Ovarian steroids are known to be important in maintaining vaginal tissue, and evidence is mounting that imbalances in the hormonal milieu contribute to vaginal pathophysiology. To date, limited data are available on the effects of hormone deprivation and replacement on vaginal tissue morphology and vaginal innervation. The goal of this study was to assess the dynamic changes in vaginal tissue structure in response to sex steroid hormone deprivation and administration. Female Sprague-Dawley rats were either kept intact (controls) or ovariectomized. Ovariectomized animals were treated with vehicle, estradiol, testosterone, progesterone, or a combination of estradiol plus testosterone or progesterone. Histological techniques, including stereological analysis and immunohistochemistry for localization of neuronal markers, were used. Ovariectomy produced a significant decrease in epithelial height that was restored with estradiol replacement. Interestingly, a subphysiological dose of estradiol resulted in hyperplasia of the vaginal epithelium and nonvascular smooth muscle. Neither testosterone nor progesterone had a significant effect on epithelial height or muscularis thickness. However, testosterone treatment resulted in a significant increase in small adrenergic nerve fibers. Addition of either testosterone or progesterone to estradiol mitigated but did not abolish the effects of estradiol alone. This study demonstrates that estradiol and testosterone have differential effects on vaginal tissue parameters and that ovarian hormones are critical for the maintenance of genital tissue structure. Present observations also suggest that combined replacement regimens may be required for an optimal physiological response. (Endocrinology 147: 61–69, 2006)

The vagina is a key organ in the peripheral genital arousal response. Limited data are available on the effects of menopause and hormone replacement therapy on tissue remodeling and vaginal innervation. Decreases in ovarian steroids because of surgical or natural menopause are known to induce structural changes in the vagina and contribute to genital pathophysiology (1, 2). Forsberg (1) reported shortening and narrowing of the human vagina accompanied by loss of vaginal folds with advancing age. In addition, the epithelial surface becomes flattened and keratinized. Zaino (3) demonstrated that, with the onset of menopause, there was a reduction of the number of layers in the vaginal epithelium, including a loss of intermediate cells, which results in an overall reduction in epithelial height. Boreham et al. (4) reported that in women with pelvic organ prolapse, vaginal smooth muscle content was most diminished in menopausal women who had not received estrogen replacement.

In laboratory studies, Park et al. (2) and Kim et al. (5) both have reported a significant decrease in vaginal epithelial height and smooth muscle tissue in ovariectomized rabbits. Similar findings have been reported in rats and nonhuman primates (6, 7). In an early study into the effects of steroid administration on the epithelium, Huggins et al. (8) reported that testosterone administered sc (0.1–4.0 mg/d) stimulated production of mucus but not keratin in superficial cells of the rat vagina. Estrone (1 μg/d), on the other hand, caused the superficial layer of the epithelium to disappear, with a concurrent increase in basal cells and keratinization. Higher doses of estrone (100 μg/d) caused only an increase in basal cells and keratin formation, whereas combining testosterone (1 mg) and estrone (1 μg) caused epithelial stratification with a decrease in mucoprotein formation and cessation of keratin production (8). These findings indicate differential roles of sex steroid hormones in the vagina.

Most studies of cyclical changes in the vagina have focused on the epithelium. At present, little is known about changes in the lamina propria or muscularis in either humans or animal models in response to hormonal insufficiency to warrant the drawing of any conclusions or formulation of hypotheses. Using immunohistochemical techniques to determine the distribution of protein gene product 9.5 (PGP 9.5), a general neuronal marker, Hilliges et al. (9) investigated the innervation of the human vaginal mucosa. In this extensive study, surgical specimens were obtained from anterior and posterior fornices and the anterior vaginal wall at the bladder neck and the introitus regions from six women, both pre- and postmenopausal. Nerve fibers within the epithelium were seen only in specimens from the introitus, with most fibers ending after penetrating two thirds the height of the epithelium. There was no evidence of intraepithelial fibers in any of the other regions studied. The authors reported networks of smaller fibers just below the epithelium and that such fibers were more prevalent in the distal regions. In addition, small fibers were more numerous in the anterior vs. posterior walls. Lastly, innervated blood vessels were seen in the deep submucosal layers of all regions studied.

Differential Effects of Estradiol, Progesterone, and Testosterone on Vaginal Structural Integrity

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Abbreviations: AChE, Acetylcholinesterase; NOS, nitric oxide synthase; PGP 9.5, protein gene product 9.5; RO, reverse osmosis H2O; TH, tyrosine hydroxylase.

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A number of studies have attempted correlating hormonal status with changes in neurotransmitter content or innervation density in the vagina. In an early study of vaginal adrenergic nerves in ovariectomized and estradiol-treated rabbits, Sjoberg (10) reported that there was a marked increase in the density of adrenergic nerves and noradrenaline content over the intact controls. In contrast, Ting et al. (11) reported an increase in nerve density in the absence of estradiol. Using histochemistry to detect acetylcholinesterase (AChE) and noradrenaline in sections of the rat vagina, Adham and Schenk (12) noted variations in both the total number of stained fibers and the relative densities of the nerve subtypes throughout the various stages of the estrous cycle. Specifically, they reported that both cholinergic and adrenergic nerve fibers were most numerous in estrus and least numerous during diestrus, with an intermediate number of fibers during metestrus and proestrus. In addition, they found that cyclic variation of AChE-positive fibers was most pronounced in the subepithelial plexus, whereas cyclic variation of adrenergic innervation involved primarily the muscularis.

Functional studies appear to corroborate these findings. Berkley et al. (13) reported that afferent fibers responding to vaginal distension or mechanical stimulation of the vaginal wall were observed most frequently during proestrus in rats. Another study demonstrated that afferent fibers of the pelvic nerve responding to vaginal distension were most sensitive at proestrus, when mating occurs (14). Berman et al. (15) demonstrated that both endothelial nitric oxide synthase (NOS) and neural NOS expression in cycling rats was highest during proestrus and lowest during metestrus. In addition, withdrawal of estradiol via ovariectomy significantly reduced both types of NOS, whereas subsequent estradiol replacement therapy resulted in an increase in their expression (15). In contrast, Al-Hijji et al. (16) investigated the extent to which vaginal smooth muscle response in ovariectomized rats was dependent on nitric oxide and reported that estrogen down-regulated NOS activity whereas treatment with progesterone significantly increased NOS activity.

Clearly there is evidence that sex steroids affect vaginal tissue structure and may play a role in neurotransmitter expression. However, a comprehensive study of vaginal morphology and innervation as related to hormone deprivation and administration is necessary to provide a baseline for new hypotheses regarding the role of sex steroids in maintaining the structure and functional integrity of the vagina. Thus, the goal of this study was to assess dynamic changes in vaginal tissue structure with hormone deprivation and administration. Although not included in this report because of the extensive amount of the data, a unique aspect of this work is the fact that it was carried out in parallel with immunohistochemical and biochemical determination of hormone receptor expression and physiological measurements of vaginal blood flow, which allowed direct correlation of all aspects of the data.

**Materials and Methods**

**Hormonal manipulation of animals**

Mature female Sprague Dawley rats (250–300 g) were purchased from Charles River Laboratories (Wilmington, MA). Animals were either kept intact (n = 8) or bilaterally ovariectomized. On postoperative d 14, osmotic pumps were implanted sc in ovariectomized animals through a dorsal midline incision between the scapulae. The pumps delivered continuous infusions of vehicle or sex steroids as shown in Table 1.

After 2 wk, vaginal smears were taken for cytological staging of the estrous cycle, and the animals were euthanized. This protocol was approved by Boston University’s Institutional Animal Care and Use Committee.

**Cytological staining of vaginal smears**

The vaginal smears taken from all animals before euthanasia were transferred to slides, stained, recoded, and read by a licensed cytologist for unbiased staging of the estrous cycle. Half of the unoperated control animals were determined to be in metestrus, and the remainder was divided between diestrus, proestrus, and estrus.

**Tissue procurement and processing**

Tissue was harvested from eight control animals and six animals in each experimental group except the E45 group. The urethra and rectum were dissected from the anterior and posterior walls of the vagina, respectively, and the entire vagina was removed and quickly weighed. A midline incision was made in the anterior wall of the vagina, from cervix to vulva, and the tissue was positioned flat for improved fixation. A small notch was made at the distal border to allow for proper orientation throughout tissue processing. The tissue was immediately immersed in 10% neutral buffered formalin and stored at 4°C for 24–72 h. After fixation, vaginal specimens were further divided in half lengthwise so each sample used in this study represented half the circumference of the vagina with equal portions of the anterior and posterior walls.

Tissue was then embedded in paraffin and subsequently sectioned at 5 μm using a rotary microtome. For each staining protocol, slides were taken from both the upper and lower vagina at consistent intervals.

**Modified Masson’s trichrome staining**

To allow for discrimination of muscle fibers from surrounding connective tissue, and to improve visualization of the epithelium, a modified Masson’s trichrome stain was used. For each animal, five nonse-

**TABLE 1. Infusion protocol and plasma concentration values**

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Infusion</th>
<th>Plasma concentration (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>V</td>
<td>6</td>
<td>Polyethylene glycol 300 (vehicle)</td>
<td>Estradiol: 13.4 ± 1.3</td>
</tr>
<tr>
<td>E5</td>
<td>6</td>
<td>Estradiol (5 μg/d, subphysiological dose)</td>
<td>19.7 ± 1.4</td>
</tr>
<tr>
<td>E15</td>
<td>6</td>
<td>Estradiol (15 μg/d, physiological dose)</td>
<td>31.2 ± 3.6</td>
</tr>
<tr>
<td>E45</td>
<td>4</td>
<td>Estradiol (45 μg/d, supraphysiological dose)</td>
<td>48.5 ± 6.0</td>
</tr>
<tr>
<td>T</td>
<td>6</td>
<td>Testosterone (11 μg/d)</td>
<td>15.4 ± 0.8</td>
</tr>
<tr>
<td>P</td>
<td>6</td>
<td>Progesterone (300 μg/d)</td>
<td>ND</td>
</tr>
<tr>
<td>ET</td>
<td>6</td>
<td>Estradiol (5 μg/d) + testosterone (11 μg/d)</td>
<td>ND</td>
</tr>
<tr>
<td>EP</td>
<td>6</td>
<td>Estradiol (5 μg/d) + progesterone (300 μg/d)</td>
<td>ND</td>
</tr>
</tbody>
</table>

In intact control rats, estradiol concentration was 36.8 ± 5.9 pg/ml, and testosterone was 139.2 ± 26.2 pg/ml. ND, Not determined.
sequent slides, evenly distributed along the length of the vagina, were processed using the following procedure. After deparaffinization, slides were postfixed in Bouin’s fixative for 1 h, and slides were placed in 4% ferric ammonium sulfate for 5 min at 50 C. Then slides were rinsed in water filtered by reverse osmosis (RO water) and placed in 1% hematoxylin for 1 min at 50 C, followed by differentiation in 2% ferric ammonium sulfate. After a 5-min rinse in RO water, slides were immersed in 0.1% acid fuchsin for 1 min and again rinsed in RO water. Next, slides were placed in 1% phosphomolybdic acid for 10 min. Lastly, they were stained with a solution of 0.25% aniline blue and 0.5% phosphomolybdic acid for 90 sec. After a final rinse in RO water, slides were dehydrated in graded ethanol, cleared in Citrusolv, and overslipped with Permount.

**Immunostaining procedure for PGP 9.5 and tyrosine hydroxylase (TH)**

Immunohistochemistry was performed using the peroxidase Vectorstain ABC method (Vector Laboratories, Burlingame, CA) and Gill’s hematoxylin counterstaining. Before immunohistochemistry, deparaffinized tissue sections were subjected to antigen retrieval by autoclaving them in 0.1 M citrate buffer (pH 6.0) for 5 min at 250 C and 20 atm pressure. Endogenous peroxidase activity was quenched with a solution of 0.3% hydrogen peroxide in methanol for 30 min at room temperature. After washing for a total of 15 min in three changes of PBS with Tween (PBS/Tween), endogenous binding sites for secondary antibodies were blocked using 3% normal serum in PBS. The normal serum used in any given immunolocalization was always that of the host species in which the secondary antibody had been raised. Slides were incubated with the blocking serum in a humidified chamber for 90 min at 37 C. Next, the primary antibody was applied and incubated in a humidified chamber overnight (19–22 h) at 4 C. After incubation with the primary antibody (1:200 dilution) (PGP 9.5 from Biogenesis, Kingston NH; TH from Chemicon, Temecula, CA), slides were washed with three changes of PBS/Tween for a total of 15 min, incubated with biotinylated secondary antibody (1:200 dilution) (PGP 9.5 from Biogenesis, Kingston NH; TH from Chemicon, Temecula, CA), slides were washed with three changes of PBS/Tween for 30 min at 37 C, and washed with three changes of PBS/Tween for a total of 15 min. The ABC complex (avidin-biotin-horseradish peroxidase) was then applied and allowed to develop for 10 min for visualization of immunoreactive sites. After a final wash, slides were counterstained with Gill’s hematoxylin. A negative control (omission of the primary antibody) and positive control (staining of rat brain sections) were carried out simultaneously with each immunohistochemical reaction.

**Image acquisition**

For morphological analyses, slides were reviewed and the clearest section on each slide was photographed at $\times 100$ or $\times 200$ using the camera feature of the Bioquant system (Bioquant Image Analysis, Nashville, TN; Nikon 5600, Q Image Retiga). Images were imported into Image J, a public domain Java image-processing program available from the National Institutes of Heath (http://rsb.info.nih.gov/ij), and subjected to additional analysis as outlined below.

**Epithelial height and muscularis area measurements**

Five trichrome images from each animal were used for morphometric assessment of the epithelium and muscularis. The areas of the epithelium and muscularis were estimated using the Cavalieri approach (17). In this method, a single point is used to represent a unit of area, and by counting points associated with the feature of interest, an estimate of area is made. Using the transparent overlay feature in Image J, a grid was generated and placed on each image (Fig. 1A). Each individual point in the grid generated by Image J for this analysis represented an area of 2500 $\mu$m$^2$. Because of variations in the length of vaginal wall in each field (e.g. sections positioned diagonally on the slide had more visible epithelium than those oriented more vertically), a correction for length needed to be made. Therefore, after calibration, the basement membrane in each field was traced, and a linear measurement was obtained using the length measurement feature in Image J. The estimated area for the region of interest was then divided by the basement membrane length measurement to obtain an area-to-length ratio for each region. The area-to-length ratio can be interpreted as the thickness or height of the epithelium. Group differences were tested for statistical significance by performing an ANOVA and a Tukey’s multiple comparison post hoc test. It is important to note that in applying this stereological method to the muscularis, only actual muscle bundles were measured, and intervening connective tissue trabeculae were excluded.

**Nerve fiber length density: PGP 9.5 and TH**

Using images obtained from slides immunostained for PGP 9.5 (five sections per animal), the method of point counting described above was used to determine area of the muscularis and lamina propria. Then, a grid overlay with randomly distributed uniform cycloid arcs was placed over each image, and immunoreactive nerve fibers intersecting the cycloids were marked and counted (Fig. 1B). Use of cycloid grids aids in determining the length of linear features when sections are not randomly oriented (18, 19). The cycloid arc plug-in feature in Image J (http://rsb.info.nih.gov/ij/plugins/grid-cycloid-arc.html) creates the appropriate grid and displays the parameters such as cycloid length per point. Length density (linear length per unit volume) was calculated according to the formula used for determining similar tissue parameters by Artacho-Perula and Roldan-Villalobos (19):

$$ \text{Length density} = \frac{2 \times \text{number of intersections with cycloid}}{\text{thickness of section} \times \text{(number of points)} \times \text{(cycloid length per point)}} $$

Group differences were tested for statistical significance by performing an ANOVA as well as a Tukey post hoc test.
**Results**

**Effects of ovariectomy and hormone treatment on morphological characteristics of the epithelium**

In control animals, the stratified squamous epithelium consisted of approximately six to eight layers of cells. Individual cells in the basal layer contained large round or oval basal nuclei. In the intermediate zone, the cells appeared more flattened and the long axis of the nuclei had become parallel to the basement membrane and epithelial surface. The nuclei of cells in this intermediate zone were lighter in appearance than the nuclei of cells in the basal layer. When nuclei were visible in superficial cells, they were flattened, darker, and more condensed. The orientation of the nuclei remained horizontal, although they were darker and more condensed than those of cells in the subjacent layer. In a few animals, keratohyalin granules could be seen in the most superficial layers.

Four weeks after ovariectomy, there was a significant thinning of the epithelium, which was reduced to one or two cell layers. This was confirmed by morphometric analysis showing a significant decrease in area-to-length ratio (a measure of epithelial thickness) for group V when compared with the control group (Figs. 2 and 3). Individual cells possessed more condensed nuclei and very little surrounding cytoplasm. The basal cells were cuboidal, and their nuclei were nearly square in profile, rather than round or oval, as seen in the control group. Consistent with the decreased number of cell layers, there was no gradual transition to anucleated, squamous cells at the epithelial surface. Occasionally more than a single epithelial cell layer was present, and the superficial layer contained flattened cells, often with highly condensed nuclei. Essentially, the epithelium had changed in character from stratified squamous to low cuboidal, reduced in many areas to a single layer of basal cells.

Estradiol administration restored the thickness of the vaginal epithelium after ovariectomy. Morphometric analysis revealed that in all three estradiol-treated groups, the mean area-to-length ratio of the epithelium was significantly higher than in ovariectomized animals receiving vehicle alone (Fig. 3). Interestingly, the epithelium was thickest in animals receiving a subphysiological dose of estradiol (group E5; \( P < 0.01 \) vs. controls), whereas tissue was most similar to that of control animals in groups treated with physiological (E15) or supraphysiological (E45) doses of estradiol. Administration of estradiol (5 \( \mu \)g/d) to ovariectomized animals caused distinct thickening of the epithelium, which appeared hyperplastic and contained approximately 10–12 layers of cells (Fig. 2). Basal epithelial cells were oval and tightly packed and, as in the controls, there was a gradual flattening of cells in the more superficial layers. The observed increase in the number of cell layers was attributed to retention of these flattened cells, the majority of which appeared to lack nuclei. In addition, there was an increase in epithelial infoldings (pegs) into the lamina propria. No epithelial hyperplasia was noted in animals treated with physiological or supraphysiological doses of estradiol (15 or 45 \( \mu \)g estradiol/d). The vaginal epithelium was composed of approximately eight to 10 cell layers and closely resembled that of the control animals in its general histological features. The basal cells were columnar, not cuboidal, epithelial intrusions into the lamina propria were relatively infrequent, and there was little or no evidence of keratohyalin granules in the superficial cells.

Administration of testosterone (11 \( \mu \)g/d) to ovariectomized animals had little effect on the mean area-to-length ratio of the epithelium (Fig. 3). The epithelium was one to three cells thick. Basal cells were cuboidal and compact with relatively condensed nuclei and very little cytoplasm. The juxtaluminal layer consisted of interspersed round and flattened cells with dark, condensed nuclei. Epithelial morphology was similar to that observed in the ovariectomized animals receiving vehicle only. The mean epithelial area-to-length ratio in animals treated with progesterone (300 \( \mu \)g/d) was not significantly different when compared with the...
of the muscularis. There were, however, some notable qualitative differences in histological appearance. In vehicle-treated animals, the epithelium consisted of a single layer of cuboidal cells, whereas in the progesterone-treated group, there was a shallow stratified squamous epithelium, three to four cells in thickness.

Coadministration of testosterone (11 μg/d) or progesterone (300 μg/d) with estradiol (5 μg/d) prevented the notable hyperplasia seen in ovariectomized animals given the same dose of estradiol alone. Both combined treatment regimens resulted in a vaginal epithelium similar to that observed in intact controls (Fig. 2). Morphometrically, the thickness of the epithelium in estradiol/testosterone- and estradiol/progesterone-treated groups was higher than in ovariectomized animals receiving vehicle (P < 0.001), lower than ovariectomized animals given subphysiological estradiol alone (P < 0.001), and not significantly different compared with unoperated controls (Fig. 3). These findings are consistent with the near-normal microscopic appearance of the vaginal epithelium in animals given the combined hormone treatments. In these groups, the basal cells were columnar with vertically oriented oval nuclei. There was a progressive flattening of cells toward the luminal surface with the nuclei becoming more squamous and horizontally oriented, and there were no observable granules in the superficial epithelial cells. As in control animals, there were only sparse epithelial infoldings into the lamina propria.

**Effects of ovariectomy and hormone treatment on morphological characteristics of the muscularis**

In control animals, the muscularis was well defined and consisted of both circular and longitudinal smooth muscle fibers. Individual muscle fibers were packed closely into bundles, separated from one another by thin connective tissue septa. The bundles were largest and most well defined in the upper portions of the vagina, whereas in the lower portions of the vagina, the bundles were smaller and less distinct. In sections from the most distal portions of the vagina, striated muscle fibers could occasionally be observed, and these were presumed to belong to the pelvic floor musculature.

Four weeks after ovariectomy, there was a decrease in the cross-sectional area of the muscularis measured in sections from animals receiving vehicle only, although the difference was not statistically significant compared with controls (Figs. 4 and 5). This was determined using the point counting method as previously described in which only actual muscle fibers were measured. Although the width of the muscularis layer appeared unchanged by morphometry, there was more connective tissue between the muscle fiber bundles in the ovariectomized vehicle group than in the controls. Interestingly, there was often a halo around the nuclei of the muscle cells in this group, but the significance of this observation is unknown.

Of the three groups treated with estradiol, the most pronounced effect on the muscularis was noted with the lowest dose, which produced a significant increase in the area/length ratio when compared with both control and ovariectomized groups. Higher doses of estradiol decreased this exaggerated effect, resulting in a muscularis area closely resembling that of the control animals. In animals receiving 5 μg estradiol daily, there was a significant increase in muscularis area as compared with both the control group and the ovariectomized group receiving vehicle only (Figs. 4 and 5). There were differences in the morphological appearance as well. Individual muscle fibers appeared enlarged compared with those in the controls and there was less connective tissue between the bundles. Administration of higher doses of estradiol (15 and 45 μg) also resulted in an increase in mean muscularis area when compared with the ovariectomized group receiving vehicle (P < 0.05). Although the measured area was again higher than in the unoperated control group, the difference was not statistically significant and the morphology of the muscularis in these groups was similar to that in controls.

There was no statistical difference in the area of the muscularis measured in sections taken from ovariectomized an-
imals without hormone replacement and those treated with testosterone (11 μg/d) or progesterone (300 μg/d); nor did any of these groups differ significantly from the controls (Figs. 4 and 5). Although the area of muscle fibers was similar among the groups, there were morphological differences. In the testosterone-treated group, muscle fiber bundles were separated by slightly more connective tissue than observed in the control animals. In ovariectomized animals receiving progesterone, there was a large amount of connective tissue between the fiber bundles, even greater than that observed in the ovariectomized animals receiving vehicle only; also, individual muscle fibers appeared slightly smaller than those in control animals.

When testosterone (11 μg/d) was coadministered with estradiol, there was less of an effect on the muscularis than with estradiol alone, although the difference was not significant with respect to control, ovariectomized, or estradiol-treated animals (Fig. 5). This suggests that testosterone may antagonize or block the effects of estradiol in this tissue. A more pronounced damping of the estradiol effect was noted when progesterone was coadministered with estradiol, and in this case, the decrease was statistically significant (P < 0.01 vs. estradiol alone), suggesting that progesterone attenuates the response of vaginal smooth muscle to estradiol. In the combined hormone therapy groups, the mean muscularis area and morphology were similar to that observed in controls (Fig. 4).

**Effects of ovariectomy and hormone treatment on PGP 9.5-positive nerve fibers**

Immunostaining for PGP 9.5, a general neuronal marker, permits visualization of the nerve network in the vagina. The largest nerve bundles could be seen in the adventitia, usually accompanying large blood vessels. Ganglion cells were also found occasionally in the adventitia. From these large nerve trunks, fibers extended radially inwards toward the muscularis and lamina propria where many smaller bundles of PGP-positive fibers were located. Isolated fibers were seen scattered throughout the muscularis, whereas more dense networks of fibers were evident in the lamina propria. Although fibers were both longitudinally and transversely oriented in relation to the long axis of the vagina, the majority appeared transversely oriented. Occasionally, fibers could be seen just beneath the basement membrane of the epithelium and in some instances, very fine, tortuous fibers were observed among the epithelial cells reaching upward into the middle layers of cells (Fig. 6A). There were also prominent perivascular plexuses on large vessels in the adventitia and on the mid-sized vessels in the muscularis and lamina propria. No qualitative regional differences were noted in the basic pattern of nerve distribution between the upper and lower vagina.

Neither ovariectomy, nor subsequent treatment with estradiol (5 μg and 15 μg/d), produced any notable effect on the length density of PGP 9.5-immunoreactive fibers (Fig. 7). In addition, no obvious differences in the orientation or distribution of the PGP-positive fibers were observed. In ovariectomized animals, testosterone administration (11 μg/d) significantly increased the density of PGP-positive fibers compared with ovariectomized and intact controls (Fig. 7).

**Effects of ovariectomy and hormone replacement on TH-immunoreactive nerve fibers**

Immunostaining for TH in control animals revealed TH-positive, presumably adrenergic, nerve fibers throughout the vaginal wall. Generally, the pattern of fibers was similar to that observed for PGP-positive fibers, but the majority of...
TH-positive nerve fibers was in the muscularis, rather than the lamina propria. In addition, there were no TH-positive intraepithelial fibers noted.

Steroid hormone administration had the same effects on TH-positive, adrenergic fibers as on PGP-immunostained fibers described above. There was no significant difference in fiber length density in ovariectomized animals when compared with controls (Fig. 7). Administration of estradiol and progesterone, alone or in combination, had no effect on TH-positive fiber length density; nor was there any appreciable difference in animals receiving a combination of estradiol and testosterone (Fig. 7). The only significant change noted in the adrenergic innervation occurred in the group receiving testosterone alone (Fig. 7). In these animals, there was an increase \( P < 0.001 \) vs. controls) in the length density of TH-immunoreactive nerve fibers, although the distribution of fibers did not vary from that observed in unoperated controls.

**Discussion**

In this study, we investigated the effects of ovariectomy and hormone treatment on vaginal tissue architecture. Ovariectomy exerted a powerful effect on the vaginal epithelium, which was reduced to a single layer of low cuboidal cells corresponding roughly to the original stratum germinativum. This dramatic structural alteration likely signifies changes in the functional properties of the epithelium. For example, lack of stratification renders the epithelium susceptible to abrasion, supporting clinical findings in which the epithelium is reported to be more fragile after menopause (20). A lower degree of protection against bacterial infection (1) may be anticipated, not only because the epithelium is thinner and more easily torn, but also because it has lost its ability to produce glycogen and so aid in maintaining a low pH in the vaginal fluid. Major changes in the vaginal epithelium might be linked to disturbances in vaginal sensation, such as those noted after menopause (21). Not only is there a thinning of the physical barrier between the lumen and the rich plexus of fine nerve fibers in the lamina propria, but intraepithelial nerve terminals may be compromised as a result of destratification. It is possible that alterations in sensory function after menopause may also be related to this significant change in epithelial morphology, because intraepithelial nerve fibers are likely truncated when the layers of the epithelium are diminished.

Examination of the vaginal muscularis demonstrated only slight muscular atrophy in ovariectomized compared with control animals. This is in contrast to earlier reports in which significantly greater atrophy of the muscularis was noted after menopause in women and ovariectomy in rabbits (4, 5). The discrepancy may be because of the relatively short time after ovariectomy in the present study, which may not have allowed significant changes in the muscularis to develop in these animals. Furthermore, this is the first study to use stereological measurements to determine the amount of smooth muscle, independent of its investing connective tissue. Simple measures of muscularis thickness do not accurately reflect changes in the relative proportions of muscle bundles and connective tissue. In addition, in the human studies, tissue samples were primarily taken from the upper portion of the vagina, whereas in this study, muscularis measurements were taken at uniform intervals throughout the entire length of the vagina. Finally, the muscularis of the rat vagina is not as robust as that seen in larger animals, such as rabbits and primates, making changes in overall muscle volume less likely.

Estradiol replacement reversed most changes in tissue morphology noted in this study after ovariectomy. Interestingly, the subphysiological dose of estradiol increased such parameters above the control levels. The epithelium was notably hyperplastic and the area-to-length ratio of smooth muscle in the vaginal wall was significantly greater than that seen in controls. Previous studies have shown that the estradiol receptor is up-regulated in the vagina of ovariectomized rats and this elevated level of estradiol receptor expression is maintained in ovariectomized animals infused with a subphysiological dose of estradiol (22). Therefore, increased receptor expression in conjunction with exogenous hormone administration may be responsible for the enhanced proliferative effects of estradiol. When physiological or supraphysiological doses of estradiol were administered to ovariectomized animals, the tissue properties appeared similar to those of the control group, indicating that the
trophic effects of estradiol on the vagina are dose dependent. There appears to be a threshold level of estradiol needed to decrease vaginal estrogen receptor expression and therefore minimize any increase in bound hormone. These data are supported by previous evidence that weak estrogens are more efficacious in the vagina, whereas stronger estrogens have greater effects on the uterus (1).

Physiological and biochemical measurements may contribute to the understanding of the functional impact of the morphological data noted above. Specifically, ovariectomized animals treated with only vehicle showed significantly decreased vaginal blood flow compared with controls (22). However, in animals treated with a subphysiological dose of estradiol, vaginal blood flow significantly exceeded controls, whereas blood flow in animals treated with a higher (physiological) dose of estradiol was not appreciably different from that in intact controls (22). In addition, Kim et al. (5) reported that androgen treatment in ovariectomized rabbits facilitates vaginal smooth muscle relaxation.

Administration of a physiological dose of testosterone to ovariectomized animals did not significantly change the area-to-length ratios in the epithelium or muscularis when compared with ovariectomized animals. These findings seem to rule out the possibility of direct regulation by testosterone acting through its own specific receptors. They also exclude indirect effects dependent on the aromatization of androgen in the vagina under these experimental conditions. If significant amounts of testosterone had been aromatized locally, vaginal tissue morphology in the testosterone-treated group should have more closely resembled that in estradiol-treated animals, and this was not the case. For the most part, progestosterone treatment failed to restore morphological changes induced by ovariectomy. Quantitative measurements of epithelial thickness were not statistically different in ovariectomized animals receiving progestosterone as opposed to vehicle. However, the epithelium in the progestosterone-treated animals was partially stratified, rather than low cuboidal, as was seen in animals receiving only vehicle. This suggests that progestosterone may play a role in maintaining epithelial integrity. Testosterone or progestosterone, when coadministered with estradiol, reduced the estradiol-induced proliferation. This suggests that progestosterone and testosterone possess properties antagonistic to those of estrogen. Progesterone and estradiol are known to have opposing effects in the uterus (23, 24), and a similar antagonistic effect between estradiol and testosterone has been reported in other systems, such as bone resorption in men (25) and aromatase gene expression in rat testes (26).

Of note in this study, there was only a 10% difference between the length densities of nerve fibers staining for PGP 9.5 (a general neuronal marker) and TH-positive fibers. This indicates that the majority of nerve fibers in the vaginal wall are TH positive, although they may not be exclusively adrenergic. Immunohistochemical studies by Giraldi et al. (27) have demonstrated that other neurotransmitters are colocalized with TH-positive fibers and that the density of cholinergic and adrenergic nerve fibers is similar. Also, innervation was most pronounced in the distal part of the rat vagina with the most robust plexuses of nerve terminals encircling vaginal arteries (27).

Our data indicate that PGP- and TH-immunoreactive fiber length density did not change significantly after ovariectomy or after estradiol administration to ovariectomized animals. This suggests that estradiol is not a key mediator of nerve fiber density. However, both PGP- and TH-immunoreactive fiber length densities were significantly increased in vaginal tissue from animals treated with testosterone when compared with intact control animals, an effect lost when testosterone was combined with estradiol. Although this has not been reported previously in vaginal tissue, there is some evidence in the literature that testosterone promotes nerve growth. Black et al. (28) reported that androgen treatment of female mice produced a 20-fold increase in nerve growth factor mRNA in the submandibular gland. In rats, Levy et al. (29) reported that testosterone treatment induced generation of thick nerve processes and an early onset of myelination, mainly of peripheral myelin, in fetal spinal cord slices. Furthermore, androgens are thought to be critical to maintaining nerve myelination and structure in male erectile tissue (30). In other studies, it has also been reported that nerve fiber density increases with increasing estradiol concentrations, (10, 12). In contrast, a recent study reported that nerve density increased in the absence of estradiol (11). In the later study, there was a relatively short duration of both ovariectomy (7 d) and estradiol replacement (7 d), and nerve fiber density was assessed only in sections from the middle portion of the vagina. These factors may account for the disparity between these previous reports and our current data.

In summary, ovariectomy produced a dramatic effect in vaginal morphology, and these changes were reversed by the administration of estradiol. In addition, it appears that there is a dose-dependent response to estradiol, with subphysiological doses producing hyperplasia of the epithelium and nonvascular smooth muscle. Although progesterone and testosterone do not appear to have a great influence on vaginal morphology, a unique effect of testosterone was the increase in small adrenergic nerve fibers. Physiological and biochemical observations, in conjunction with the morphological data presented, suggest that sex steroid hormones are important modulators of tissue parameters that may ultimately affect vaginal hemodynamics and arousal.

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