UTERINE SMOOTH MUSCLE FIBERS IN CASTRATE AND ESTROGEN-TREATED RATS

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The relationship between the ovarian hormones and the uterus is well established by numerous studies (1). It has been shown that estrogen treatment of ovariectomized animals has a profound physiological effect on the myometrium. In the castrate rat, for example, the uterus is electrically and mechanically quiet (2–5). On the other hand, estrogen or diethylstilbestrol treatment will result in spontaneous and rhythmic electrical and mechanical activity.

The purpose of this report is to relate the observed morphological changes in fine structure to the known functional changes which occur in the smooth muscle fibers of the uterus of castrate rats following hormonal treatment. Since action potentials in uterine smooth muscle suitably primed with estrogen propagate from cell to cell, one can test in a unique way the structural relationships which may exist to facilitate this passage. Three possible structural means for impulse propagation have been suggested in the literature and are as follows: (1) via protoplasmic continuities (6–9); (2) ephaptic or electrical spread across the intercellular space of varying dimension (10–13); and (3) via specialized membranous junctions (8, 9, 14–18). Previous studies, however, have been concerned with smooth muscle whose function was not varied physiologically in a decisive way, and it has been difficult to evaluate the results except by indirect evidence. In an abstract (18), the present author reported that in the osmium tetroxide-fixed, estrogen-primed uterus, numerous areas of close uniform membrane apposition between adjacent smooth muscle fibers are found. These areas were interpreted as resembling the nexus or tight junction described in other studies in which permanganate fixation was employed.

MATERIALS AND METHODS

25 sexually mature, ovariectomized white rats were used in this study. 9 wk *after* ovariectomy, one (0.5 mg) diethylstilbestrol pellet was inserted subcutaneously in the dorsal hind quarter in each of 13 animals, and 50 μg of estradiol was injected subcutaneously in the dorsal hind quarter in each of seven animals. One group of five animals was castrated, but was untreated hormonally. These animals served as experimental controls.

One control, two diethylstilbestrol-treated rats, and one estradiol-treated rat were sacrificed daily for a period of 5 days.

In addition, the five remaining ovariectomized animals which were treated initially with either diethylstilbestrol or estradiol were kept for a period of 9 wk and then sacrificed for study. These animals served as additional experimental controls. Before fixation, the uterus was tested for contractility either by electrical stimulation or by topical application of oxytocin (50 μU/ml). The uterus of each animal was divided into equal halves for light and electron microscopy.

Light Microscopy

One-half of each uterus was fixed in acetic acid-formalin (1%:10%) in mammalian saline. After fixation, dehydration in ethyl alcohol, clearing in cedarwood oil, and embedding in paraffin, 5-μ sections were prepared. The sections were stained with toluidine blue and eosin and with Ehrlich's hematoxylin and eosin.

Electron Microscopy

The second half of the uterus was sliced into thin, transverse sections which were placed in 1% osmium tetroxide in Hanks' balanced salt solution for 1 hr, or in 1% potassium permanganate in Hanks' balanced salt solution for 1 hr. After rinsing and dehydrating, the tissue was embedded in Epon 812. Ultrathin sections were cut with an LKB ultrotome Microtome. The sections were mounted on formvar and carbon-coated copper grids, stained with lead hydroxide, and examined in an RCA EMU 3G electron microscope.

RESULTS

Gross Microscopic Structure of the Uterus from Ovariectomized Rat (Control)

Fig. 1 illustrates the cross-sectional structure of the uterus from an ovariectomized rat (9 wk). Two distinct layers of smooth muscle are evident in this section. The width of the outer layer varies in thickness from 1.5 to 2.0 mm in this section, which has been enlarged photographically 25

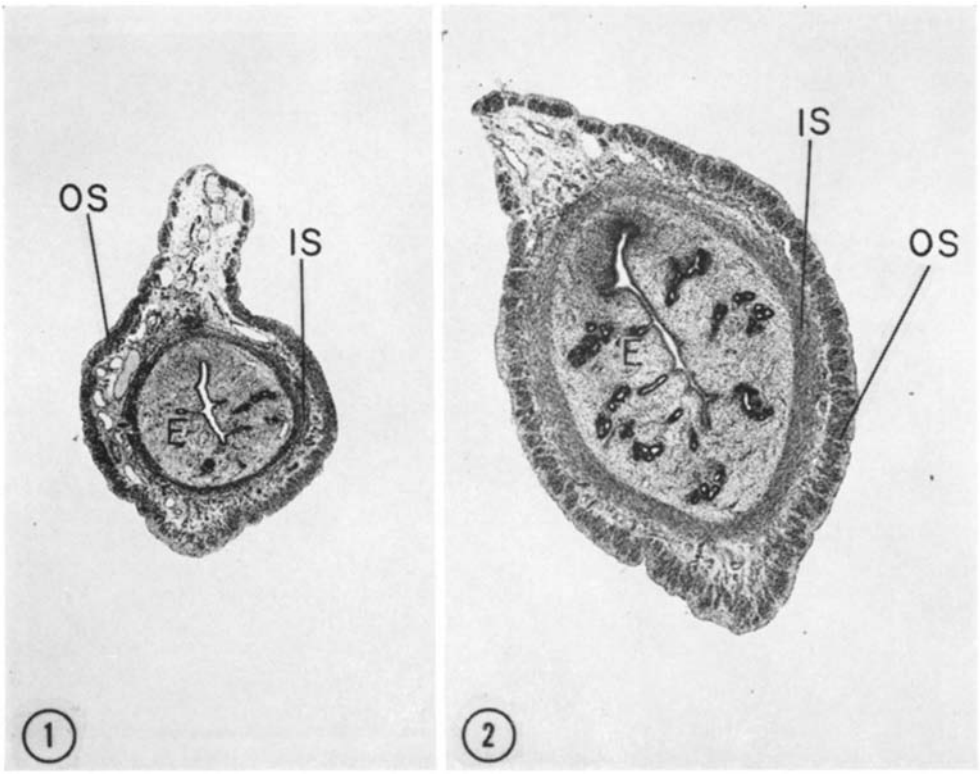

FIGURE 1 Cross-section of the uterus from an ovariectomized rat (9 wk). Control. The myometrium of this hormonally untreated uterus displays two distinct muscle layers. The outer smooth muscle layer (*OS*) varies in thickness from 1.5 to 2.0 mm in this photographically enlarged section. The inner layer (*IS*) is separated from the outer layer by enlarged blood vessels and connective tissue. The inner layer varies in thickness from 0.5 to 3.0 mm. This uterus did not contract when stimulated electrically or by topical application of oxytocin. Hematoxylin and eosin stain, 5-μ section. × 25.

FIGURE 2 Cross-section of the uterus from an ovariectomized, dietylstilbestrol-treated (5 days) rat. Experimental. The whole uterus has undergone a marked hypertrophy after 5 days of hormone treatment as outlined in Methods. This animal was a littermate of the animal from which the section in Fig. 1 was obtained. The animals were of comparable size and weight. The outer smooth muscle layer (*OS*) varies in thickness from 2.5 to 4.0 mm in this photographically enlarged section. The inner smooth muscle layer (*IS*) varies in thickness from 2.5 to 4.0 mm. The connective tissue space between the two muscle layers is markedly reduced. This uterus responded to electrical and chemical (oxytocin) stimulation. Hematoxylin and eosin stain. 5-μ section. × 25.

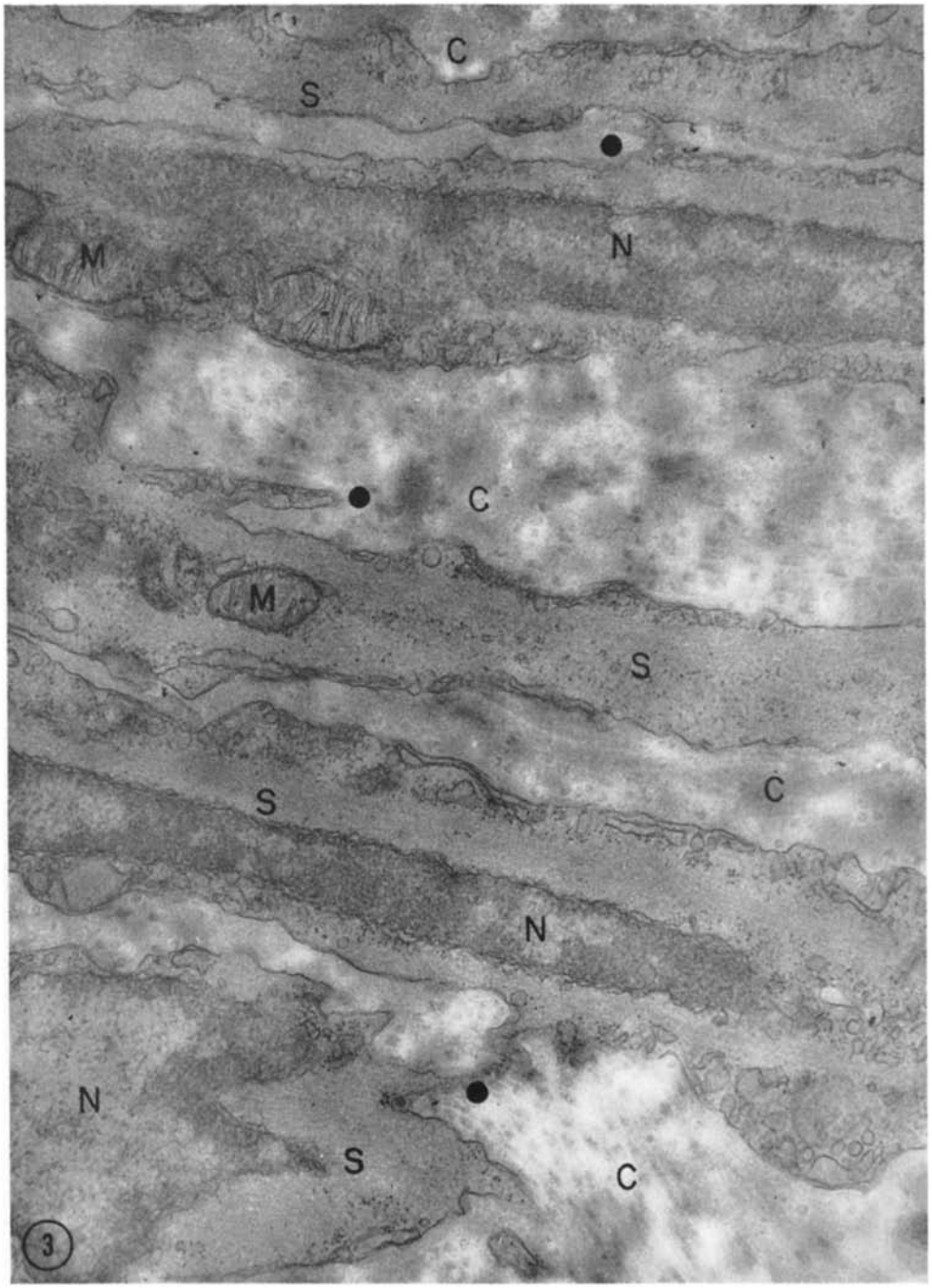

FIGURE 3 Uterus of control, untreated, ovariectomized rat (9 wk). The smooth muscle fibers are elongated and thin. In a few places, small projections (indicated by black dots) traverse the interfiber space which is irregular and filled with collagen. Regions of close cell contact are invariably small. This uterus did not contract when stimulated electrically or by the topical application of oxytocin. *C*, collagen; *M*, mitochondria; *N*, nucleus; *S*, sarcoplasm. OsO_4 fixation. × 22,000.

times. The inner layer ranges from 0.5 to 3.0 mm in width.

The uterus from this animal did not contract when stimulated electrically or by the topical application of oxytocin.

The section in Fig. 1 was taken from the middle of the right uterine horn.

Gross Microscopic Structure of the Uterus from Ovariectomized Rats (9 Wk) that Received Diethylstilbestrol Treatment (5 Days)

Fig. 2 illustrates the hypertrophy of the uterus following hormonal treatment. Two distinct layers of smooth muscle are also evident in this section. In this case, the width of the outer layer varies in thickness from 3 to 4 mm in this section, which has been enlarged photographically 25 times. The inner layer ranges from 2.5 to 4 mm in width.

This animal was a littermate of the control animal whose uterus is shown in Fig. 1. The two animals were of comparable size and weight.

The uterus from this treated animal contracted when stimulated electrically and by the topical application of oxytocin.

The section in Fig. 2 was taken from the middle of the right uterine horn.

Structure of Smooth Muscle Fibers in the Uterus of Ovariectomized Rats (9 Wk)

Light microscopic examination of the uterus stained by the classical toluidine blue and eosin method reveals the myometrium to be generally eosinophilic (acidophilic), suggesting that little

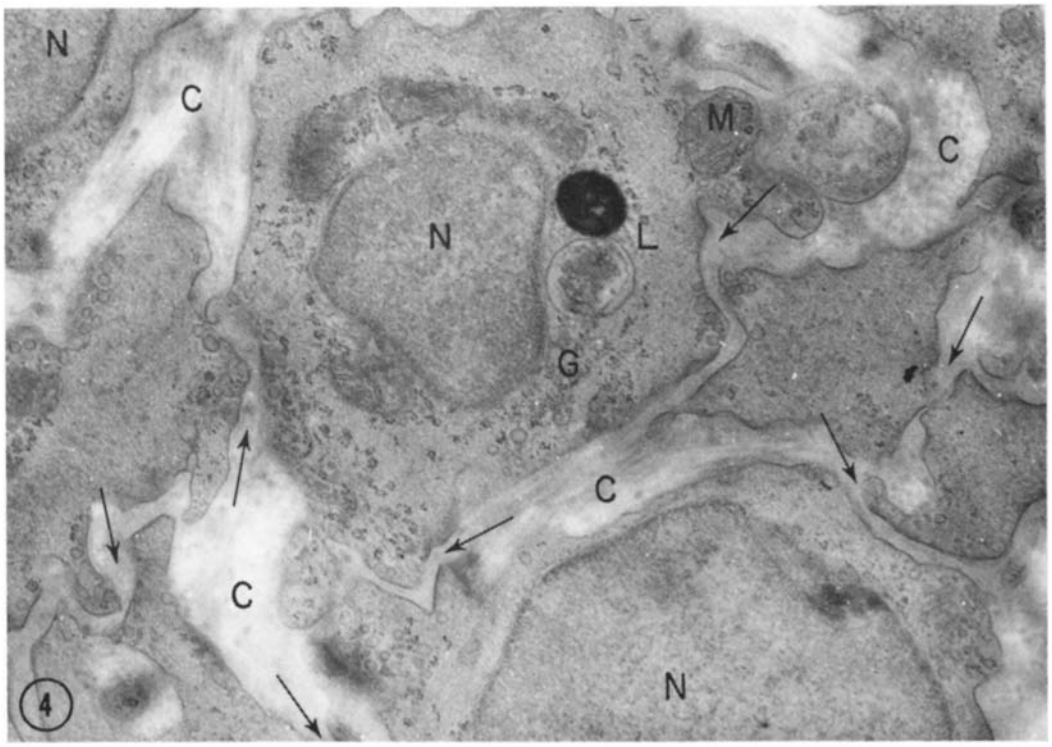

FIGURE 4 Uterus of control, untreated ovariectomized rat (9 wk). This cross-section illustrates the irregular shape of the smooth muscle fibers and demonstrates regions in which even a slight reduction in the interfiber space could result in interfiber junctions of the type shown in Fig. 6 (indicated by arrows). The interfiber space is variable and filled with collagen. Although the central fiber contains numerous granules, only a small number of these are assumed to be ribosomes on the basis of their staining reaction (light microscopy), configuration, and size. The granules appear to be primarily glycogen. This uterus did not contract when stimulated electrically or by the topical application of oxytocin. *C*, collagen; *M*, mitochondria; *N*, nucleus; *L*, lysosomes; *G*, granules. OsO_4 fixation. × 25,500.

cytoplasmic ribonucleoprotein is present in the smooth muscle fibers. This finding was confirmed by an electron microscopic study of the tissue. Although cytoplasmic granules are found in the muscle fiber, their size and distribution pattern suggest that they are primarily glycogen. Nevertheless, ribosomes are found which are characteristically 150 A in diameter. They are arranged in chains or clusters or are membrane-bound. The smooth muscle fibers appear to be, more or less, elongated and spindle shaped. The nuclei are found in the central portion of the attenuated fibers. The surface contours are not invariably smooth, but may possess thin projections which may approach adjacent muscle fibers. The space between adjacent fibers may be as little as 500–1,000 A. The intercellular space is thus highly irregular and is filled with collagen. The nuclei generally do not have well developed nucleoli. In the perinuclear space may be found mitochondria, lysosomes, ribosomes, and elements of poorly developed ribosome-studded sarcoplasmic reticulum and agranular sarcoplasmic reticulum. Glycogen granules are found scattered throughout the sarcoplasm and beneath the sarcolemma. Numerous pinocytotic vesicles are found associated with the sarcolemma. See Figs. 3 and 4.

Structure of Smooth Muscle Fibers in the Uterus of Estradiol- and Diethylstilbestrol-Treated Castrate Rats (5 Days)

In sharp contrast to the control uterus, the hormonally treated uterus of the ovariectomized

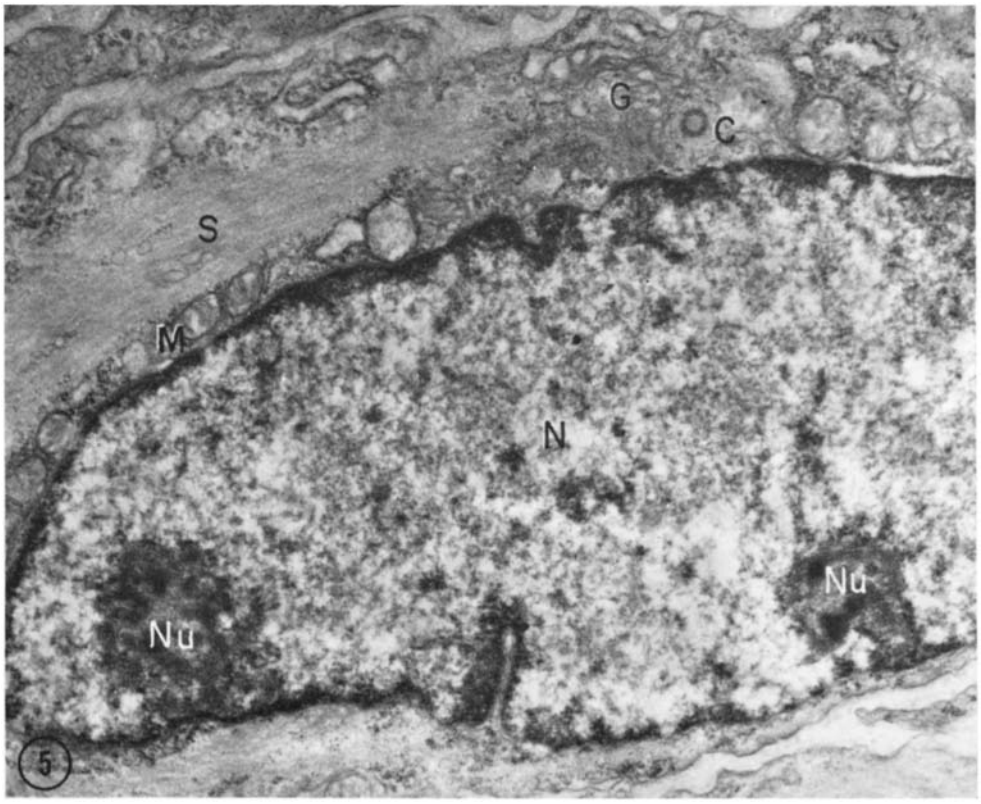

FIGURE 5 Uterus of experimental, diethylstilbestrol-treated (5 days) ovariectomized rat. The smooth muscle fibers in the myometrium reveal evidence of hypertrophy in both nucleus and sarcoplasm (Fig. 6). The most obvious change in the nucleus is the development of one or more nucleoli which tend to be closely associated with the nuclear membrane. In the sarcoplasm, centrioles are found, although divisional figures are not found. A Golgi apparatus is also found near the nucleus in these fibers. This uterus responded by contraction when stimulated electrically or by the topical application of oxytocin. *C*, centriole; *G*, Golgi apparatus; *M*, mitochondria; *N*, nucleus; *Nu*, nucleolus; *S*, sarcoplasm. OsO_4 fixation. × 27,000.

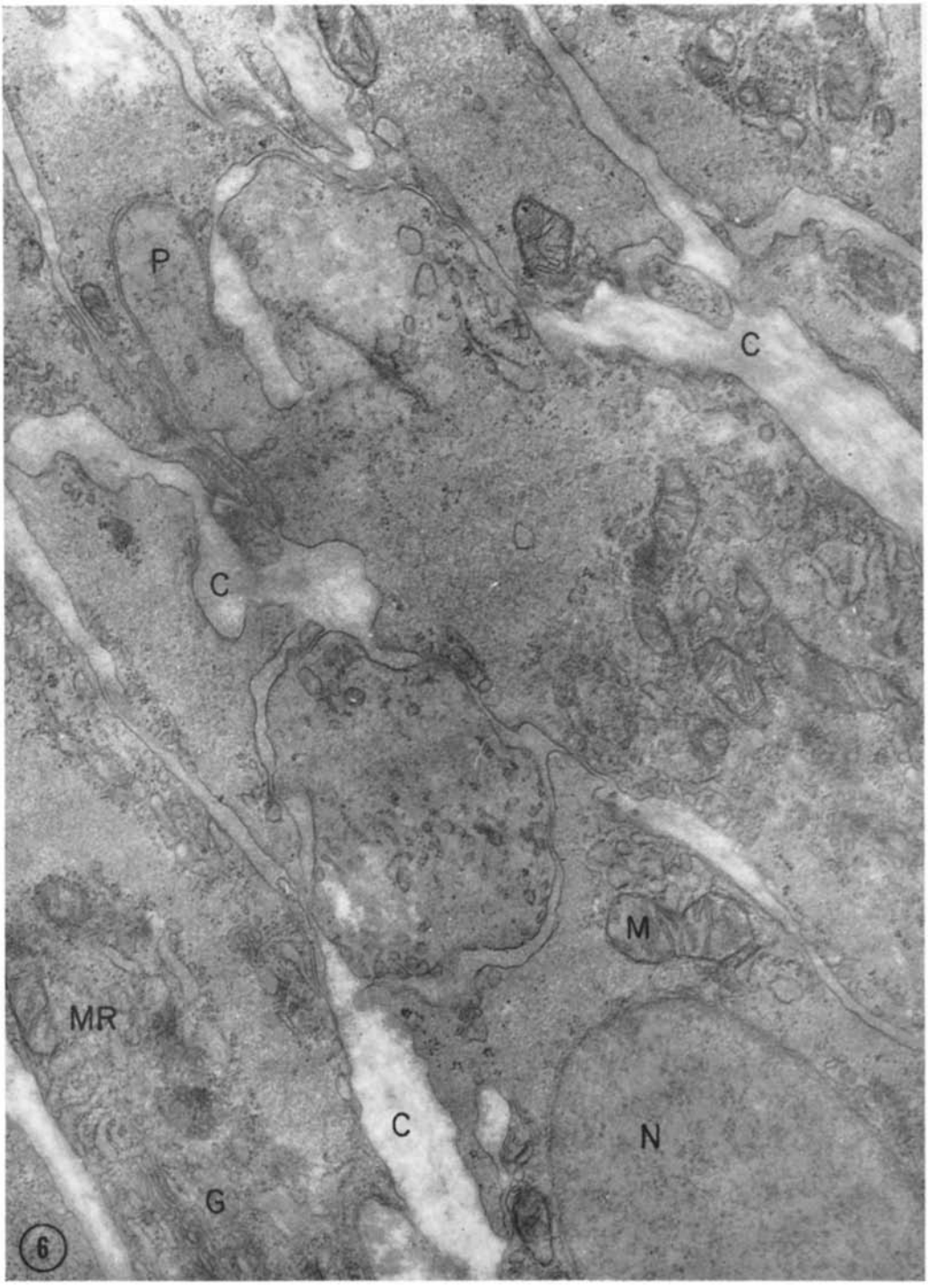

FIGURE 6 Uterus of experimental, diethylstilbestrol-treated (5 days) ovariectomized rat. Notice the elaborate development of sarcoplasmic organelles including the Golgi apparatus, ribosomes, and granular reticulum. The cells are hypertrophying, and the intercellular collagenous spaces are reduced as shown by a large cell process (P) at the upper left. The cell projections produce an extensive, close (cell-to-cell) membrane apposition. This uterus responded to electrical and chemical (oxytocin) stimulation. *C*, collagen; *G*, Golgi apparatus; *M*, mitochondrion; *MR*, ribosome-studded reticulum; *N*, nucleus; *P*, cell process; *S*, sarcoplasm. OsO_4 fixation. × 32,000.

rat is markedly basophilic, as revealed by light microscopy with the toluidine blue and eosin stain, suggesting the presence of abundant sarcoplasmic ribonucleoprotein. Electron microscopic examination confirms the hypertrophy of the smooth muscle fibers which now appear more angular in shape. The nuclei of these fibers contain highly developed nucleoli which tend to be eccentrically placed near the nuclear membrane. At the nuclear poles, a well developed ribosome-studded sarcoplasmic reticulum and free ribosomes are found. In addition, a system of agranular (Golgi) membranes is highly developed. Centrioles are frequently encountered in the hormone-treated muscle fibers, but no evidence of fiber divisions is observed (Figs. 5 and 6).

As the smooth muscle fibers enlarge, bulblike processes develop from them and extend into the interfiber space. These processes reduce the interfiber space, their membranes coming into close and uniform apposition with the membranes of adjacent fibers, and the processes are frequently

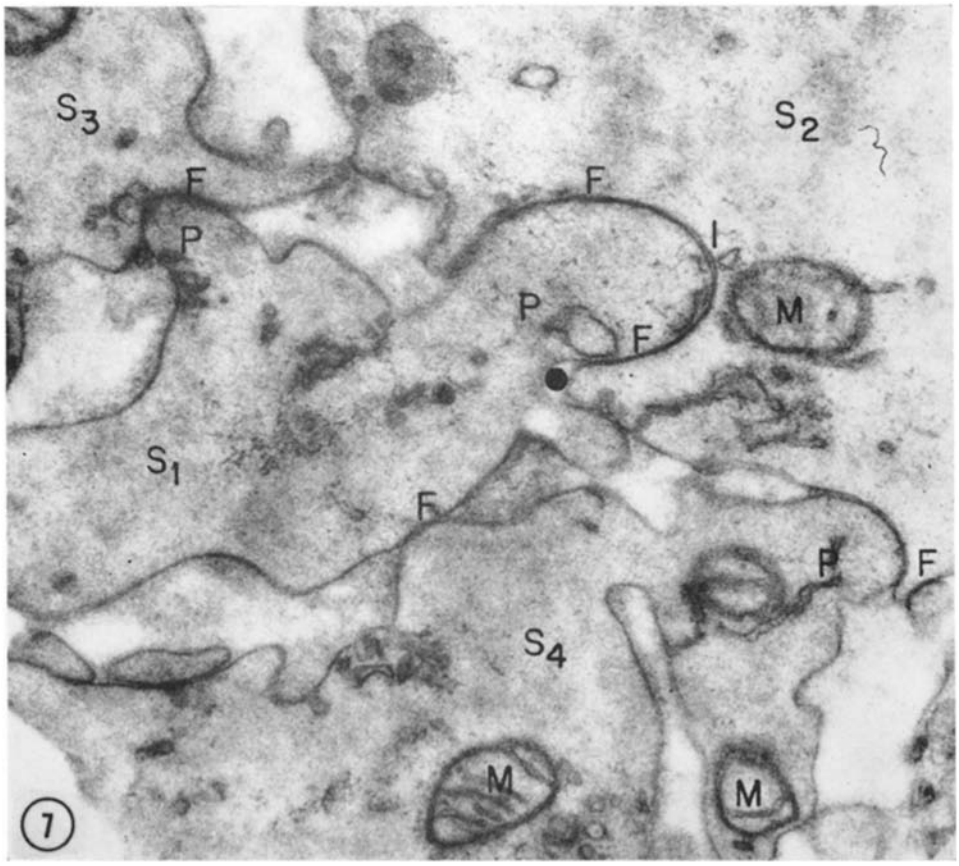

FIGURE 7 Uterus of experimental, diethylstilbestrol-treated (5 days) ovariectomized rat. Although permanganate fixation both obliterates much of the fine structure within the smooth muscle fiber and produces over-all changes in fiber shape, the relationship between adjacent fibers appears to be somewhat similar to that described in Fig. 6. Large fiber processes are found which may indent adjacent fiber surfaces (*P*). In other places, point contacts appear as between S_1 and S_4. The nature of the membrane contact is a membrane fusion (*F*). A membrane fusion is illustrated at higher magnification in Fig. 8 (a point marked by black dot is a reference point). Not all fusions are complete, and hence two distinct membranes (*I*) may be seen between S_1 and S_2. This uterus responded by contraction when stimulated electrically or by the topical application of oxytocin. *F*, membrane fusions; *I*, incomplete membrane fusion; *M*, mitochondria; *P*, bulbous processes; S_1–S_4, smooth muscle fibers. Permanganate fixation. × 42,500.

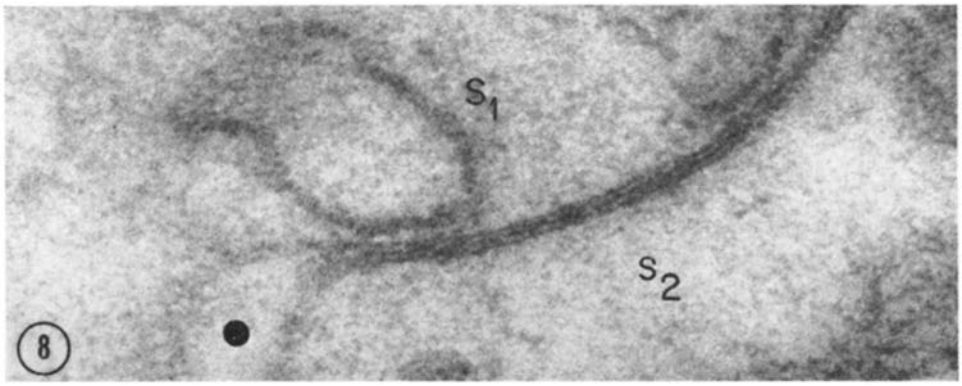

FIGURE 8 Uterus of experimental, diethylstilbestrol-treated (5 days), ovariectomized rat. High magnification detail of Fig. 7, illustrating the membrane fusion between smooth muscle fibers S_1 and S_2. The black dot is a reference mark for Figs. 7 and 8. × 190,000.

encircled by the adjacent fibers. In these special regions of intimate fiber apposition, the membranes of the two fibers are separated by a rather uniform gap of 200–250 A in osmium tetroxide–fixed tissue (Fig. 6).

With permanganate fixation, much of the fine structure within the smooth muscle fibers is obliterated. Nevertheless, the membranous components such as the sarcolemma, mitochondria, and endoplasmic reticulum remain intact. A muscle fiber (S_1) with a bulblike process comparable to those described above can be seen in Fig. 7. This process is in intimate contact with an adjacent fiber (S_2). The contact appears as a membrane fusion of the type that has been termed nexus or tight junction (Fig. 8).

A total of 200 cellular junctions was recorded in both osmium tetroxide– and permanganate-fixed estrogen-primed (physiologically active) uterine smooth muscle of castrate rats. Each smooth muscle fiber in the estrogen-primed uterus may be in membranous contact with as many as four adjacent fibers as seen in a single thin section. In addition, the interfiber junctions were found to occur randomly along the muscle fiber surface. In the unprimed (nonfunctional) uterine smooth muscle of the castrate rat (9-wk duration), there was no evidence of well defined cellular junctions like those found in the physiologically active tissue.

DISCUSSION

The results of the present study indicate that the formation of interfiber junctions which occurs as a result of the action of estrogenic hormones on the uterus may account for the transuterine conduction of electrical and mechanical activity. Further, the present results indicate that the type of interfiber junction found depends upon the method of fixation employed. Osmium tetroxide fixation yields close uniform membrane apposition while permanganate fixation results in the formation of membrane fusions (tight junction or nexus). It is not an easy matter, at this time, to determine which method yields the true nature of the membranous relationship or junction in this tissue. The solution of this problem will depend upon additional studies designed to clarify the striking difference due to the fixation methods employed here. In this regard, the technique employed by Trelstad, Revel, and Hay (19) in a study of tight junctions between cells in tissue of the early chick embryo is significant, since both close and tight junctions were reported in that tissue.

In earlier studies, Laguens and Lagrutta (8) and Silva (9) reported three types of junctions in the human uterus and in the rat uterus, respectively. These interfiber junctions include (1) close membrane apposition, (2) membrane fusion, and (3) protoplasmic continuity. Membrane fusion and protoplasmic continuity were rather infrequently found, whereas close membrane apposition occurred most frequently. Unfortunately, those authors provided little data relating to the frequency or location of the junctions, the precise physiological state of the tissue, or the specific effect of the fixatives (osmium tetroxide and per-

manganate) on the type or frequency of the junction found.

It appears clear from the present study that when the uterus is electrically and mechanically quiet, very few cellular junctions exist between the smooth muscle fibers. When the uterus hypertrophies under the influence of estrogen, however, numerous, large areas of uniformly close membrane apposition are found after osmium tetroxide fixation whereas nexuses or tight junctions are found after permanganate fixation. Similar junctions have been reported in other smooth muscle, in cardiac muscle, and at the electrical synapses of the nervous system (20–22). In view of the present observations, it is difficult to conceive of any structural relationship between smooth muscle fibers other than highly specialized areas of smooth muscle fiber membrane apposition or membrane fusion as being primarily involved in the electrical transmission or conduction from fiber to fiber which permits the synchronous mechanical activity of the uterus at parturition. Although protoplasmic continuities have been reported to occur rather infrequently (8, 9), their role in the conduction process cannot be disregarded.

The wave form of the propagated electrical activity in many smooth muscle bundles (e.g., taenia coli, intestinal circular, and uterine) exhibits considerable variability. The results of the present study suggest that uterine smooth muscle fibers are changing in size and shape and in their relationship to adjacent fibers. In addition, marked changes in the metabolic activity occur in the muscle fibers, as judged by the differences in the organelle profiles found in uterine smooth muscle fibers of castrate untreated and castrate estrogen-treated rats. The increasing numbers of cellular junctions and the size, shape, and location of these junctions of all types which form as a result of estrogen treatment may also contribute to the variability of the pattern (size, shape, and speed) of the propagated electrical activity in the uterus.

Bozler (23) has classified smooth muscles into two main groups: (1) those which are activated solely by motor nerves (e.g. vas deferens); and (2) those which may be rhythmically active because of their inherent myogenic automaticity (e.g., gastrointestinal and urinary tracts). It has been shown by Bozler (2), Caspo (3), Marshall (4), and Melton (5) that the smooth muscle of the uterus, *suitably primed by estrogen*, can be classified as a smooth muscle whose rhythmic activity is of myogenic origin. However, it may be useful to consider the uterus in a third, distinctive category, for several reasons. First, it is possible that uterine smooth muscle may actually be activated and driven by the autonomic nervous system (9, 24). Second, the uterus is mechanically and electrically inactive or shows uncoordinated, weak, or localized contractions during certain periods of the estrous cycle and during the gestation period of pregnancy. Third, uterine smooth muscle becomes highly reactive physiologically with rhythmic, well coordinated, strong, and generalized contractions, *only* experimentally during hormonal treatment or naturally during parturition when hormonal action on the tissue results in the formation of numerous interfiber junctions. When hormonal levels decline, the uterus involutes and becomes relatively quiescent, as stated above. The muscle of the uterus is thus functionally unique. It may be useful to add a third physiological category to Bozler's original classification of mammalian smooth muscles: (3) those in which conduction and mechanical activity are dependent upon hormonal interactions (e.g., uterus).

SUMMARY

The smooth muscle fibers in the uterus of estrogen-treated, castrate female rats undergo changes which can be related to changes in their physiological properties. In the uterus, which is treated for 2–5 days with either estradiol or diethylstilbestrol, marked changes occur in the nuclei and sarcoplasm of smooth muscle fibers as seen after osmium tetroxide fixation. The nulceoli become highly developed and tend to be eccentrically located near the nuclear membrane. Centrioles can be found near the nuclei, although no divisional figures are found. Golgi apparatus and ribosome-studded sarcoplasmic reticulum are prominent features of the hypertrophying muscle fibers.

The interfiber space is reduced by the development of bulblike processes from muscle fibers which come into close association with adjacent muscle fibers. With osmium tetroxide fixation these appear as intimate and uniform membrane apposition. With permanganate fixation, the areas of membrane apposition described above appear as membrane fusions.

In the absence of estrogen, the uterus is electri-

cally and mechanically quiet and there is no evidence of well defined cellular junctions like those found in the physiologically active tissue.

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