MODULATION OF UTERINE MORPHOLOGY AND GROWTH BY ESTRADIOL-17β AND AN ESTROGEN ANTAGONIST

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ABSTRACT

The estrogen antagonist CI628 maintains sustained hypertrophy of the uterine epithelium and the synthesis of many proteins including peroxidase. CI628 is a progestogen, inducing secretion of the protein by surface epithelial and glandular cells. CI628 is a connective tissue mitogen, inducing DNA synthesis in fibroblasts and the endothelium. CI628 and estrogen share these properties mentioned above. Estrogen, however, induced moderate growth of the mucosa within a 24-h period and massive hyperplasia of the mucosa within a 24-h period therafter. CI628 alone, or in combination with estradiol, does not have a mitogenic effect on the mucosa, and in fact blocks the mitotic response normally induced by estrogen alone.

Estrogen is an essential requirement for growth and differentiation of the uterus, cervix, vagina, breast, and some mammary tumors (Jensen, 1965; Jensen and DeSombre, 1972). Pharmacological interference by antiestrogens blocks growth of these tissues (Duncan et al., 1963; Callantine et al., 1966; Lerner et al., 1966; Jensen et al., 1972; Heuson et al., 1972). These drugs, including nafoxidine (Upjohn 11,100), CI628 (Parke-Davis), and MER 25 (ethamoxytriphetol), vary in their estrogenic effect and block estrogen action by competitive binding to the estrogen receptor protein in the cytosol and nucleus (Callantine et al., 1968; Jensen et al., 1972). The effect of estradiol-17β and Parke-Davis CI628 on uterine growth is the subject of this report.

MATERIALS AND METHODS

Virgin female rats of the Sprague-Dawley strain at 21–23 days old were used in these experiments. Animals in experimental groups of six were given daily subcutaneous injections of: (a) estradiol-17β (0.1–0.4 μg in 0.1 ml glycerol); (b) Parke-Davis CI628 at concentrations vary-ing between 50 and 500 μg/0.1 ml glycerol; and (c) CI628 followed by 0.4 μg estradiol-17β 30 min later. Animals comprising the control group were given daily injections of 0.1 ml glycerol.

Animals in experimental and control groups were sacrificed at 12-h intervals. Animals under light ether anesthesia were perfused with cold Formalin-glutaraldehyde fixative (Karnovsky, 1965) via the aorta for 15–30 min. The intact perfused uteri were excised at the vagina and oviduct and immersed in cold fixative for an additional 30 min. Each uterine horn was cut into three segments (a) postoviductal, (b) corpus, and (c) adcervical, as per the diagram:
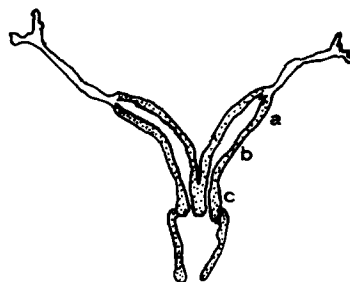
The uterine tissue was postfixed in osmium tetroxide, dehydrated in graded alcohols, and processed for electron microscopy. Semithick plastic sections (1-2 μm thick) cut with an MT2 ultramicrotome (Ivan Sorvall, Inc., Newtown, Conn.) were stained with toluidine blue and photographed with an Olympus microscope. The dimensions of the uterine epithelium, of the width of the submucosa, and of muscle layers in all segments (a-c), after all treatments, and at 12-h time intervals within a 96-h period, were calculated with a Filar eyepiece micrometer gauge (Bausch & Lomb, Inc., Rochester, N.Y.).

Experiments with [³H]Thymidine

[methyl-³H]Thymidine (sp act 15 Ci/mM), obtained from New England Nuclear, Boston, Mass., was injected (a) into the saphenous vein of rats under light ether anesthesia, or (b) directly into the uterine lumen of laparotomized females. In the latter group, fluid in the lumen was withdrawn before injection of <0.1 ml of the radioactive tracer. [³H]Thymidine was injected into rats at 6-h intervals after injection of estrogen, antagonist, or antagonist plus estrogen. The radioactive pulse period per animal varied between 3 and 30 min. Uteri were excised from the anesthetized rats, rinsed in physiological saline solution, and then fixed by immersion in Formalin-glutaraldehyde fixative (Karnovsky, 1965). In other experiments, uteri were excised from animals that were perfused for 15-30 min via the aorta with cold fixative. The uterine segments were postfixed in osmium tetroxide and processed for electron microscopy by routine procedures.

Autoradiographic Procedures

Autoradiographic techniques, according to Granboulan (1965), were used. Radioactive tissue embedded in 1-μm thick, unstained plastic sections was fixed to glass slides. Kodak NTB-2 (Eastman Kodak Co., Rochester, N.Y.) and Ilford L4 emulsions (Ilford Ltd., Ilford, Essex, England) were used to coat the sections. After 7-14 days of exposure, the emulsion was developed in Microdol X for 4 min at 18°C, rinsed in water, and fixed in 30% sodium thiosulphate for 5 min. Dried sections were then mounted in Permount or glycerol, placed on cover slips, and examined with an Olympus microscope. Photographs of experimental and control sections were compiled as a montage to reconstruct uteri in transverse sections. The labeled cells in the endometrium, submucosa, and muscle layers were counted from these light micrographs.

RESULTS

The Uterine Mucosa of the Immature Rat

All segments of the uterus were lined by low columnar epithelial cells, showing little variation in height from region to region (Figs. 1 and 3).

These cells, with an average height of 23 μm, possessed centrally or basally placed nuclei and a poorly developed system of granular endoplasmic reticulum. Although mitotic figures were seen occasionally in the mucosa, the degree of tritiated thymidine incorporation over a 15-min pulse period was insignificant.

Uteri of immature rats injected with vehicle (glycerol) were observed at 24, 36, 48, and 72 h postinjection. The endometrial epithelium for all segments had an average height of 21-25 μm and were identical to epithelial cells of uteri in immature uninjected rats.

24-h Changes

THE RESPONSE TO ESTRADIOL-17β: After estradiol injection, the epithelium in segments (a) and (b) showed a growth increase by 33% over the immature uterine mucosa, while a 54% increase was observed for the epithelium of the adcervical segment (c) (Figs. 2 and 4). Few mitotic figures appeared in the mucosa.

After a 15-min pulse period with [³H]thymidine, the bulk of radioactivity appeared over nuclei of cells in the submucosa and myometrium. Few grains appeared over the surface or glandular epithelium. Close scrutiny of these autoradiographs further demonstrated that nuclei from endothelial cells of the submucosal blood vessels and connective tissue cells incorporated most of the tritiated thymidine.

INJECTION OF ESTROGEN PLUS INHIBITOR: Cells in segments (a), (b), and (c) showed, respectively, increases in height of 54%, 60%, and 77% over cells in similar segments of the immature uterus (Figs. 2 and 5). The glandular or surface epithelial cells incorporated little thymidine over a 15-min pulse period; the bulk of the label occurred over nuclei in the submucosa and myometrial layers.

INJECTION OF INHIBITOR ALONE: Cells in segment (a) hypertrophied extensively, showing a 101% increase in height over control cells of the immature uterus. Cells in segment (b) showed a less dramatic hypertrophic response (only 56% increase in height) while cells in segment (c) increased in height by only 36% (Figs. 2 and 6). Endothelial and connective tissue cells incorporated most of the tritiated thymidine during the first 24 h after treatment with C1628.

48-h changes

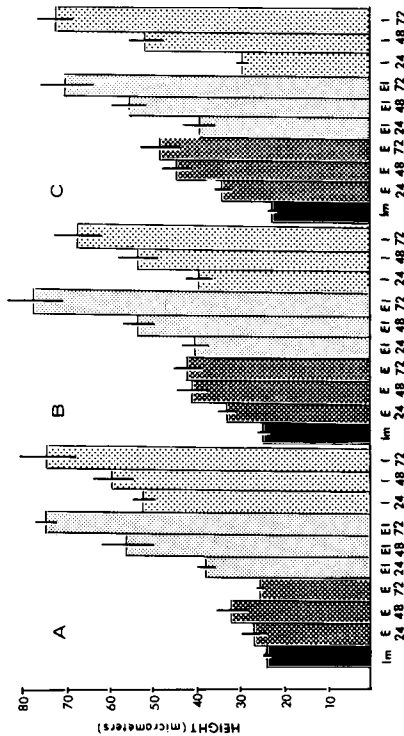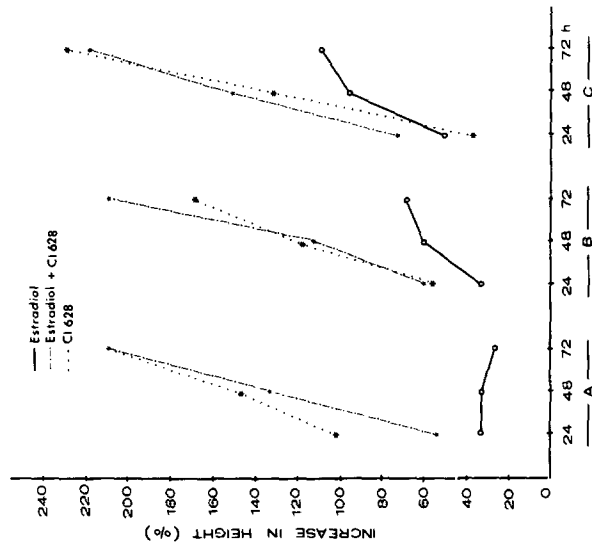INJECTION OF ESTROGEN: The 48-h response to estrogen was characterized by a 33%

FIGURE 1 This diagram illustrates the variation in height of uterine epithelial cells of the immature rat before or after administration of estradiol-17β (0.4 μg) alone, E; inhibitor (500 μg CI628) 30 min before estradiol-17β (0.4 μg), EI; or inhibitor (500 μg CI628) alone, I. Injectants were administered subcutaneously in 0.1 ml glycerol at 24-h intervals to 21-day old female rats. The average epithelial cell heights expressed in micrometers are shown for the postoviductal (A), medial (B), and adcervical (C) uterine segments.

FIGURE 2 The percentage increase in height of the uterine epithelium of rats treated with estradiol, inhibitor plus estradiol, and inhibitor alone over that of immature rats injected only with glycerol is illustrated in this graph.
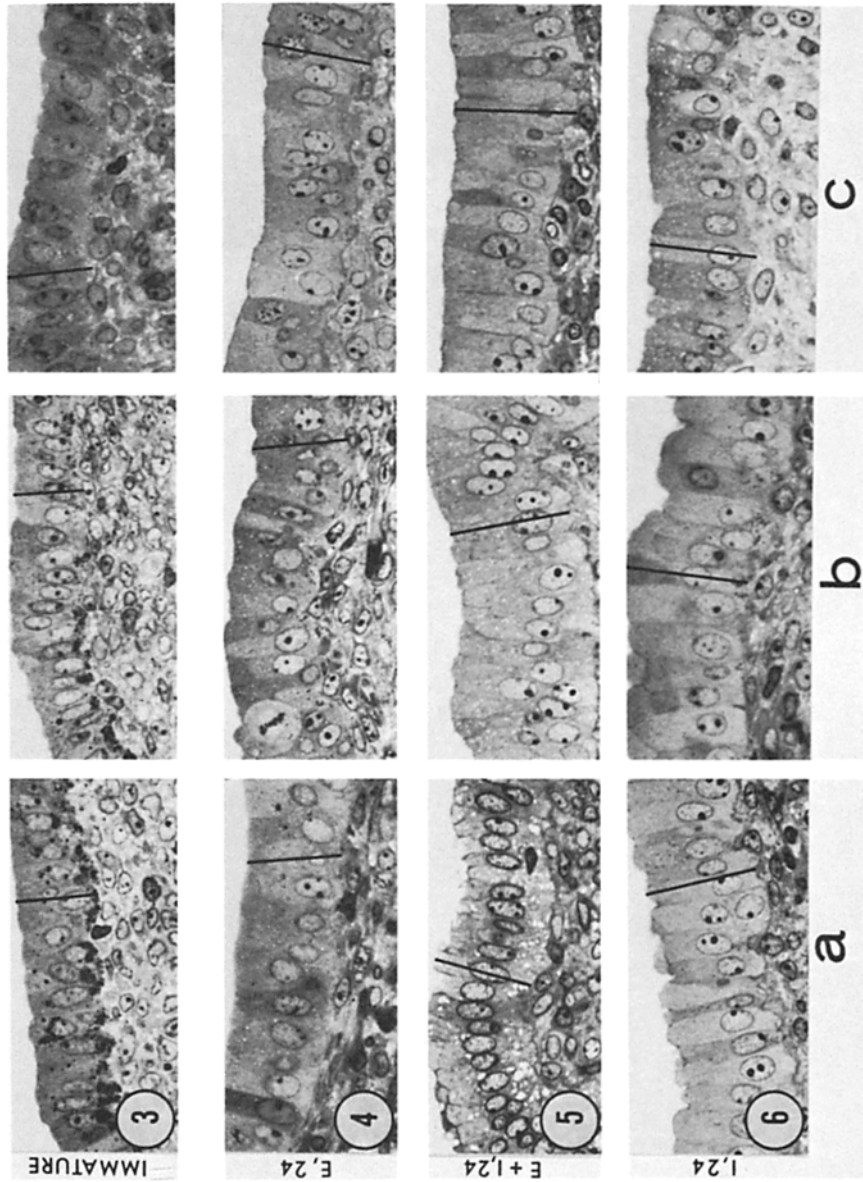
FIGURES 3–7 and 9–13   All light micrographs represent randomly taken, transverse sections through the endometrium at the postoviductal segment (a), the corpus, or medial segment (b), and the adcervical segment (c). All photographs, except where otherwise indicated, were taken at the same magnification and printed at the same enlargement. × 430.

FIGURE 3   The endometrium of the immature rat (21 days old) uterus is shown. The low columnar epithelial cells have an average height of 23 μm and show little variation from segment to segment.

FIGURES 4–6   Transverse sections show the endometrium 24 h after immature rats were injected with estradiol (Fig. 4), inhibitor and estradiol (Fig. 5), and inhibitor alone (Fig. 6). The epithelial cells, after all treatments, are markedly hypertrophied, showing variations from 33% to 100% increase in height over epithelial cells from the similar segments of the endometrium of immature rats. Rats treated with inhibitor and estradiol, or with inhibitor alone, showed most significant increase in height of the uterine epithelial cells.

E,48

a
7

b

c

E+I,48

9

SM

Glands

M

8

I,48

a
10

b

c
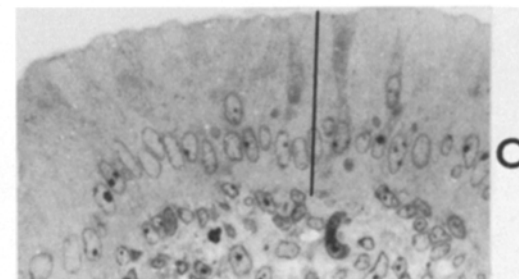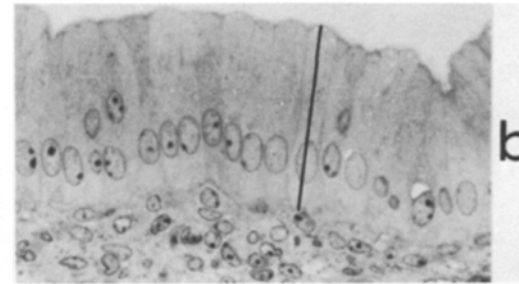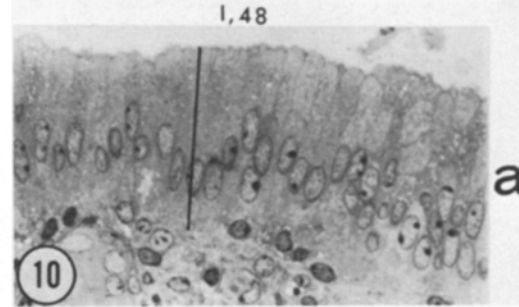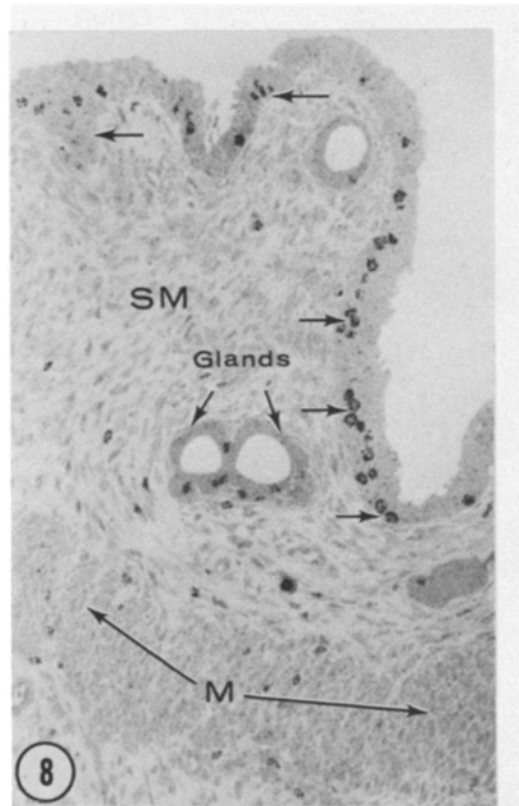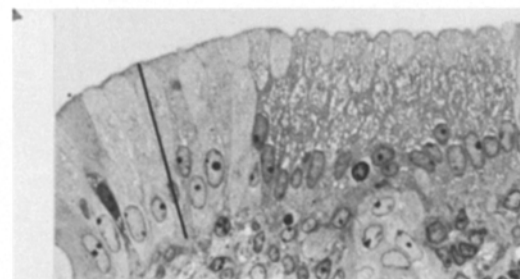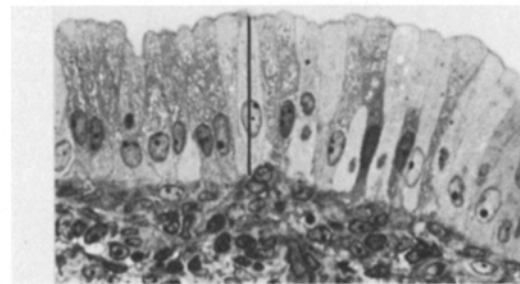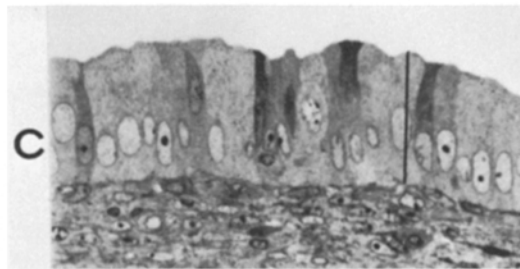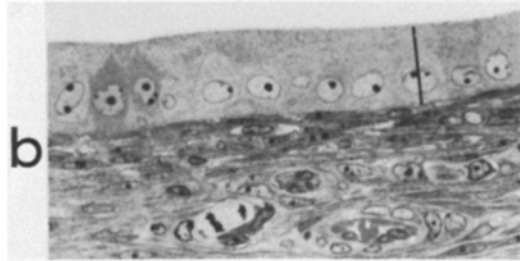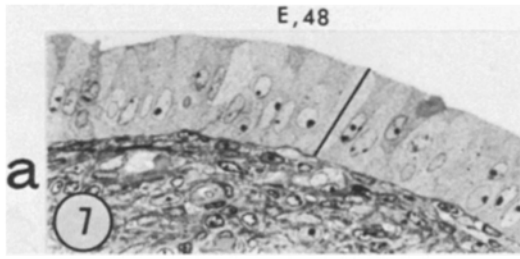
increase in height of cells in segment (a), 60% in segment (b), and a 95% increase in segment (c) (Figs. 1, 2, and 7). The elongate cells in segment (c) displayed extensive heterogeneity in cell density. Increased mitosis appeared in cells of all segments; these figures were, however, more prominent in cells of segments (a) and (b). After a 15-min pulse period, the bulk of the radioactivity appeared over nuclei of epithelial cells lining the lumen and in some glands (Fig. 8). In contrast to the 24-h images, few of the connective tissue cells incorporated tritiated thymidine.

INJECTION OF ESTRADIOL PLUS INHIBITOR: Further increases in the height of epithelial cells in all segments of the uterus ensued after injection of estrogen plus antagonist (Figs. 1, 2, 9) for 48 h. The cells reached an average height of 54 μm, showing increases in height of 133% for segment (a), 112% for segment (b), and 150% for segment (c). The epithelium incorporated no tritiated thymidine; the bulk of the label appeared over connective tissue cells of the submucosa.

INJECTION OF INHIBITOR: The uterine epithelial cells increased significantly in height over the 24-h stage after a second injection of C1628 (Figs. 1, 2, 10). Their secretory apparatus was now also well developed and all cells were positive for peroxidase activity. Tritiated thymidine was incorporated mainly by connective tissue cells of the submucosa.

72-h Changes

INJECTION OF ESTROGEN: The uterine lumen was now filled with fluid. Cells in segment (a) showed great variation in height, reaching an average height of 26 μm. This reflected only an 8% increase in height over similar cells in segment (a) of the immature rat uterus (Figs. 1, 2, 11). Cells in segments (b) and (c) showed, respectively, a 68% and a 118% increase in height over controls (Figs. 1 and 2). These cells possessed moderately well-

developed granular endoplasmic reticulum. At 72 h after injection of estradiol-17β, few epithelial cells were seen in mitosis or incorporated tritiated thymidine into their nuclei; few submucosal and myometrial cells were labeled.

INJECTION OF ESTROGEN PLUS INHIBITOR, AND INHIBITOR ALONE: Cells in segment (a) reached an average height of 74 μm, reflecting an increase in height of 208% over similar cells in the immature uterus. The maximum absolute increases in height occurred in cells of segment (b) of the estrogen plus inhibitor series (Figs. 1, 2, 12, 13) while the greatest percent increase (227%) was represented by cells in segment (c) of the inhibitor-treated animals. These cells did not incorporate tritiated thymidine and few, if any, cells in the submucosa incorporated label at this time.

Changes in the Submucosa and Myometrium

In all segments of the uterus, extensive variations in the thickness of the submucosa and muscle layers were observed after injection of estradiol. Inhibitor- and estrogen-, as well as inhibitor-treated rats had submucosa that were thickened in all segments (Fig. 14). Except in segment (a), the myometrium (Fig. 15) showed an increase in thickness after all treatments (i.e. with estrogen and inhibitor).

Effect of Inhibitor on Uteri of Cycling Rats

Mature cycling rats treated chronically, 6 days per wk, with inhibitor (1 mg/kg C1628) showed diestrus vaginal cytology. Unlike castration, which effected uterine regression to a small, atrophic organ, inhibitor-treated rats showed an initial decrease in uterine weight which was then maintained for the 10-wk treatment period (Table I). Furthermore, the uteri of the inhibitor-treated

FIGURE 7 Transverse sections through the uterine epithelium show the 48-h response to two physiological doses of estradiol-17β. These markedly hypertrophied cells display extensive heterogeneity in staining.

FIGURE 8 48 h after injection of estradiol into an immature rat. After a 15-min pulse with [^{3}H]thymidine, silver grains are located mainly over epithelial cells (arrows), over glandular cells, and over a few cells in the submucosa (SM) and muscle zones (M). × 130.

FIGURES 9-10 Epithelial cells of inhibitor plus estradiol- or inhibitor-treated immature rats showed a marked increase in height over rats treated with estradiol alone (Fig. 7).
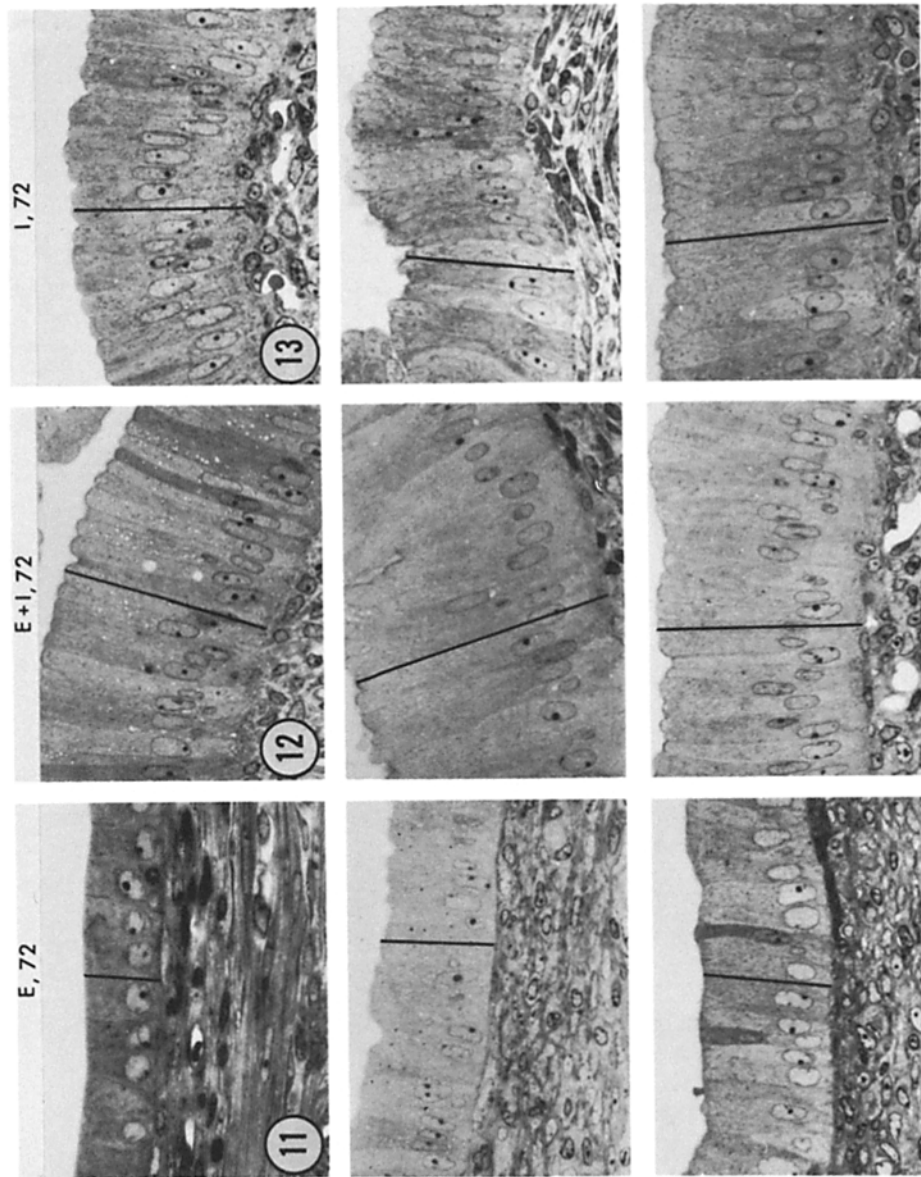
FIGURES 11–13 Comparisons in height of the uterine epithelium of rats treated for 72 h with estradiol, inhibitor plus estradiol, and inhibitor alone are made in these light micrographs. The lumen of uterus of rats receiving estradiol alone is filled with fluid, while uteri after estradiol plus inhibitor and inhibitor alone have little fluid and their lumens are filled with enormously hypertrophied epithelial cells (Figs. 12, 13). Cells in segment (c) of the inhibitor-treated animals show as much as a 227% increase in height over cells in the similar segment of the immature uterus.
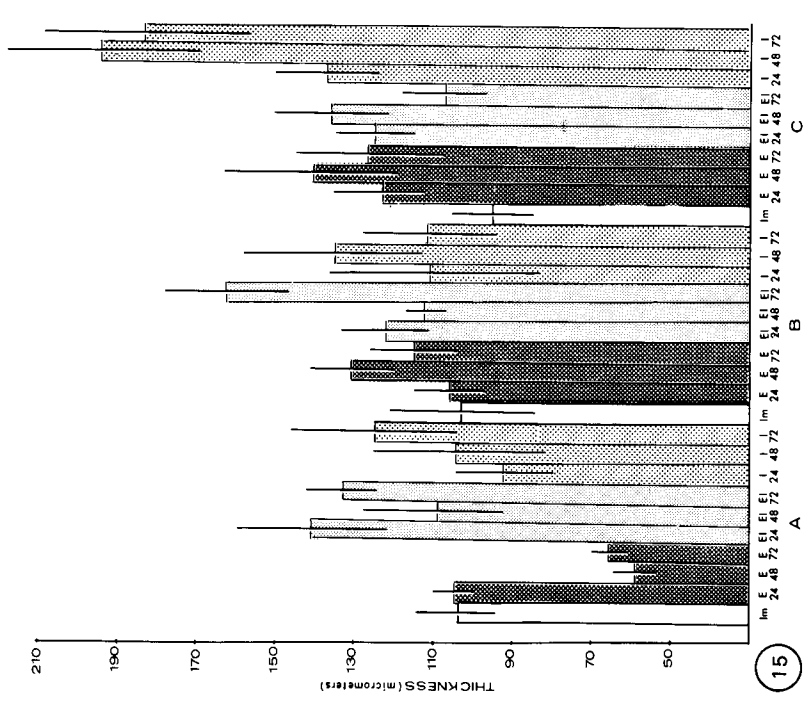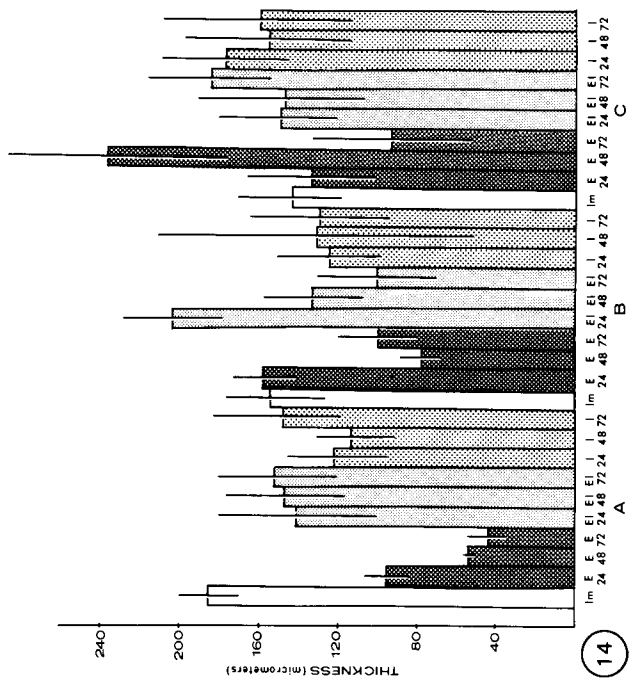
FIGURES 14–15   Diagrams illustrate the variation in thickness of the submucosa (Fig. 14) and myometrum (Fig. 15) in the three uterine segments after treatments for a period of 24–72 h with estradiol-17β, estradiol plus CI628, and CI628 alone.

TABLE I

Uterine Wet Weight (mg) in Cycling, Castrated, and Inhibitor-Treated Female Rats

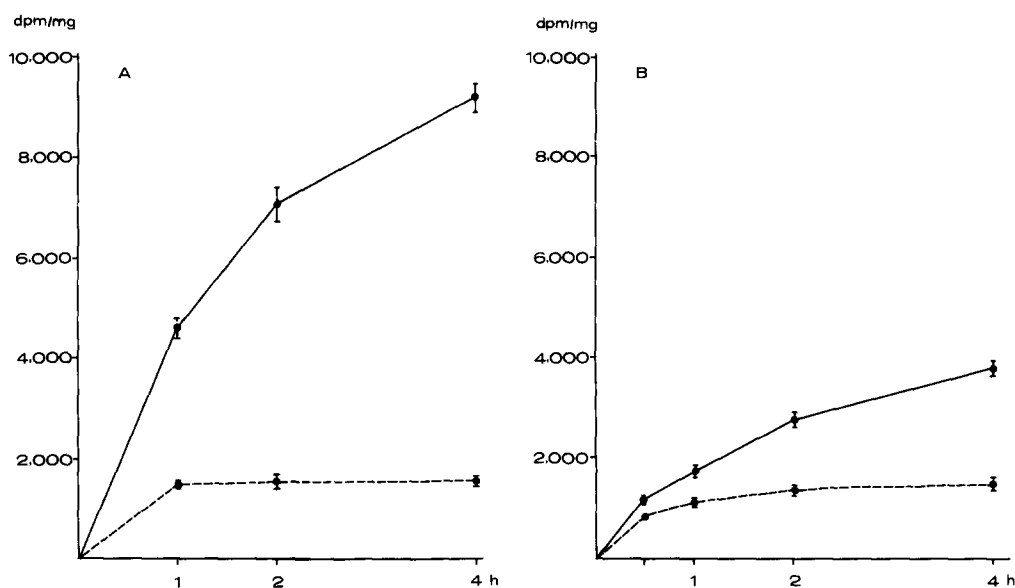| | Control | | | | |
	E + ProE	DiE + MetE	Composite mean	C1628	Castrate
wk					
1	411 ± 2	299 ± 6	344 ± 19	216 ± 7	—
2	470 ± 23	332 ± 2	442 ± 26	209 ± 5	—
4	468 ± 24	346 ± 5	419 ± 24	209 ± 9	—
10	516 ± 47	373 ± 23	430 ± 32	203 ± 9	80 ± 4

FIGURE 16   In vitro uptake of tritiated estradiol by slit uterine horns of castrate *A* or C1628-treated *B* rats. 50-day old rats were either ovariectomized or treated with C1628 (1 mg/kg, 6 days per wk) for 10 wk. Uteri were removed 24 h after the last injection; horns were slit longitudinally and stirred in beakers containing 500 ml 0.1 nM [6,7-^{3}H]estradiol-17β (57 Ci/mmol) alone (——), or with 10^{-5} M Parke-Davis C1628 (-----) in Krebs-Ringer-Henseleit glucose buffer at 37°C. At indicated time points, five uterine horns were removed, rinsed in buffer, dried, weighed, and combusted to tritiated water which was counted at 27% efficiency. Results are expressed as mean dpm per milligram dry weight with bars showing the standard deviation. Uptake with excess (C1 628 [---]) shows nonspecific association of estradiol with the tissue.

intact rats showed a markedly depressed ability to concentrate tritiated estradiol in vitro (Fig. 16) consistent with the reduced amounts of cytosol estrogen receptor determined by sedimentation analysis of uteri of inhibitor-treated rats.

DISCUSSION

Our results show that the various segments of the immature rat uterus change significantly in response to estrogen, antagonists, or combinations of both. Estrogen or antagonist initiates an immediate hypertrophic response by the uterine epithelium in all segments of the uterus. This hyper-

trophic response is correlated with hyperplastic response by cells in the submucosa, the endothelium having the most actively DNA-synthesizing and dividing cells.

Between 24 and 48 h, the estrogen-stimulated mucosa reaches its peak of mitotic activity; these cells are moderately hypertrophied and show some development of the protein synthetic and secretory apparatus. By 72 h, the mitotic response had ceased; the epithelial cells increased in size somewhat and developed a more extensive secretory apparatus.

The endometrial endothelium of rats given inhibitor does not actively divide, but instead,

continues to hypertrophy with significant development of its protein synthetic and secretory apparatus. These cells seem to be somewhat overstimulated and their vacuolated appearance resulted from extensive dilation of the cisternae of the granular endoplasmic reticulum The mitotic response of the submucosal cells diminished by 72 h after treatment with inhibitor or with inhibitor plus estrogen.

The studies in the intact rat are consistent with both antiestrogenic and progestational activities for CI628. The uterine weights were significantly reduced from those of untreated cycling rats, while the epithelial cells showed marked secretory changes. The lack of growth response to the inhibitor, which binds to the estrogen receptor, appears consistent with an effect on receptor levels. The lower concentration of cytosol receptor in such uteri is reflected in their decreased ability to concentrate estradiol in vitro.

Previous studies have shown that the administration of nafoxidine (Upjohn 11,100) markedly reduced the in vivo uptake of estradiol-17β by uterine tissue (Jensen et al., 1972), and inhibited growth of the uterus (Jensen, 1965). Although U11,100 A has been reported (Lee, 1974) to be unable to antagonize the mitogenic response of the mouse uterine luminal epithelium to estradiol, it is clear from this study that the antiuterotropic effect of CI628 resides in its ability to block the mitotic response of the rat uterine endometrium to estrogen.

It is speculated, therefore, that (a) the inhibitor blocks estrogen action by reducing the amount of receptor available for association with estrogen probably both by direct competition for the receptor and indirectly by limiting receptor synthesis; (b) it displays its estrogenic effect by inducing transcription for specific proteins (e.g., peroxidase) that usually appear after estrogen stimulation; (c) it displays its progestogenic effect by stimulating secretion of the newly synthesized protein; and (d) it displays its antiuterotrophic effect by repressing hyperplasia of the uterine mucosa.

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