Histochemical Study of Estrogenic and Antiestrogenic Effects of Clomiphene Citrate on Glycogen Synthesis by Myometrium and Luminal Epithelium of Rat Uterus [1]

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ABSTRACT Glycogen localization in the myometrium and luminal epithelium of the rat uterus was studied by the periodic acid-leucofuchsin technique after ovariectomized rats had been treated with various regimens of 1.0 μg estradiol dipropionate and 50.0 μg clomiphene citrate. Three regimens were used: (a) one or three dosages of clomiphene, alone or in combination with one or three dosages of estradiol; (b) a single dosage of clomiphene before a single dosage of estradiol; (c) a single dosage of clomiphene after a single dosage of estradiol.

The estrogenic effect of clomiphene on the myometrium was less than that of estradiol. Clomiphene suppressed myometrial glycogen accumulation induced by estradiol when administered with estradiol, six hours before the hormone, or as long as 24 hours after estradiol. The luminal epithelium responded differently to estradiol and clomiphene: the number of luminal epithelial cells containing glycogen strikingly increased after clomiphene treatment but not after estradiol treatment. A few scattered cells contained glycogen 24 hours after one dosage of the drug. Forty-eight hours after a single dosage or after three dosages of the drug administered one per day, every luminal epithelial cell contained glycogen. The effect of clomiphene on the luminal epithelium may be either a unique action of the drug or an abnormal response of the tissue, similar to that reported for high doses of estradiol. This effect of clomiphene on the luminal epithelium may possibly be a factor in the drug's ability to block blastocyst implantation in the rat.

Among the many uterotrophic effects of estradiol is its ability to increase uterine glycogen synthesis. In the rat this has been clearly demonstrated biochemically (Boettinger, '46; Walaas, '52; Bitman et al., '65; Gregoire et al., '67) and histochemically (Bo and Atkinson, '52; Rosenbaum and Goolsby, '57). During the estrous cycle and after treatment of ovariectomized rats with small doses of estradiol, the glycogen is located only in the myometrium (Bo and Atkinson, '52), but large doses of estradiol also induce glycogen accumulation in the luminal epithelium (Bo and Atkinson, '53; Rosenbaum and Goolsby, '57). Many substances inhibit estradiol-induced uterine glycogen synthesis: progesterone (Cecil and Bitman, '68), cortisol (Bitman and Cecil, '67), cycloheximide (Bitman et al., '66; Cecil and Bitman, '67), actinomycin (Valadares et al., '68), and epinephrine (Kostyo and Leonard, '55).

Clomiphene citrate (2- p- (2-chloro-1, 2-diphenylvinyl) phenoxy-triethylamine dihydrogen citrate), a nonsteroidal drug used clinically to induce ovulation, has a weak estrogenic effect and a partial antiestrogenic effect on the wet weight increase of the rat uterus (Holtkamp et al., '60; Roy et al., '64b). Similar results were found with the mouse (Clitheroe, '66) and gerbil (Kaul and Ramaswami, '68).

We wished to more precisely determine clomiphene's estrogenic and antiestrogenic

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effects on a specific biochemical constituent that responds to estradiol. Therefore, we undertook a histochemical study of uterine glycogen synthesis to see whether there was any difference in the localization of the glycogen after clomiphene and estradiol treatment. While our investigation was in progress, clomiphene was reported to increase total uterine glycogen and to partially inhibit estradiol-induced glycogen synthesis (Wood et al., '68; Mohla and Prasad, '68). Later, Mohla and Prasad ('69b) showed that clomiphene also inhibited the glycogen increase stimulated by histamine-releaser compound 48/80. Our results confirm these previous biochemical results of Wood et al. ('68) and Mohla and Prasad ('68) and extend them by showing that clomiphene affects the myometrium and luminal epithelium differently than estradiol and that the drug decreases the estradiol-induced glycogen accumulation of the myometrium even when administered after estradiol.

MATERIALS AND METHODS

Virgin female young adult rats of the Holtzman strain were provided with water and Purina lab chow *ad libitum*, housed four to a cage in a constant temperature room (70°F), and exposed to 14 hours of light per day. They were bilaterally ovariectomized under metophane anesthesia and nine to ten days later were divided into groups of four.

Three experiments were designed to study the effects of one or three dosages of clomiphene administered alone or in combination with one or three dosages of estradiol, the effect of a single dosage of clomiphene given before a single dosage of estradiol, and the effect of a single dosage of clomiphene given after a single dosage of estradiol. The experimental designs are given in tables 1 to 3. The doses administered were 50.0 μg clomiphene citrate [2]/0.1 ml 5% acacia by gavage, and 1.0 μg of estradiol dipropionate [3]/0.1 ml of cottonseed oil, sc. The vehicles were tested individually and neither had a glycogenic effect. The first dosage in all treatment schedules was given between 8:00 and 9:00 AM, and all animals receiving a combined treatment were killed 24 hours after final treatment with drug or hormone.

At autopsy the uteri were quickly removed, trimmed of excess fat and mesentery, cut into 8–12 small pieces, and fixed 36–48 hours in an ice cold mixture of nine parts of 80% alcohol and one part of 10% formalin, saturated with picric acid. The tissue was embedded in paraffin and sectioned transversely at 7 μ. Three adjacent sections from two different areas of the block were mounted individually on glass slides. One section was stained with hematoxylin and eosin, the second was treated by the periodic acid-Schiff (PAS) technique (McManus, '48), and the third was treated with 1% malt diastase for 30 minutes at room temperature (70–72°F) before the PAS procedure. Liver sections were included in each staining tray as a technique control. Basic fuchsin (Fisher Scientific Company, C.I. 42500) was used to prepare the leucofuchsin.

The slides were coded so the sections could be graded without knowledge of the treatment. Myometrium and luminal epithelium were graded separately. The glycogen grade for the myometrium reflects our impression of the response of the treated animals relative to the ovariectomized controls. The grade is on a 0–5+ scale based on increased glycogen accumulation in the outer longitudinal layer of the myometrium, for this layer responds better to a glycogenic stimulus than does the inner circular layer. The luminal epithelium on the other hand was graded on a 0–4+ scale, which represents increasing numbers of epithelial cells containing glycogen relative to the number of cells in the section. The response varied from a few cells containing glycogen (1+) to every cell having it (4+). Because of the different criteria used to evaluate the two areas, comparisons between the myometrium and epithelial grades should not be made. Representative sections of most of the grades are shown in plates 1 and 2.

RESULTS

The uterine horns of the clomiphene-treated animals were larger, more hyperemic, and appeared to have more luminal

[2] The clomiphene citrate and estradiol dipropionate were supplied through the courtesy of The W. S. Merrell Company, Cincinnati, Ohio and Ciba Pharmaceutical Company, Summit, New Jersey, respectively.

[3] See footnote 2.

fluid than those of the ovariectomized controls. However, the uteri of animals treated with estradiol could not be distinguished grossly from those treated with clomiphene. The drug and hormone also caused hypertrophy of the luminal and glandular epithelium.

When present, endometrial glycogen was located only in the luminal epithelium and not in the glandular epithelium. In these cells the glycogen was located apically and basally. Surrounding the endometrial glands were a few cells containing glycogen which resembled the "glycogen-bearing cells" described by Rosenbaum and Goolsby ('57), but their distribution and number did not appear to be related to treatment. These cells are probably neutrophilic leucocytes. Glycogen granules were located primarily in the outer longitudinal layer of the myometrium and the number of granules varied with the treatment. However, the glycogen response of the inner circular layer to the various treatments was slight and did not appear to differ between groups.

The results of the first experiment (table 1) showed that a single dosage of clomiphene had an estrogenic and an antiestrogenic effect on myometrial glycogenesis. However, clomiphene's estrogenic effect in increasing the amount of glycogen was less than that of estradiol (compare figs. 7, 8, 10). Clomiphene caused glycogen accumulation in the luminal epithelium (fig. 3) whereas estradiol induced little or none (fig. 2). The drug decreased the myometrial glycogen increase induced by estradiol (fig. 9) when the two were administered concurrently.

After three dosages of clomiphene, estradiol, or the combination (groups 5, 6, 7) myometrial glycogen accumulation was unchanged from that seen after one dosage of each. However, three dosages of clomiphene did cause a striking increase in the number of luminal epithelial cells having glycogen (fig. 5), for every cell contained the polysaccharide. The same response of the epithelium was observed in rats treated with three dosages of the combination (fig. 6).

The results of the second experiment (table 2) show that clomiphene decreases the myometrial glycogen accumulation induced by estradiol when given before the hormone. A six-hour clomiphene pretreatment (group 13) decreased the glycogen to about the same amounts as the clomiphene control (group 10), but no decrease could be detected after the 24-hour clomiphene pretreatment (group 15). As seen in figures 1, 3, 4, and 5 a single dosage of clomiphene induced a time-dependent increase in the number of luminal epithelial cells having glycogen (groups 2, 10, 11, 12). The maximum response of the luminal epithelium, seen 48 hours after a single dosage, was similar to the response observed after three dosages of the drug (group 5). A similar time-dependent increase was observed in the epithelium of

TABLE 1

Glycogen response of the myometrium and luminal epithelium of ovariectomized rats treated with one or three 50.0 μg dosages of clomiphene citrate (C), either alone or in combination with one or three 1.0 μg dosages of estradiol dipropionate (E). The dosages were given once per day for three days

Group [1]	Treatment	Autopsy time [2]	Glycogen grade [3]	
			Myometrium	Luminal epithelium
1	None	24	+	−
One dosage				
2	C	24	+++	+
3	E	24	+++++	−
4	C+E	24	++++	+
Three dosages				
5	C	72	+++	++++
6	E	72	+++++	−
7	C+E	72	++++	++++

[1] Four rats/group.
[2] Hours after the first treatment.
[3] Glycogen grades explained under Materials and Methods.

TABLE 2

Effect of a single 50.0 μg dosage of clomiphene citrate (C) given 6, 12, or 24 hours before a single 1.0 μg dosage of estradiol dipropionate (E) on the glycogen response of the myometrium and luminal epithelium of the uterus in ovariectomized rats

Group [1]	Treatment	Autopsy time [2]	Glycogen grade [3]	
			Myometrium	Luminal epithelium
Controls				
8	None	24	+	—
9	E	24	+++++	—
10	C	30	+++	++
11	C	36	+++	+++
12	C	48	+++	++++
Experimentals				
13	C 6 hours before E	30	+++	++
14	C 12 hours before E	36	++++	+++
15	C 24 hours before E	48	+++++	++++

[1] Four rats/group.
[2] Hours after the first treatment.
[3] Glycogen grades explained under Materials and Methods.

TABLE 3

Effect of a single 50.0 μg dosage ofclomiphene citrate (C) given 6, 12, or 24 hours after a single 1.0 μg dosage of estradiol dipropionate (E) on the glycogen response of the myometrium and luminal epithelium of the uterus in ovariectomized rats

Group [1]	Treatment	Autopsy time [2]	Glycogen grade [3]	
			Myometrium	Luminal epithelium
Controls				
16	None	24	+	—
17	C	24	+++	+
18	E	30	++++++	—
19	E	36	++++++	—
20	E	48	++++++	—
Experimentals				
21	E 6 hours before C	30	+++	+
22	E 12 hours before C	36	+++	++
23	E 24 hours before C	48	+++	+++

[1] Four rats/group.
[2] Hours after the first treatment.
[3] Glycogen grades explained under Materials and Methods.

the animals receiving estradiol after clomiphene (groups 13, 14, 15).

The third experiment (table 3) showed that a single dosage of clomiphene given 6, 12, or 24 hours after a single dosage of estradiol decreased myometrial glycogen accumulation induced by estradiol (groups 21–23). The amount of myometrial glycogen in the experimental groups was about the same as that of the clomiphene control. Glycogen was seen in the luminal epithelium only in the clomiphene-treated animals. However, in this experiment estradiol appeared to have a glycogenic effect, for the epithelial response increased with the 12 and 24 hours estradiol pretreatment.

DISCUSSION

Our results support the biochemical observations (Wood et al., '68; Mohla and Prasad, '68) that clomiphene has estrogenic and antiestrogenic effects on uterine glycogen synthesis. However, we found that the localization of the glycogen and the glycogen response of the myometrium and luminal epithelium differed after clomiphene and estradiol treatment. Therefore, our results indicate that: 1. clomiphene has a weak estrogenic effect on myometrial glycogenesis, 2. the drug has a strong estrogenic effect on glycogen accumulation in the luminal epithelium, and 3. clomiphene's antiestrogenic effect when

given before, with, or after estradiol is restricted to the myometrium. Because the doses, specific timing, and experimental conditions used in the previous biochemical studies and in our histochemical study differed, definite comparisons are not entirely valid. However, some interesting speculations can be made from the results of the two methods of glycogen analysis.

Our results confirm those of Wood et al. ('68) that uterine glycogen increases with increasing dosages of clomiphene, but we found the increase was limited to the luminal epithelium and that the myometrial response did not increase after one and three dosages of the drug. Using a biochemical technique, Wood et al. ('68) found no inhibition of estradiol-induced glycogenesis by multiple dosages of clomiphene, and concluded that the inhibition was masked by the inherent estrogenicity of clomiphene. However, using the histochemical method, we did find a suppression of estradiol-induced myometrial glycogen by multiple dosages of clomiphene, but it was associated with a large increase in epithelial glycogen (group 7). Because only total uterine glycogen was measured by Wood et al. ('68), it is possible that the epithelial response was large enough to mask the inhibition of myometrial glycogen.

Wood et al. ('68) and Mohla and Prasad ('68) noted that total uterine glycogen was inhibited when clomiphene was given with or before estradiol. In our experiments giving clomiphene with or before estradiol, we found the inhibition to be only in the myometrium, for the drug always increased epithelial glycogen. In addition we found that clomiphene decreased the myometrial glycogen accumulation induced by estradiol when the drug was administered after the hormone. Clomiphene's mechanism of action has been postulated to be one of competitive inhibition at the site of the estradiol receptor (Roy et al., '64b,c; Eisenfeld and Axelrod, '67; Kato et al., '68; Dipietro et al., '69). There are also reports that clomiphene acts as an antihistamine under certain conditions (Schlough and Meyer, '69; Mohla and Prasad, '69b). Since estradiol had induced the maximum myometrial glycogen response before clomiphene was given (group 23), competitive inhibition is not necessarily the mechanism of action in this experiment. Under these conditions clomiphene may be stimulating glycogenolysis and in this way decreasing the estradiol-induced glycogen response in the same way that epinephrine does (Kostyo and Leonard, '55).

The large amount of glycogen in the luminal epithelium after clomiphene treatment was surprising. Glycogen is not present in these cells at any stage of the estrous cycle nor after treatment of ovariectomized rats with small doses of estradiol (1.0 μg/rat) (Bo and Atkinson, '52). However, large doses of estradiol (10.0–20.0 μg/rat) induce glycogen accumulation in the luminal epithelial cells (Bo and Atkinson, '53; Rosenbaum and Goolsby, '57; Bo, '59). The dose of estradiol we used (1.0 μg) induced little or no glycogen synthesis in the luminal epithelium, but Bitman et al. ('65) found that this dose does stimulate maximum uterine glycogen synthesis in the rat.

In the first experiment the increased epithelial response to clomiphene depended on the number of dosages of the drug, and in the second it depended on the time after a single dosage. Since the timing of the two experiments overlapped, it is not clear whether the epithelial response depends chiefly on time or dosage. The dose of clomiphene given was 50 fold that of estradiol. However, the effect of clomiphene on glycogen synthesis in the myometrium and luminal epithelium did not reflect this difference in dose levels, for the drug was less effective than a 1.0 μg dose of estradiol in stimulating myometrial glycogenesis but far more effective on the luminal epithelium. The reason for this apparent difference in sensitivities of the two tissues to the drug and hormone is not known.

Clomiphene blocks blastocyst implantation in the rat, and its postulated mechanisms of action in this regard have been reviewed by Lopez-Escobar and Fridhandler ('69) and Schlough and Meyer ('69). Since glycogen is not seen in the luminal epithelium during the estrous cycle or after treatment of ovariectomized rats with small doses of estradiol, the glycogenic effect of clomiphene on the luminal epithlium may be a nonphysiologic response of these cells to the drug. Because this one component of the epithelial cells is altered to such a great degree, clomiphene may

also induce other biochemical changes in these cells. Therefore, it is possible that this strong estrogenic effect of clomiphene on the luminal epithelium may be a contributing factor in the drug's ability to block implantation in the rat, for the alteration of the normal function of these cells may make them resistant or unreceptive to blastocyst attachment and invasion. Enders and Schlafke ('69) have investigated and reviewed the importance of the luminal epithelium during the early stages of implantation in the rat and other species.

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PLATE 1

EXPLANATION OF FIGURES

Photomicrographs of sections of the luminal epithelium and surrounding endometrium from ovariectomized rats treated as indicated. All sections were treated by the periodic acid-Schiff technique.

1 Section treated with malt diastase before the PAS procedure. No glycogen granules are present in the luminal epithelium. Grade 0. ×400.

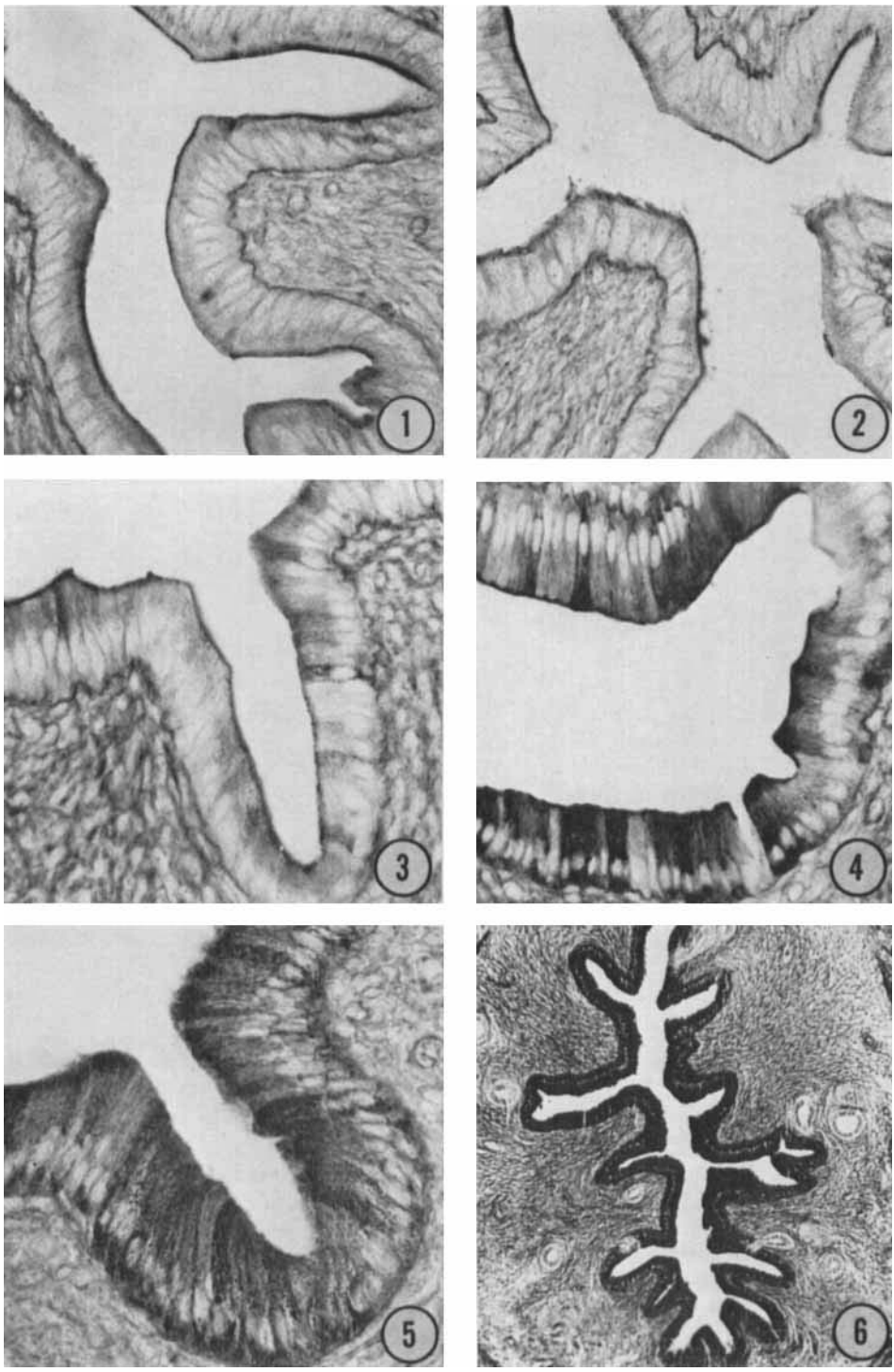
2 No glycogen is present in the epithelial cells. Grade 0. The rat was killed 24 hours after one dosage of estradiol. ×400.

3 Only a few scattered epithelial cells contain glycogen. Grade 1. The animal was killed 24 hours after one dosage of clomiphene. ×400.

4 Nearly every epithelial cell contains glycogen granules located both apically and basally. Grade 3. The rat was killed 36 hours after one dosage of clomiphene. ×400.

5 Every epithelial cell contains large amounts of glycogen and there is some hypertrophy of the cells. Grade 4. The rat was killed 24 hours after three dosages of clomiphene on three successive days. ×400.

6 Every luminal epithelial cell contains glycogen but there is none in the glandular epithelium. Grade 4. The rat was killed 24 hours after three dosages of clomiphene and estradiol. ×45.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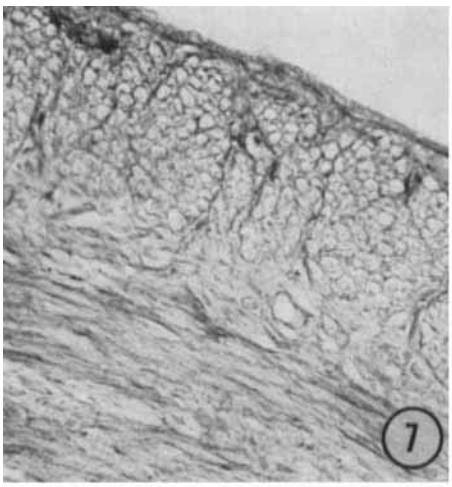

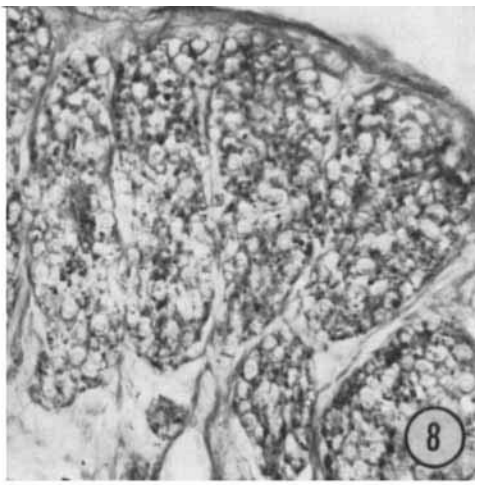

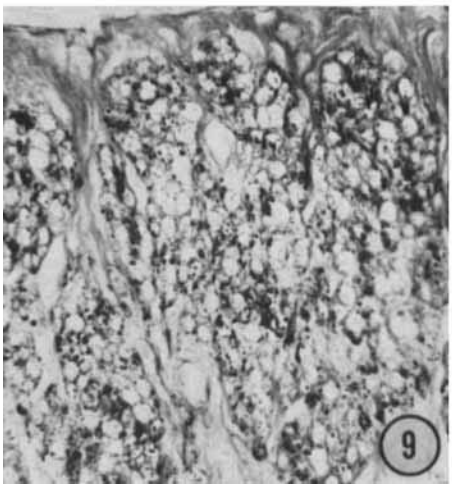

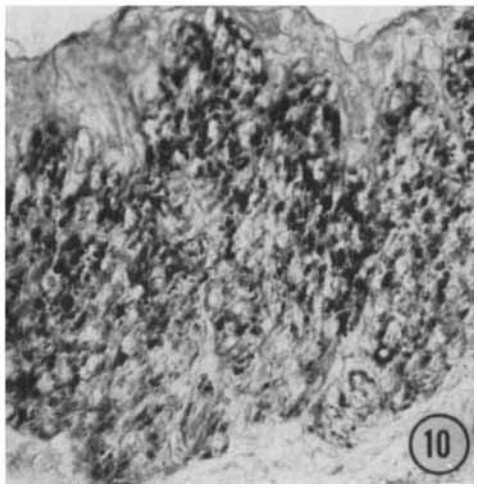

EXPLANATION OF FIGURES

Photomicrographs of the myometrium of rat uteri from ovariectomized rats treated as indicated. All sections were treated by the periodic acid-Schiff technique.

7 No glycogen is present in either layer of the myometrium. Grade 0. The section was treated with malt diastase before the PAS procedure. ×400.

8 A few small glycogen granules are scattered throughout the outer muscle layer. Grade 3. The rat was killed 24 hours after one dosage of clomiphene. ×400.

9 Many small glycogen granules and a few larger ones are evident. Grade 4. The rat was killed 24 hours after one dosage of clomiphene and estradiol administered concurrently. ×400.

10 Many small glycogen granules are seen scattered between numerous large granules. Grade 5. The rat was killed 48 hours after a single dosage of estradiol. ×400.