen (1.4–4  $\mu$ g/0.1 ml) dissolved in glycerol were also injected into immature females or mature castrates (110–150 g).

Rats either were given a single dose of antagonist, estrogen, or antagonist plus estrogen, or else were given injections at 6-h intervals. At least six rats were used at varying time periods between 60 min and 96 h after hormone or antagonist injection.

Progesterone and testosterone at doses of 1–2 mg in 0.1 ml glycerol were injected into immature rats in order to examine the effect of other steroid hormones. Control experiments also involved the injection of glycerol or saline into immature and ovariectomized females.

### *Studies with Cycling Rats*

The stage of the estrous cycle was judged by light microscope observations of vaginal smears. Animals were induced into a state of constant estrus by exposure to prolonged periods of light, according to procedures described by Lawton and Schwartz (1967).

The uterine fluid from rats during the various phases of the estrous cycle and during constant estrus was collected via a syringe, the needle of which was inserted into the lumen of the uterus. The fluid was either used at once, or else stored at 0°C until needed.

### *Demonstration of Endogenous and Exogenous Peroxidase Activity*

Biopsy samples of mammary carcinoma and tissues from cycling rats (uteri, cervix, vagina, hypothalamus, anterior pituitary, ovaries) were excised under ether anesthesia and immersed in a mixture of cold 4% glutaraldehyde-1% formaldehyde in 0.1 M cacodylate buffer, pH 7.2 (Karnovsky, 1965). In other experiments these tissues were excised from ether-anesthetized rats that had been perfused for 30–45 min with the same fixative. The tissues were washed for 8 h in cacodylate buffer, cut into 50–100- $\mu$ m thick sections and placed in Tris-HCl buffer at pH 7.2. The slices were then incubated for 30–45 min in medium composed of 10 mg 3,3-diaminobenzidine-tetra HCl in 10 ml Tris-HCl buffer (pH 7.0) containing 0.3% H<sub>2</sub>O<sub>2</sub>. Some sections were incubated in medium lacking H<sub>2</sub>O<sub>2</sub>.

For the demonstration of exogenous peroxidase activity, 0.1 ml of 0.9% NaCl containing horseradish peroxidase (Sigma, Type VI) at a concentration of 1 mg/ml was injected via the saphenous vein into immature females and into immature females receiving physiological doses of estrogen (estradiol-17 $\beta$ , diethylstilbestrol), or antagonist for periods of 60 min–72 h. Animals were sacrificed at various time intervals between 3 and 30 min after injection of the tracer. The tissue was then fixed *in situ* in anesthetized rats, excised, and immersed for 45–60 min in cold fixative. After an 8-h rinse in cacodylate buffer, 100- $\mu$ m thick slices of tissue were incubated in the DAB-H<sub>2</sub>O<sub>2</sub> medium described above.

### *Microscope Techniques*

The aldehyde-fixed, incubated, and postosmicated tissues were dehydrated in graded ethanol-water solutions and embedded in Epon. Sections, either unstained or stained for 1 min with lead citrate, were examined in the Hitachi HU 11C or Siemens Elmiskop 101 electron

microscope. Semithick sections, 1–2  $\mu\text{m}$ , were photographed with an Olympus microscope.

### *Analyses of Rat Uterine Fluid*

Fluids were taken from uteri of cycling rats in estrus and constant estrus, and from immature females 72 h after administration of estradiol-17 $\beta$  or diethylstilbestrol. In some experiments, fluid was passed through a Millipore filter (Millipore Corp., Bedford, Mass.) (0.45  $\mu\text{m}$  pore size), while in other experiments the natural fluid was used after centrifugation to remove cell debris.

The protein content of the fluid was determined by the method of Lowry et al. (1951), with a Heath 700 series spectrophotometer.

The highly sensitive colorimetric peroxidase assay technique using DAB as hydrogen donor was used to measure peroxidase activity in uterine fluids (Herzog and Fahimi, 1973). Horseradish peroxidase (Sigma Type VI) and lactoperoxidase dissolved in distilled water or in 0.05 M phosphate buffer at concentrations of 0.05–0.5  $\mu\text{g}/\text{ml}$  served as reference control samples.

## RESULTS

### *Cytochemical Localization of Endogenous Peroxidase Activity in DMBA-Induced Rat Mammary Carcinoma*

The growing estrogen-dependent rat mammary carcinoma is composed of well-formed alveoli, ductules, and interalveolar fibrous connective tissue. The large glandular cells surround a wide lumen that contains secretory product and sloughed cell processes. The cells possess basal nuclei and a well-developed secretory apparatus consisting of supranuclear Golgi cisternae, granular endoplasmic reticulum, and apical secretory vesicles.

Intense endogenous peroxidase activity is present in the cisternae of the nuclear envelope, some parts of the Golgi complex, and in cisternae of the granular endoplasmic reticulum of the growing hormone-dependent tumor (Figs. 1, 2). Numerous vesicles and dilated cisternae of the endoplasmic reticulum in the apical cytoplasm of acinar cells contain peroxidase-positive material. The material in the lumina of the alveoli and ductules stains intensely for peroxidase activity (Fig. 2, inset). Not all cells of the alveoli (of the hormone-dependent tumor) stained positively for endogenous peroxidase activity (Fig. 1 A), as moderately reactive and unreactive cells lined the acini.

The regressing tumor of the castrate female contains flattened acinar cells and a poorly developed secretory apparatus. The acinar cells are

entirely negative for peroxidase activity (Fig. 3 A), as are the cells of hormone-independent tumors which continue to grow after ovariectomy. In some glandular cells the peroxidase-positive material is present in single vesicles and vacuoles in acinar cells (Fig. 3) of the regressing tumor. Acinar cells of the growing tumor in castrates receiving injections of estrogen stain intensely positive for endogenous peroxidase activity (Fig. 4).

### *Peroxidase Activity in the Cervical and Vaginal Epithelium of the Estrogen- and Antagonist-Treated Immature Rat, and in the Cycling Rat*

The stratified squamous epithelium of the vagina and cervix proliferate to form a keratinized epithelium after stimulation with estradiol-17 $\beta$  or diethylstilbestrol, and after treatment with antiestrogens (CI628, Upjohn 11,100). Only the intermediate and superficial layers of the vaginal epithelium stain positively for endogenous peroxidase activity. The reaction appears 12–72 h after the injection of physiological doses of estradiol-17 $\beta$  into immature rats (Fig. 5, 5 A, Table I). The peroxidase activity is present in the nuclear envelope, in the cisternae of the granular endoplasmic reticulum, and in isolated vesicles in the cytoplasm. The Golgi cisternae are weakly stained. The more flattened superficial cells contain large vacuoles with peroxidase. Peroxidase-positive material also appeared to coat the extracellular surfaces of the superficial layers of the vaginal epithelium.

Cervical and vaginal cells from animals treated with CI628 or Upjohn 11,100 underwent some proliferation and keratinization. By 72 h, however, the superficial cells were mucinogenic. Peroxidase staining was also less intense than after treatment with estrogen, but its localization in the nuclear envelope and in cisternae of the granular endoplasmic reticulum was prominent.

Our results suggest that estrogens and antagonists stimulate the intermediate and superficial cells of the vaginal and cervical epithelium to synthesize and secrete peroxidase.

### *Uterine Peroxidase: Induction by Estradiol-17 $\beta$ Diethylstilbestrol, Antiestrogens, and during the Estrous Cycle of the Rat*

The distribution of endogenous peroxidase activity in the epithelial and glandular cells of the

uterus of immature females after treatment with estradiol and CI628 has been reported by Churg and Anderson (1974). In closely correlated studies reported here, we show that the synthetic estrogen, diethylstilbestrol, and the estrogen antagonist, nafoxidine, also induce synthesis and secretion of peroxidase in the uterine epithelial cells of the immature rat. Peroxidase activity appears within 12–24 h after treatment with estrogen and estrogen-antagonists (see Table I). The nuclear envelope, the cisternae of the granular endoplasmic reticulum, and some regions of the Golgi apparatus are involved in the elaboration, storage, and transport of the peroxidase (Figs. 6, 7). Peroxidase staining is intense in the epithelium of the estradiol-17 $\beta$ -treated animal, in the estrogen plus CI628- or nafoxidine-treated rat, and after diethylstilbestrol (Fig. 6, insets). The epithelium of the immature uterus was devoid of peroxidase activity (Fig. 6, inset no. 3).

Not all cells of the uterine epithelium synthesized and stored peroxidase (Churg and Anderson, 1974). Marked heterogeneity in peroxidase storage appeared after treatment with the synthetic agents CI628 (Fig. 8) and diethylstilbestrol (Fig. 9). In all cases, however, the normal and synthetic estrogen and the inhibitors to estrogen stimulated synthesis and secretion of the peroxidase within 24 h after injection into immature rats. Increment in cell mass was associated with stored enzyme in the mucosal lining. Progesterone and testosterone injections did not stimulate peroxidase synthesis in immature or ovariectomized rats (Table I), suggesting that induced peroxidase is not a nonspecific response to the injection of steroid hormones.

The distribution of peroxidase in the uterine, vaginal, and cervical mucosa of the various stages of estrus in the rat was also examined (Table II). Our results show that endogenous peroxidase was only present in the epithelium of the uteri of rats during proestrus, estrus, and constant estrus. Sections from the hypothalamus and the anterior pituitary consistently show no peroxidase reaction product under the conditions of this study.

#### *Analysis of the Uterine Fluids of the Rat*

By use of the recently developed DAB colorimetric procedure for assaying peroxidase (Herzog and Fahimi, 1973), the relative kinetic activities of horseradish peroxidase, lactoperoxidase, and uterine fluid peroxidase were examined. It is clear that uterine fluid peroxidase, under the conditions of our experiments, was far less active than the

purified peroxidases from plants and milk (Table III). Our data provide firm evidence, however, for a uterine fluid peroxidase in rats exposed to estrogens.

#### *Effect of Estrogen and Inhibitors on the Movement of Horseradish Peroxidase from the Interstitium to the Uterine Lumen*

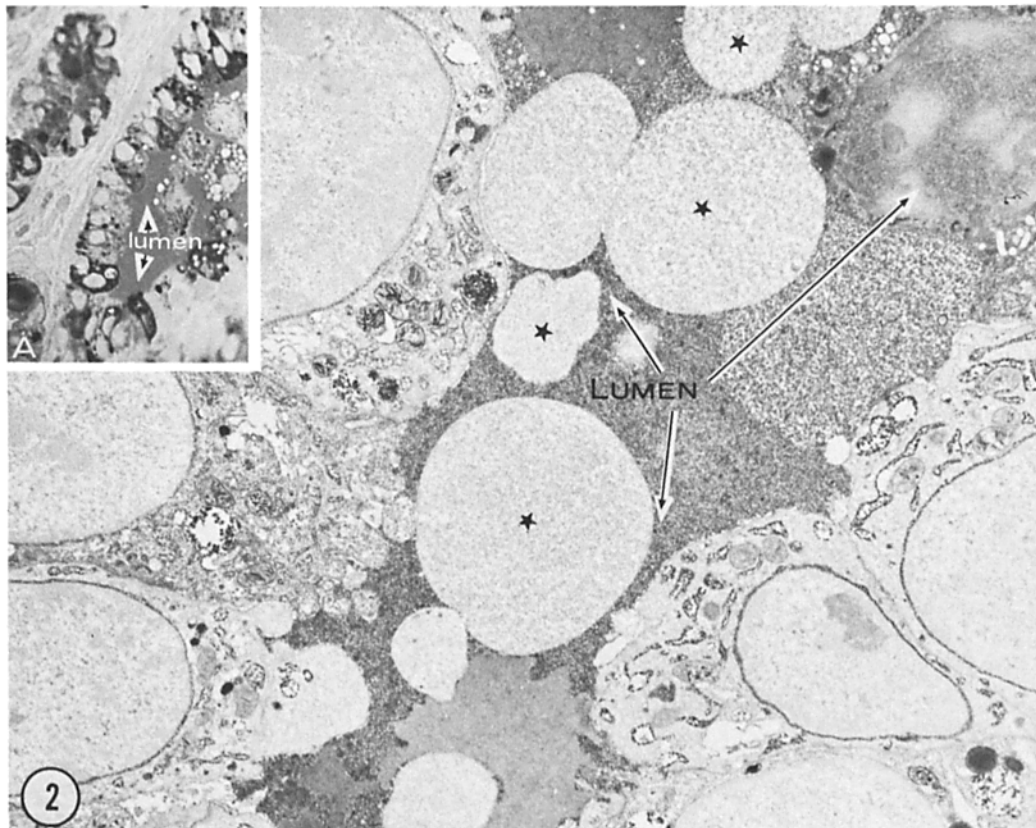
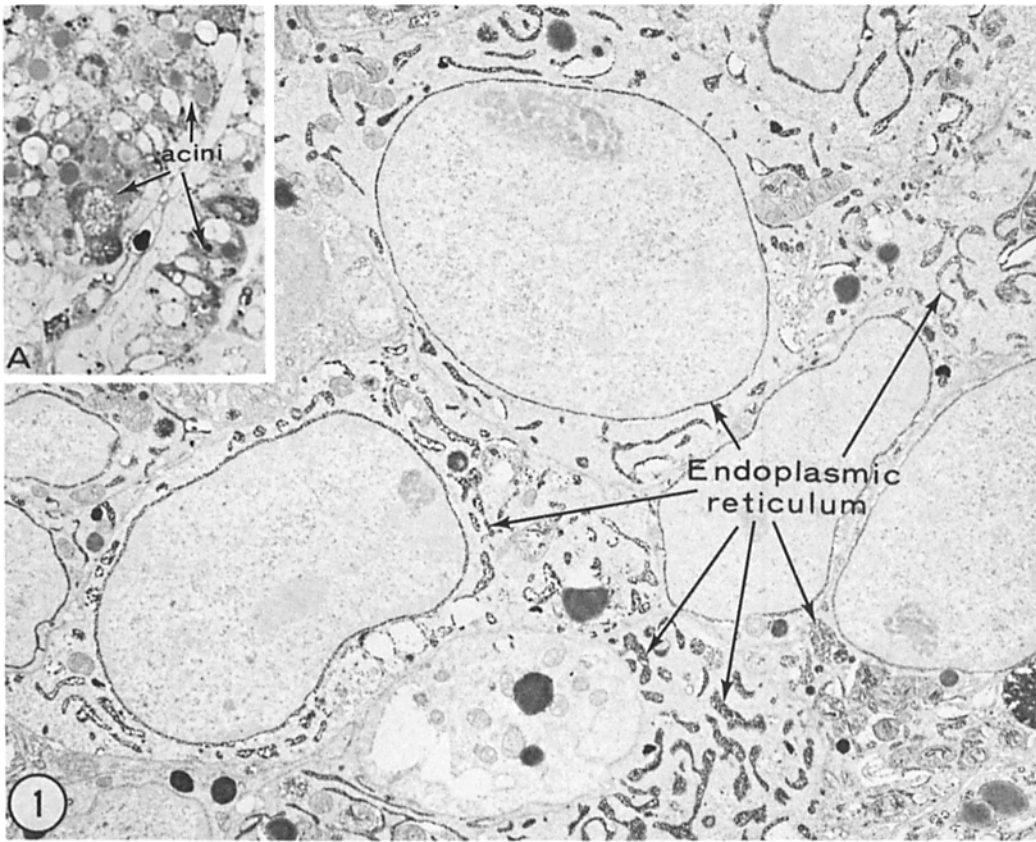
The possibility exists that a blood-borne peroxidase could reach the uterine lumen via movement across junctions of the uterine mucosa. To test this possibility, horseradish peroxidase was injected for varying time intervals into the blood of animals treated with estradiol-17 $\beta$ , diethylstilbestrol, and the antiestrogens. The uterine vasculature of immature and treated animals was readily permeable to passage of peroxidase; within 3 min the exogenous peroxidase filled the interstitium between epithelial cells (Figs. 10–12). At 72 h after injection of diethylstilbestrol into immature rats, intracellular endogenous peroxidase and extracellular horseradish peroxidase are visualized by the DAB procedure (Fig. 9). Extracellular peroxidase reaches the apical junctional complexes, but does not permeate them. At earlier periods after estradiol-17 $\beta$  treatment (6–12 h) when no endogenous peroxidase is visible in the cells, horseradish peroxidase may be seen within the extracellular space within 3–30 min after its injection, blocked by junctions (Figs. 10–12). Small intracellular vesicles with reaction product probably represent sites of uptake and possible transport of blood-borne horseradish peroxidase through the epithelium (Fig. 10). Exogenous peroxidase does not permeate the junctions between gland cells of the uterus after treatment with estradiol-17 $\beta$ .

#### *Does Uterine Fluid Peroxidase Originate in the Oviduct?*

To answer this question the oviducts of laparotomized immature and ovariectomized mature rats were ligated. After time had been allowed for healing (3–14 days), the rats were subsequently injected with physiological doses of estradiol-17 $\beta$  and examined 72 h later for uterine peroxidase activity. The epithelial cells and the uterine fluid contained a peroxidase that therefore appears to originate in the uterus.

#### DISCUSSION

Estradiol-17 $\beta$  and synthetic estrogen (diethylstilbestrol) administered to ovariectomized animals



play a regulatory role in transcription in estrogen-sensitive mammalian cells (Tata, 1966; Mueller et al., 1958; Williams-Ashman and Reddi, 1971). Not only do they maintain general metabolic processes, they also induce synthesis of "specific proteins" and structural differentiation in normal target cells (Kohler et al., 1967; O'Malley et al., 1969; Gorski and Notides, 1969; Barnea and Gorski, 1970; DeAngelo and Gorski, 1970), and maintain growth and proliferation in estrogen-dependent malignant tissues (Huggins et al., 1959). Specific protein synthesis precedes general protein synthesis (Hamilton, 1968), increment in cytoplasmic mass, and cellular proliferation. Our results show that tissues requiring estrogen for growth synthesize cell-specific *marker proteins* that can be used as biochemical and morphological markers for estrogen-induced differentiation.

### *Mammary Tumors*

The DMBA-induced rat mammary carcinoma, like its human counterparts, displays estrogen-dependency for growth (Dao, 1964; Young and Hallows, 1973). Regression after ovariectomy can be reversed by administration of estrogen (Dao, 1964) and after hypophysectomy by doses of prolactin (Pearson et al., 1972). This tumor is known to contain estrogen receptors (Jensen et al., 1967; Kyser, 1970; McGuire and Julian, 1971), a characteristic which is diagnostic for estrogen-dependent human mammary carcinomas (Jensen et al., 1971).

This growing hormone-dependent tumor displays the identifying characteristic of endogenous peroxidase synthesis and secretion. After castration, the tumor loses endogenous peroxidase activity which is correlated with diminution of the protein-synthetic apparatus in regressing cells described by others (Scott et al., 1967). Moreover, since peroxidase activity reappears in tumors of castrates given estrogen therapy, and is absent from growing estrogen-independent tumors, it ap-

pears that peroxidase is a marker enzyme for the estrogen-dependent DMBA rat mammary carcinoma (DeSombre et al., 1975). More extensive studies are required to verify this suggestion.

### *The Cervix and Vagina*

The cervical and vaginal epithelium undergoes changes that correspond to the development of secretory activity of the endometrium during the estrous cycle. Not only does estrogen stimulate an increase in the number of the cell layers with displacement of older superficial cells, it also induces keratinization of the intermediate and superficial layers. From direct anatomical observations it was obvious that estradiol-17 $\beta$  and diethylstilbestrol induced synthesis of peroxidase in an intermediate layer of squamous cells that stored keratin filaments in their cytoplasm. Peroxidase appeared in these cells only during periods of endogenous estrogen stimulation (estrus and constant estrus, for example) or after injection of hormone to spayed and immature rats, and was absent from castrates not receiving estrogen therapy. Nafoxidine and CI628 also stimulated peroxidase synthesis in the cervical and vaginal cells. Peroxidase activity first appeared about 12 h after injection of estradiol-17 $\beta$ , and was seen within the nuclear envelope, the granular endoplasmic reticulum, and in cytoplasmic vesicles. The material seemed to be secreted into the narrow extracellular space surrounding the intermediate and superficial cells.

### *Uterus*

Anti-estrogens like CI628 and nafoxidine usually have weak estrogenic (Duncan et al., 1963; Callantine et al., 1966) and progestational activity (Emmens and Martin, 1964). Nafoxidine administered for 6-12 wk produced in most rats a state of permanent diestrus (Heuson et al., 1972). These inhibitors administered alone or with estrogen induced hypertrophy of the uterine epithelial cells

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FIGURE 1 Diaminobenzidine-stained cells are shown in acini of the DMBA-induced rat mammary tumor of an intact rat (Fig. 1 A, 1- $\mu$ m thick section). The peroxidase-positive activity is present in the nuclear envelope, in cisternae of the endoplasmic reticulum, and in small cytoplasmic vesicles (Fig. 1). Portions of the Golgi complex are weakly stained. Fig. 1,  $\times$  6,000. Fig. 1 A,  $\times$  150.

FIGURE 2 Sections through the acinar lumen of the growing breast tumor are illustrated in these micrographs. The lumen which contains homogeneously gray peroxidase-positive material (Fig. 2, 2 A) is lined by darkly stained cells that contain peroxidase in their endoplasmic reticulum and in apical vesicles. Cytoplasmic processes from these cells are unstained (stars). Fig. 2,  $\times$  7,000. Fig. 2 A,  $\times$  150.

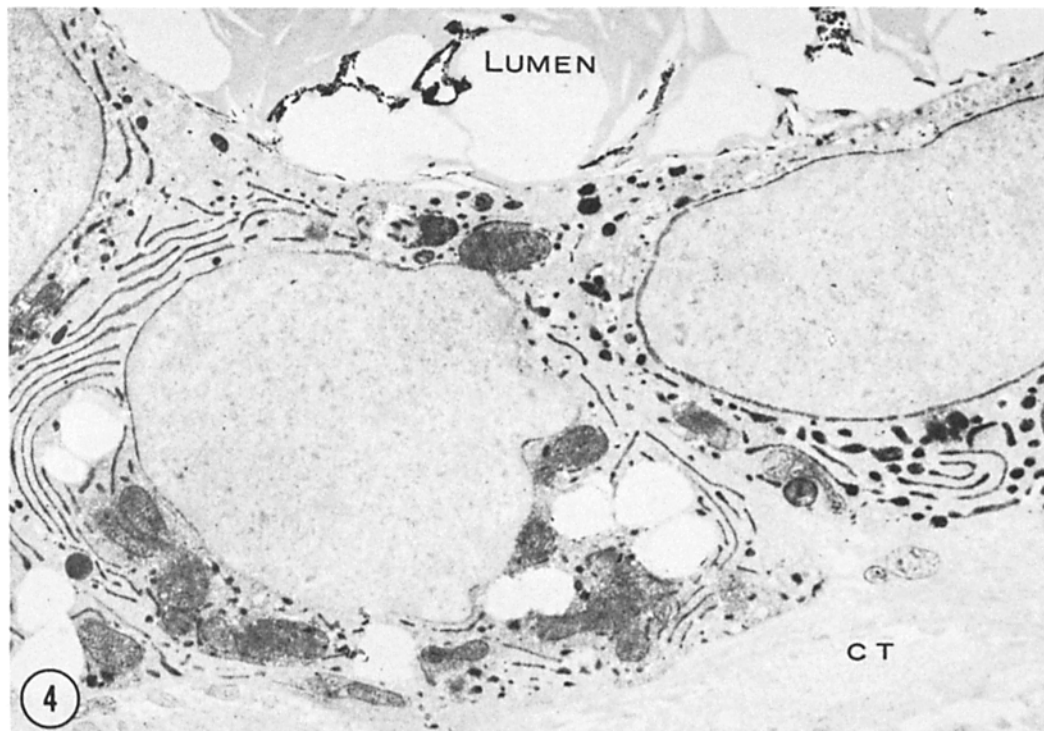
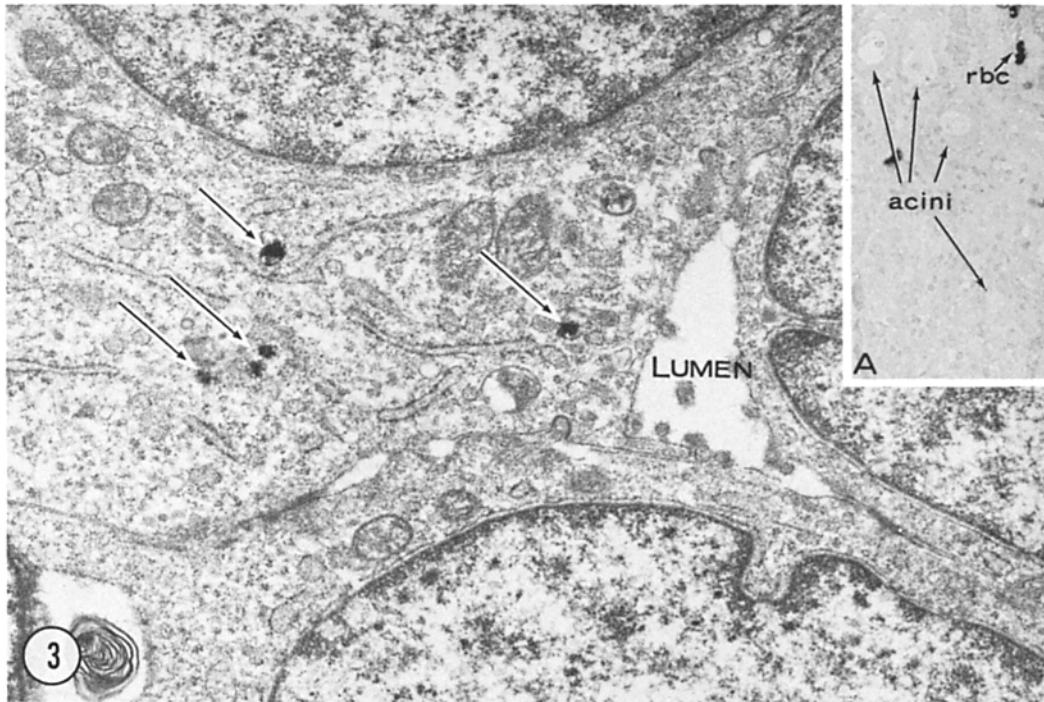


FIGURE 3 Tissues from the regressing tumor in the castrate rat are entirely unreactive for endogenous peroxidase (Fig. 3 A, red cells; erythrocytes (*rbc*) are DAB-positive). Dilated cisternae (arrows) that contain peroxidase reaction product are present at the apices of some acinar cells. Fig. 3,  $\times 16,000$ . Fig. 3 A,  $\times 150$ .

FIGURE 4 Acinar cells of the DMBA-induced rat mammary tumor of a castrate female that received daily estrogenic injections are shown here. Intense peroxidase activity is present in the cisternae of the endoplasmic reticulum. *Ct*, connective tissue.  $\times 10,500$ .

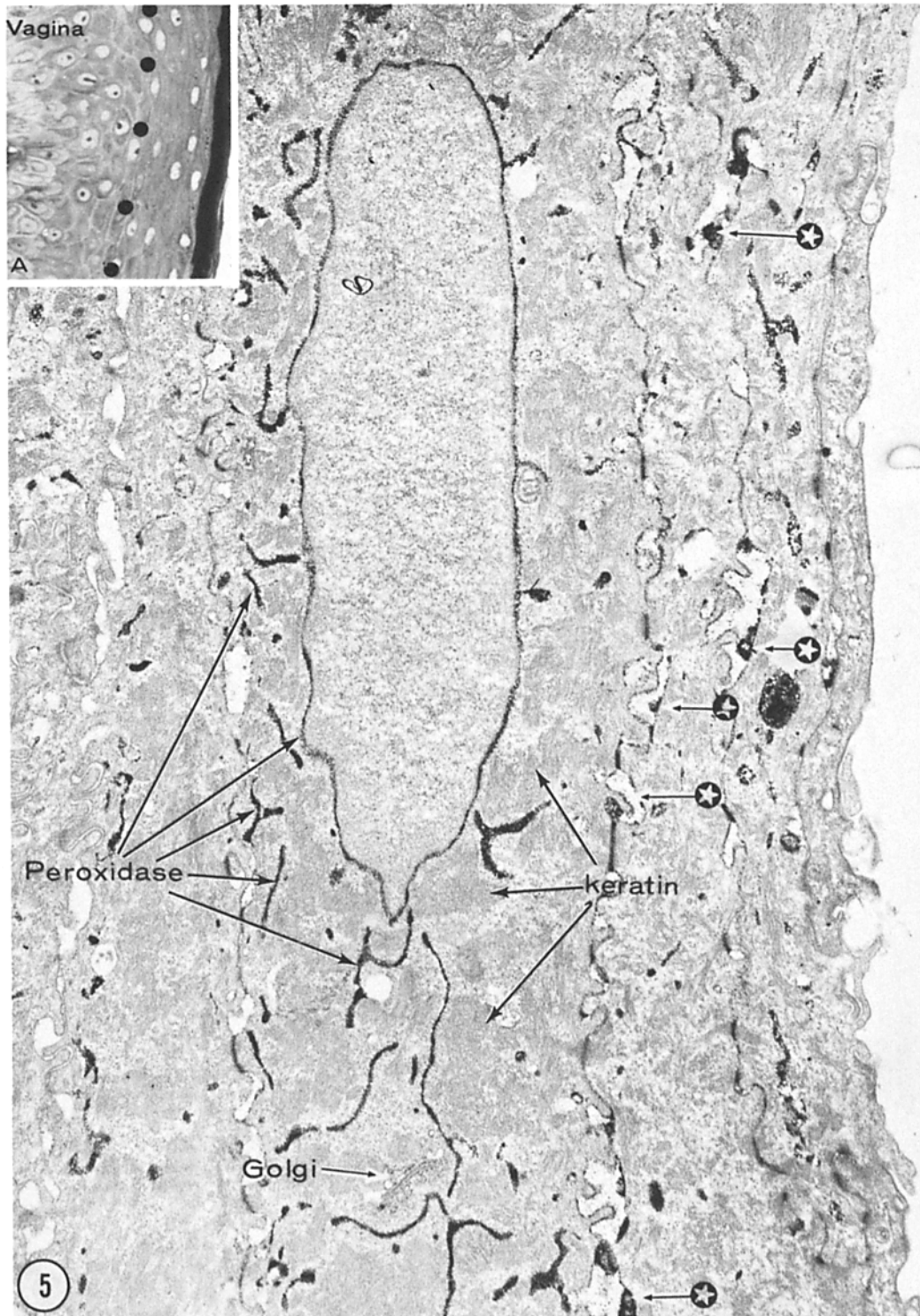


FIGURE 5 Sections through the vaginal epithelium of an immature rat that received three injections of estradiol-17 $\beta$  (0.4  $\mu$ g per injection) over a 72-h period are shown here. In the semithick toluidine blue-stained section (1- $\mu$ m thick), closed circles illustrate the intermediate layer of epithelium in which peroxidase staining first appears (Fig. 5 A). Vaginal endogenous peroxidase activity is seen in the nuclear envelope, in cisternae of the endoplasmic reticulum, and in cytoplasmic vesicles. Some peroxidase-positive material is also present on the surface of the cells in the superficial layers of the epithelium (stars).  $\times$  8,500.  $\times$  400.



TABLE I  
Distribution of Peroxidase Activity in Estrogen Target Tissues of Immature Female Rats after Injection

Tissues	Diethylstilbestrol or Estradiol-17 $\beta$ *						Upjohn 11,100 CI628†				Estradiol-17 $\beta$ Plus CI628				Other Steroids
	Imm	E <sub>6</sub>	E <sub>24</sub>	E <sub>48</sub>	E <sub>72</sub>	E <sub>96-144</sub>	I <sub>24</sub>	I <sub>48</sub>	I <sub>72</sub>	I <sub>96</sub>	EI <sub>24</sub>	EI <sub>48</sub>	EI <sub>72</sub>	EI <sub>96</sub>	Pg and T 12-96 h
Ovary	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Oviduct	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Uterus	—	—	+	++	+++	++++	+	++	+++	++	+	++	+++	++++	—
Cervix	—	—	+	++	++	++	+	++	+++	++	+	++	++	++	—
Vagina	—	—	+	++	++	++	+	++	+++	++	+	++	++	++	—
Hypothalamus	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Pituitary	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

\* E = 0.1-0.4  $\mu$ g/24 h (physiological), or \*E = 1-4  $\mu$ g or 24 h (hyperphysiological).

† I<sub>low</sub> = 50  $\mu$ g/24 h or I<sub>high</sub> = 500  $\mu$ g/24 h.

CI628 30 min Estradiol-17 $\beta$ .

Progesterone (Pg) (1 mg/24 h), Testosterone (T) (1 mg/24 h).

TABLE II  
Distribution of Peroxidase Activity in Mature Female Rats

Tissues Binding Estrogen	Proestrus	Estrus	Diestrus	Lactating	Constant estrus	Ovex	Ovex + E <sub>2</sub> *
Uterus	+	++++	—	—	+++++	—	++++
Cervix	+	+++	—	—	+++++	—	++++
Vagina	+	+++	—	—	+++	—	+++
Breast	—	—	—	+	—	—	—
Hypothalamus	—	—	—	—	—	—	—
Pituitary	—	—	—	—	—	—	—

\* Dosage of estradiol-17 $\beta$  = 0.4  $\mu$ g/24 h.

TABLE III  
Specific Activity of Hemoproteins and Uterine Peroxidase\*

Hemoprotein	U/mg/min		U/ml/min pH 7.02	U/mg protein/min
	pH 5.0	pH 7.02		
Horseradish Peroxidase (Sigma VI)	25.91 $\times$ 10 <sup>3</sup> $\pm$ 2.4 SE	5.97 $\times$ 10 <sup>3</sup> $\pm$ 0.16 SE		
Lactoperoxidase (Sigma)	0.91 $\times$ 10 <sup>3</sup> $\pm$ 0.006 SE	0.769 $\times$ 10 <sup>3</sup> $\pm$ 0.035 SE		
Uterine Fluid				
E72†			2.36 $\pm$ 0.185 SE	719 $\times$ 10 <sup>-3</sup>
Late estrus			7.93 $\pm$ 0.519 SE	813 $\times$ 10 <sup>-3</sup>
Constant estrus			11.33 $\pm$ 0.326 SE	777 $\times$ 10 <sup>-3</sup>

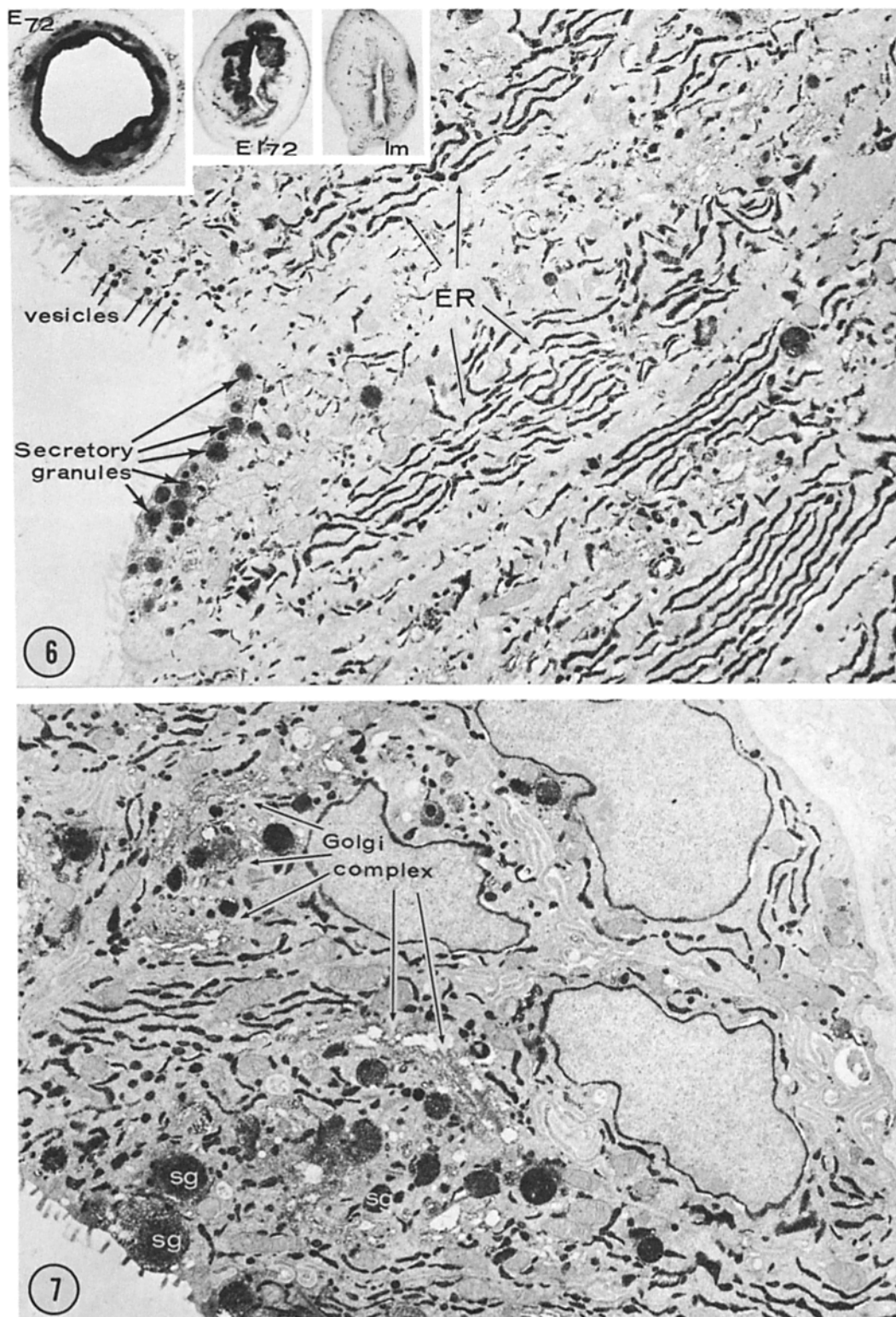
\* Colorimetric assay using 3,3'-diaminobenzidine tetrahydrochloride as hydrogen donor according to Herzog and Fahimi, (1973).

† Fluid derived from immature rats receiving three injections of 0.4  $\mu$ g estradiol-17 $\beta$  over a 72-h period.

and the accumulation of secretory material in the cells apices and in well-developed glands (Heuson et al., 1972). These investigators also attest to the weak progestational effects of nafoxidine. Our findings concur that nafoxidine and also CI628 stimulate secretory activity of the uterine mucosa; significant amounts of the material stored and secreted consist of peroxidase. Our enzyme kinetic studies on the uterine fluid verify the existence of a secreted peroxidase in the uterus.

#### Possible Functions of Hormone-Induced Peroxidases

Thyroid peroxidase is found in glands under stimulation by TSH derived from the anterior pituitary; atrophic glands lack peroxidase (Strum and Karnovsky, 1970). Thyroid peroxidase effectively catalyzes the iodination of a variety of proteins (Coval and Taureg, 1967). Since it catalyzes the iodination of tyrosyl residues in thyrox-



FIGURES 6 AND 7 The insets are photographs of 100- $\mu$ m thick vibratome sections through the uterus of rats given three injections of 0.4  $\mu$ g estradiol-17 $\beta$  in 72 h (*E72*), three injections of estradiol-17 $\beta$  (0.4  $\mu$ g) and three injections of CI628 (500  $\mu$ g) over a 72-h period (*E+I 72*), and of an immature rat given three injections of 0.1 ml glycerol over a 72-h period (*Im*). The mucosa of the estrogen- and estrogen + inhibitor-treated rats are intensely stained for peroxidase activity. The electron micrograph shows the distribution of endogenous peroxidase in the cisternae of the endoplasmic reticulum (*ER*), in apical secretory granules (*Sg*), and vesicles, and in regions of the Golgi complex of endometrial cells in an estrogen-treated rat (Fig. 6), and in a CI628-treated rat (Fig. 7). Fig. 6,  $\times$  5,000. Fig. 7,  $\times$  9,000. Insets,  $\times$  15.

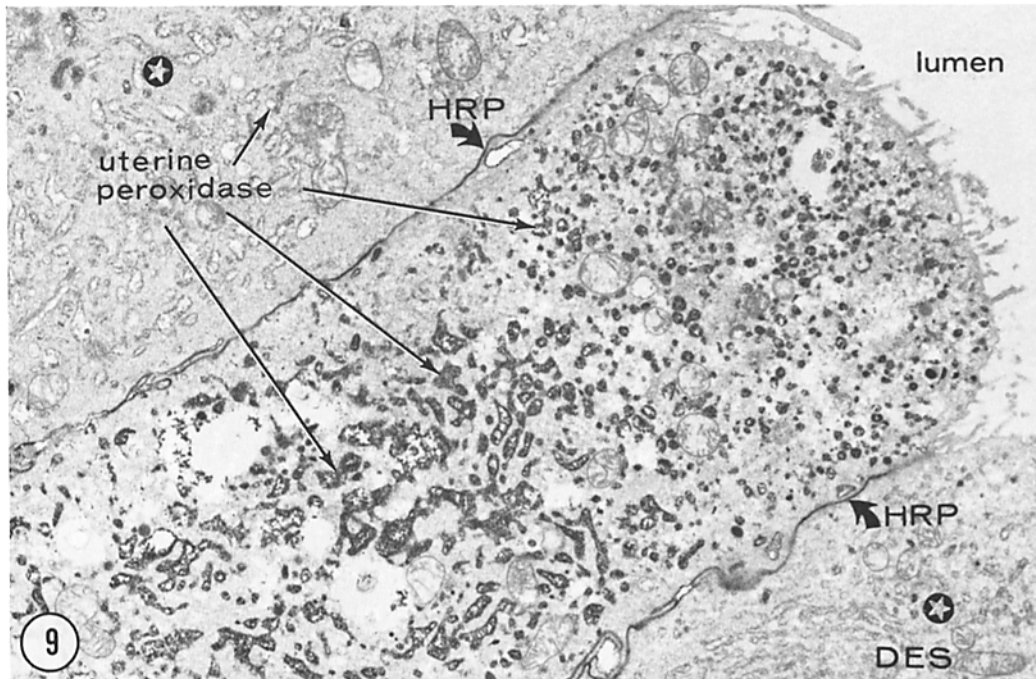
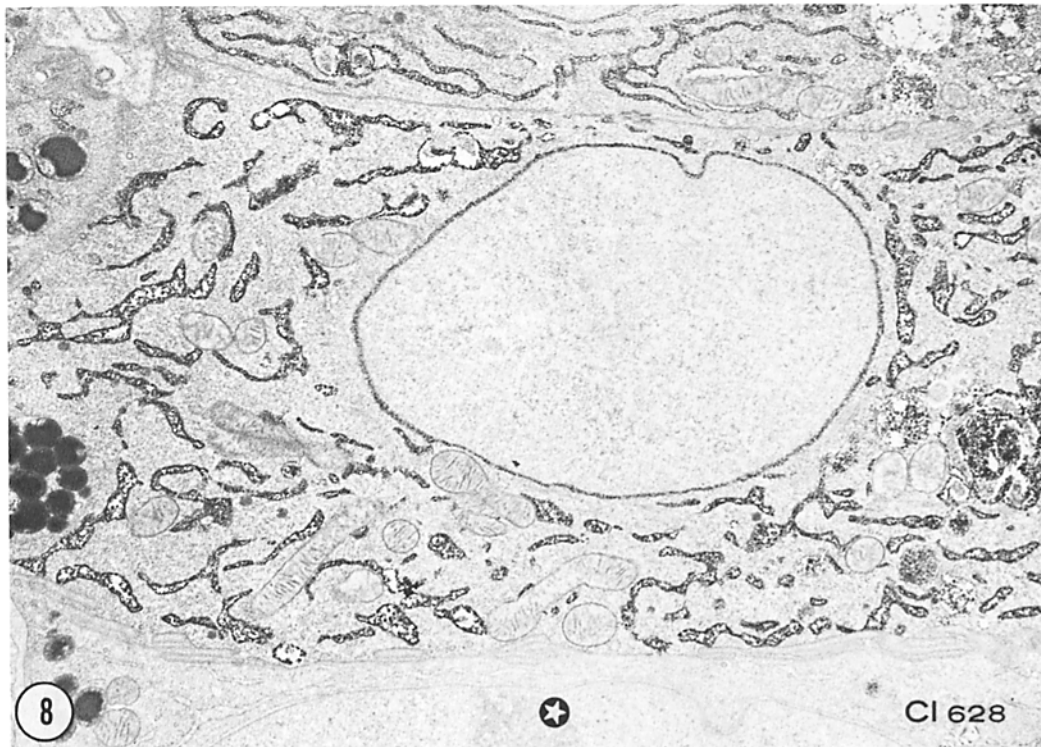
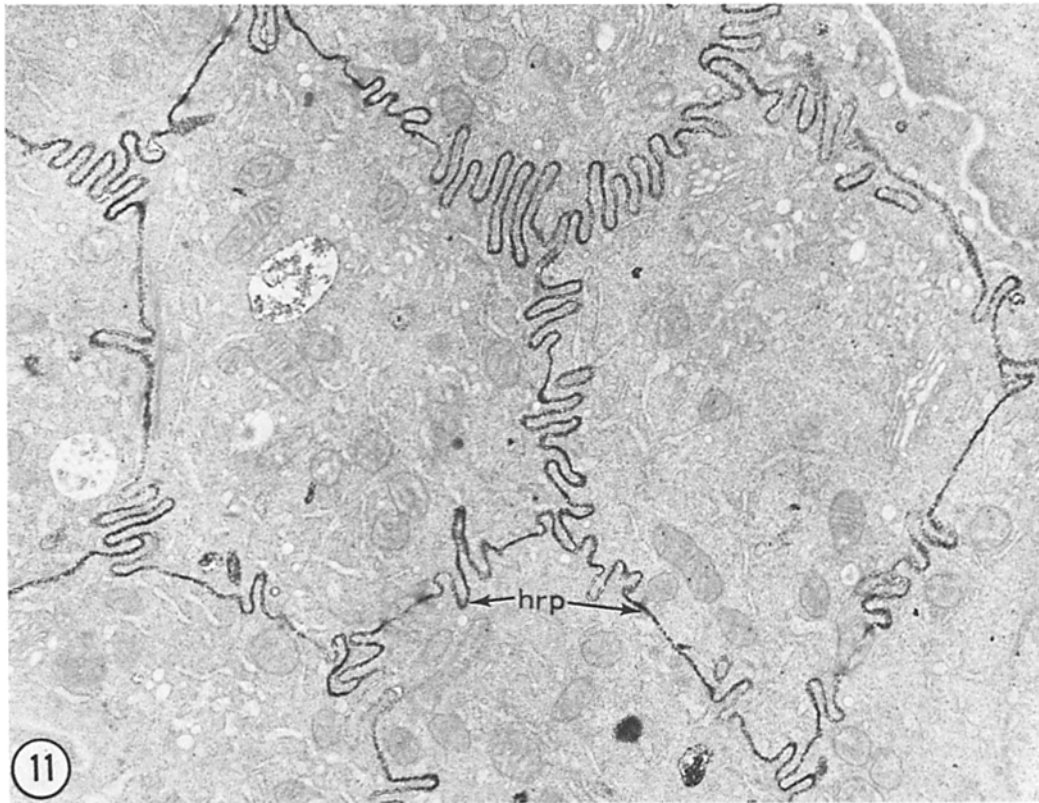
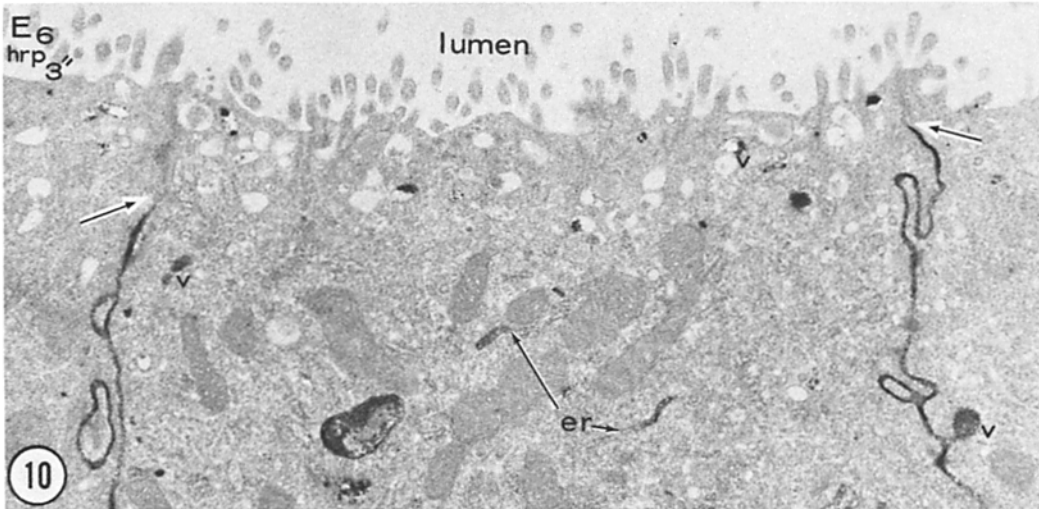


FIGURE 8 In rats given three injections of CI628 (500  $\mu\text{g}$  per injection) over 72 h, the heterogeneity of peroxidase staining in the uterine epithelium is apparent. Some cells are intensely reactive, while adjacent cells are devoid of peroxidase activity (stars).  $\times 6,500$ .

FIGURE 9 This heterogeneity of peroxidase staining is also apparent in the uterine mucosa of a rat given three injections of diethylstilbestrol (0.4  $\mu\text{g}$  per injection) in 72 h; the stars show cells weakly reactive for endogenous peroxidase. The extracellular space shows DAB-positive staining for horseradish peroxidase 3 min after injection of the tracer into the blood via the saphenous vein.  $\times 6,400$ .



FIGURES 10-12 3 min after the injection of horseradish peroxidase into immature rats treated for 6 h with  $0.1 \mu\text{g}$  estradiol- $17\beta$ , DAB-positive material (*hrp*) appears in the extracellular space surrounding epithelial cells of the uterine mucosa and in the apical cisternae (*er*). These cells possess few pinocytotic vesicles (*v*). Fig. 10,  $\times 11,500$ . Fig. 11,  $\times 17,000$ . Fig. 12,  $\times 6,000$ .

ine, its activity is essential to thyroid hormone production. Peroxidase in the endometrium could be involved in iodination of proteins or in other halogenation reactions similar to that described for the thyroid system.

Other functions have been ascribed to the peroxidases of the estrogen-stimulated uterus. Indeed, it is well established that peroxidases can catalyze the oxidation of various phenolic hormones, including estrogen, to phenoxy radicals that may secondarily bind proteins (Williams-Ashman and Johnson, 1960; Williams-Ashman and Reddi, 1971). Peroxidase in the presence of hydrogen peroxide can catalyze the conjugation of estradiol to proteins and some thiols (Klebanoff and Segal, 1960; Klebanoff, 1965; Jellinck and Irwin, 1963; Jellinck and Fletcher, 1970) as well as to DNA and other polynucleotides (Jellinck and Fletcher, 1970). Brökelmann and Fawcett (1969) and Brökelmann (1969) indicated that the amount of tritiated estradiol bound to uterine tissue could be enhanced by the addition of hydrogen peroxide to the DAB incubation medium; the radioactivity appeared over sites of tissue peroxidase. Subsequent studies by Lyttle and Jellinck (1972) suggest that peroxidase can metabolize estrogen to water-soluble by-products. Lyttle and Jellinck (1972) and Brökelmann and Fawcett (1969) suggest that uterine peroxidase may therefore function to terminate estrogen action in target tissues. This is quite conceivable, but entirely unproven to date. The question remains open: are the estrogen-induced peroxidases of the female reproductive system catalysts for estrogen metabolism; are they halogenation agents; or do they have other physiological roles?

It is well known that lactoperoxidase and myeloperoxidase in association with halides and hydrogen peroxide exert bactericidal, virucidal and fungicidal effects (Klebanoff and Luebke, 1965; Klebanoff et al., 1966; Belding et al., 1970) and possibly a spermicidal function (Smith and Klebanoff, 1970; Klebanoff and Smith, 1970). We cannot rule out the possibility that rat mammary carcinoma, vaginal, cervical, and uterine peroxidases are functioning as germicidal agents.

What appears clear is that endogenous peroxidase or one or more hemoproteins with peroxidase activity are produced in tissues dependent on estrogen for growth, and that they are secreted into the tissue lumina. Competitive growth antagonists to estrogen also induce peroxidase synthesis in the epithelial cells of these tissues. These observations

and the partial agonist effects of such antiestrogens suggest that these agents may at least partially participate in receptor-mediated gene activation of such tissues. Preliminary indirect evidence showing some nuclear estrogen receptor in uteri after treatment with nafoxidine in vivo (Rochefort et al., 1972) or in vitro (Clark et al., 1973) would be consistent with this concept. Studies with such antiestrogens to discern the induction of specific proteins as well as general trophic responses should lead to a better understanding of the role of hormones and their antagonists in gene-regulated responses of normal or neoplastic tissues.

### *The Transendometrial Transport of Enzymes*

Analyses of uterine fluids show the existence of prealbumin, albumin, and globulins similar to those present in the blood plasma (Beier, 1968; Peterson and Spaziani, 1971). Our studies, using an exogenous protein tracer (horseradish peroxidase), show that the movement of plasma-derived proteins into the uterine lumen is blocked by tight junctions between endometrial cells. Some tracer is incorporated by pinocytosis in the endometrium. A series of intracellular vesicles aggregate at the cell apices, giving the suggestion that material could be transported to the lumen by an intracellular route.

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