Effects of antiestrogens on eating, female sexual behavior, and the uptake of 3H-estradiol in the central nervous system in rats.

Edward J. Roy

University of Massachusetts Amherst

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EFFECTS OF ANTIESTROGENS ON EATING, FEMALE SEXUAL BEHAVIOR, AND THE UPTAKE OF $^3$H-ESTRADIOL IN THE CENTRAL NERVOUS SYSTEM IN RATS

A Dissertation Presented

by

EDWARD J. ROY

Submitted to the Graduate School of the University of Massachusetts in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

February 1977

Psychology
EFFECTS OF ANTIESTROGENS ON EATING, FEMALE SEXUAL BEHAVIOR, AND THE UPTAKE OF H-ESTRADIOL IN THE CENTRAL NERVOUS SYSTEM IN RATS

A Dissertation

by

Edward J. Roy

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Dr. Mark S. Fischer, Member

Dr. Bonnie R. Strickland, Psychology Department Chairperson
Acknowledgments

The energy and enthusiasm that went into my work on these projects were derived largely from the enjoyment of sharing the successes and frustrations with my friends in the lab. My appreciation of George has been continuous from beginning to end, and I have come to feel that he is a friend without losing a speck of admiration for him. And thanks be to Ruth for the special dinners, and the cookies that often sustained me through the lab camp-outs. And to Tom, the best colleague and friend. And Jeff, for supplying me with something to tell George when my own research wasn't going well, and good hearty laugh when it was. And Christie, gerbil lady with a touch of class. And Babs in the early era, for pizzas without anchovies. And Marie, for keeping me human. And Cindy, Betsy, and John, who each in his/her own way was a great technician. I would also like to thank the members of my committee for their time and cooperation, Mark Friedman, Mark Fischer, and Skip Fournier.

This research was supported in part by Research Grant NS-10873 and Research Career Development Award NS-00090 from the National Institute of Neurological and Communicative Disorders and Stroke to George N. Wade, and by Training Grant MH 11823 from the National Institute of Mental Health. MER-25 or ethamoxypriphetol, 1-(p-2-diethylaminoethoxyphenyl)-1-phenyl-2-methoxyphenylethanol, was provided by Merrell National Laboratories; CI-628, \( \mathcal{L} \)-(4-pyrrolidinoethoxy)
phenyl-4-methoxy-α'-nitrostilbene monocitrate, was provided by Parke-Davis; and nafoxidine or U-11, 100 A, 1-(2-(p-(3,4-dihydro-6-methoxy-2-phenylnapth-1-yl)-phenoxy)-ethyl) pyrrolidine, was provided by the Upjohn Company.

1 Estradiol benzoate was generously provided by the Schering Corp. Progesterone was purchased from Steraloids, Inc.
ABSTRACT

MER-25, which acts as an antiestrogen on most estrogen-sensitive behaviors and in peripheral tissues, acts only as an estrogen with respect to eating and body weight. MER-25 fails to antagonize the effects of EB on eating and body weight, while simultaneously antagonizing EB's effects on female sexual behavior, uterine growth, and vaginal cytology. Estradiol benzoate (EB) and MER-25 similarly affect eating and body weight in gonadectomized rats and gerbils. In rats, both compounds cause a transient decrease in food intake and a permanent decrease in body weight relative to controls; the eating and body weight effects of both MER-25 and EB are attenuated by progesterone; both MER-25 and EB affect females more than males; MER-25 decreased food intake when implanted in the same hypothalamic areas in which EB decreases food intake; and both EB and MER-25 reduce body weight by means independent of food intake reductions. In gerbils, both EB and MER-25 increase food intake and body weight. The decrease in eating and weight gain caused by MER-25 are not due to toxicity, as indicated by its failure to induce a learned aversion to saccharin and its failure to alter running wheel activity. These results indicate that the systems mediating the effects of estrogens on eating and body weight differ biochemically from other behavioral and somatic estrogen-sensitive systems.

In studies directed at the inhibitory behavioral actions
of antiestrogens, the hypothesis that antiestrogens compete with estradiol for binding sites in the brain was tested. Effects of antiestrogens on estradiol binding were compared with their effects on female sexual behavior. The non-steroidal antiestrogens, MER-25, CI628, and nafoxidine reduce the uptake of $^3$H-estradiol in whole homogenates and isolated cell nuclei of brain tissues and in similar paradigms block the induction of female sexual behavior. The antiestrogens were injected intraperitoneally 2 hours prior to an intravenous injection of $^3$H-estradiol, and the animals were killed 2 hours after the estradiol injection. CI-628 reduces radioactivity in whole homogenates and purified cell nuclei of cerebral cortex, hypothalamus, preoptic area-septum, as well as the pituitary. Nafoxidime reduces uptake in cell nuclei of the hypothalamus, preoptic area-septum, and pituitary. In this paradigm, MER-25 inhibits uptake in the pituitary, but not in the brain. In the analogous behavioral experiments, with antiestrogens injected 2 hours prior to an intravenous injection of unesterified estradiol, CI-628 and nafoxidine totally inhibit lordosis responding. MER-25 shows no inhibition of behavior in this paradigm. However, when MER-25 is injected 12 hours prior to the estradiol, it inhibits retention of $^3$H-estradiol at 2 hours in brain and pituitary cell nuclei, and lordosis responding is also inhibited. Additionally, the antiestrogens can apparently displace previously bound $^3$H-estradiol. When the antiestrogens are injected 2 hours prior to an injection of $^3$H-estradiol,
MER-25, CI-628, and nafoxidine all show greater inhibition of nuclear estradiol retention at 12 hours after the $^3$H-estradiol injection than at 2 hours. Analogously, when CI-628 is injected 2 hours after an intravenous injection of $^3$H-estradiol, it displaces most of the radioactivity present in hypothalamic-preoptic area nuclei at 12 hours after the estradiol injection. These results indicate that anti-estrogens can prevent or reverse the nuclear concentration of estradiol in brain cells and are consistent with a role of the cell nucleus in the induction of estrous behavior by estradiol.
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INTRODUCTION

Estradiol affects both eating and female sexual behavior in rats by actions in the central nervous system. Small implants of estradiol in the hypothalamus cause a decrease in food intake and induce sexual receptivity (Beatty et al., 1974; Dorner et al., 1968; Jankowiak and Stern, 1974; Lisk, 1962; Wade and Zucker, 1970), while implants in other regions of the brain do not. Lesions of parts of the hypothalamus reduce the sensitivity of rats to the effects of systemic estradiol injections on food intake (Beatty et al., 1975). Likewise, lesions of the diencephalon either enhance or diminish the estrogen-responsiveness of the lordosis reflex, depending on the location of the damage (Law and Meagher, 1958; Powers and Valenstein, 1972; Singer, 1968).

Anti-estrogens may be useful in analyzing the behavioral actions of estradiol. These synthetic compounds inhibit the actions of estrogens on a variety of measures. The anti-estrogen MER-25 has been studied extensively in a variety of species and has been found to antagonize the effects of estrogens on the uterus, vagina, mammary gland, euiduct, and brain (e.g., Giannina et al., 1974; Komisaruk and Beyer, 1972; Lerner, 1964; Lerner et al., 1958; McDonald, 1973; Meyerson and Lindstrom, 1968; Sodersten, 1974; Stone and Emmens, 1964; Roy and Wade, 1975). For example, estradiol-
induced uterine weight increases can be attenuated and vaginal cornification prevented by MER-25 administration. Female sexual behavior and estradiol-induced increases in locomotor activity can be inhibited by antiestrogens, as well as estradiol-induced alterations in gonadotropin release (Arai and Gorski, 1968; Labhsetwar, 1970; Roy and Wade, 1975). The compounds do not inhibit or mimic androgens or progestins (Lerner et al., 1958).
PART I

EFFECTS OF AN ANTIESTROGEN ON FOOD INTAKE AND BODYWEIGHT

There has been no published study designed to determine whether antiestrogens will inhibit estradiol's actions on eating and body weight regulation. In fact, available data suggest that the antiestrogen MER-25 might itself produce the same effects as estradiol (Beyer and Vidal, 1971; Lerner et al., 1958; Lerner et al., 1966).

In most mammalian species estradiol decreases eating and body weight in intact females and ovariectomized animals given the hormone exogenously. The effects have been most clearly characterized in rats (see Wade 1976 for review): 1) The effects on food intake of either ovariectomy (OVX) or continued replacement injections of estradiol benzoate are transient, while the body weight changes are permanent (Gentry and Wade, 1976a; Mook et al., 1972; Tarttelin and Gorski, 1971; Wade, 1975). 2) Progesterone antagonizes the action of estradiol on f.i. and BW, but has no effects of its own (Wade, 1975; Ross and Zucker, 1974). 3) Females are more sensitive than males to the effect of EB injections on food intake (Gentry and Wade, 1976b). 4) Estradiol will produce a reduction of food intake when implanted in the ventromedial hypothalamus (Beatty et al., 1974; Jankowiak and Stern, 1974; Wade and Zucker, 1970). 5) Some actions of EB on body weight reduc-
tion are apparently independent of food intake changes (Blaustein et al., 1976; Dubuc, 1976). In female Mongolian gerbils, estradiol produces effects on eating and body weight opposite to those produced in rats; injections of EB increase food intake and body weight (Maass and Wade, unpublished).

The first experiment assesses the ability of the anti-estrogen MER-25 to inhibit the effects of EB on food intake and body weight. The subsequent studies determine to what extent the effects on eating and body weight of an anti-estrogen, MER-25, parallel those of EB.

**General Method**

The subjects were Sprague-Dawley rats obtained from Charles River Breeding Laboratories (Wilmington, Mass.) and Mongolian gerbils obtained from Tumblebook Farms, (Northfield, Mass.). Animals were castrated at various times before treatment as indicated in each experiment. Gonadectomies were performed through a single ventral incision under sodium pentobarbital (Nembutal, 40 mg/kg) or methoxyflurane (metofane) anesthesia. Animals were housed individually, and the lighting cycle provided 12 hours of light and dark. Injections were given daily 1-2 hours prior to the onset of dark. Substances to be injected were dissolved or suspended in sesame oil to a final volume of 0.1 cc (except the 20 mg dose of MER-25 which was in 0.2 cc oil) and injected subcutaneously. Purina Laboratory Chow pellets were available ad libitum except where otherwise noted.
Food intake was measured daily in the rat studies and twice a week in the gerbil study to the nearest 0.1 g during the middle of the light period with spillage collected. Body weight was measured to the nearest 1 g every third day. Data were analyzed by the appropriate *t* test for between-group or within-group comparisons or by analysis of variance.
EXPERIMENT 1. COMPARISON OF THE EFFECTS OF MER-25 ON THE ACTIONS OF ESTRADIOL ON FOOD INTAKE, FEMALE SEXUAL BEHAVIOR, UTERINE WEIGHT, AND VAGINAL CYTOLOGY

This experiment tested whether MER-25 would antagonize EB's effects on eating and body weight. Two doses were tested and concurrent comparisons made with the effects on sexual behavior, vaginal cytology, and uterine weight.

Procedure

Part 1. Fourteen females averaging 250 g were ovariectomized; the following day injections were begun. Group 1 (n=5) received the oil vehicle; Group 2 (n=5) received 2 ug/day of EB; Group 3 (n=4) received 2 ug of EB and 5 mg of MER-25. Food intake was measured daily and body weight every third day. Sexual behavior was tested under red illumination during the dark phase with male rats on the 4th and 9th days of injection. Injections were continued for 22 days, and then the animals were sacrificed and uterine weights measured.

Part 2. Thirty females were ovariectomized 31 days prior to treatment. Animals were assigned to one of four groups, balanced according to baseline food intake and body weight. The animals received daily injections of oil (n=8), 2 ug EB (n=8), 20 mg MER-25 (n=7), or 2 ug EB plus 20 mg MER-25 (n=7). Food intake was measured daily, and
body weight was measured on days 3, 6, and 10 of treatment. Vaginal smears were taken daily beginning on the 3rd day of treatment. Sexual behavior was tested during the dark on days 2, 5, 9, and 10. For testing on the last day, 500 ug of progesterone was injected 4 hours prior to testing. The response of the female was rated on a scale of 0-3 after Powers and Valenstein (1972). The receptivity score was the average response to 10 vigorous mounts. At the end of treatment all animals were given an overdose of sodium pentobarbital and the uterus was removed, cleaned of fat and mesentery, blotted and weighed to the nearest 1 mg. Naso-anal length was also measured.

Results

Part 1. Food intake comparisons were based on individual means during the 3rd week when the oil group's food intake had stabilized above pre-operative levels. EB caused a reduction of food intake by more than 4 g/day, t(8)=3.68, p < .01, Table 1. MER-25 produced no attenuation of EB's effect. The EB plus MER-25 group ate slightly less than the EB group and 5 g/day less than the oil control, t(7)=3.70, p < .01. The effects of the EB and MER-25 on food intake were not additive at this dose, since the MER-25 group ate the same amount as the EB plus MER-25 group. Estrogen antagonism was likewise not seen for body weight effects (Table 1) the effects of EB and MER-25 were additive. The EB plus MER-25 group gained less than the MER-25 group, t(7)=2.48, p < .05. MER-25 caused a significant
Table 1. Effects of estradiol benzoate (EB, 2 ug/day), MER-25 (5 mg/day), EB (2 ug/day) plus MER-25 (5 mg/day), and oil vehicle on daily food intake during the third week of treatment and body weight gain during 22 days of treatment. (Mean - standard error of the mean)
<table>
<thead>
<tr>
<th></th>
<th>Food Intake, g.</th>
<th>Body Weight Gain, g.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oil</td>
<td>25.8 ± 1.0</td>
<td>64 ± 6</td>
</tr>
<tr>
<td>EB</td>
<td>21.6 ± 0.6</td>
<td>12 ± 3</td>
</tr>
<tr>
<td>MER-25</td>
<td>20.0 ± 0.9</td>
<td>22 ± 5</td>
</tr>
<tr>
<td>EB + MER-25</td>
<td>20.8 ± 0.9</td>
<td>5 ± 3</td>
</tr>
</tbody>
</table>
suppression of body weight, \( t(8) = 5.53, p < .001 \), which did not differ significantly from that produced by EB. The 5 mg dose of MER-25 did not clearly inhibit EB induced sexual behavior. It did however inhibit the uterine weight increase (EB-treated: 558 mg vs. EB plus MER-25: 314 mg, \( t(7) = 2.128, p < .05 \), one-tailed) and completely prevented uterine ballooning.

Part 2. MER-25 at the higher dose also showed no antagonism of estrogen-induced changes in weight regulation, in contrast to the inhibition of all other somatic and behavioral parameters examined (Table 2 and Figure 1). The combination of MER-25 and EB caused greater reduction of food intake than either drug or hormone alone. Animals receiving both ate significantly less than the EB group, \( t(13) = 4.55, p < .001 \), or the MER-25 group, \( t(12) = 2.19, p < .05 \). Body weight showed a similar additive effect of the hormone and antiestrogen. The EB plus MER-25 group lost significantly more weight than the EB group, \( t(13) = 3.97, p < .01 \), or the MER-25 group, \( t(12) = 3.66, p < .01 \). Body length was slightly suppressed by EB, \( t(14) = 2.85, p < .05 \), and by MER-25, \( t(13) = 2.04, p < .05 \), one-tailed. On the other hand, all other estrogen sensitive parameters measured showed inhibition by MER-25 and little or no estrogenic effects from MER-25 alone. The uterine weight response was clearly inhibited, \( t(13) = 5.75, p < .001 \), and vaginal cornification was reversed. The vaginal response was interesting in that all animals receiving EB plus MER-25 initially had
Table 2. Effects of estradiol benzoate (EB, 2 mg/day) and MER-25 (20 mg/day) alone or in combination on various estrogen-sensitive parameters. (Mean ± standard error of the mean)
<table>
<thead>
<tr>
<th></th>
<th>EB(n=8)</th>
<th>EB+MER-25(n=7)</th>
<th>MER-25(n=7)</th>
<th>OIL(n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Uterine Weight, g</strong></td>
<td>206±13</td>
<td>102±13</td>
<td>64±5</td>
<td>58±4</td>
</tr>
<tr>
<td><strong>Vaginal Smear, % Cornified</strong></td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Sexual Behavior, RQ&lt;sup&gt;a&lt;/sup&gt;</strong></td>
<td>2.31±.31</td>
<td>1.61±.17</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>(EB induced)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Sexual Behavior, RQ</strong></td>
<td>2.66±.17</td>
<td>1.99±.14</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>(EB+Progesterone induced)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Final Body Length, cm</strong></td>
<td>22.0±.1</td>
<td>22.0±.1</td>
<td>22.0±.2</td>
<td>22.5±.2</td>
</tr>
<tr>
<td><strong>Body Weight Change, g</strong></td>
<td>-12.9±1.9</td>
<td>-29.0±4.2</td>
<td>-9.6±2.5</td>
<td>+15.2±2.2</td>
</tr>
<tr>
<td><strong>Food Intake, g/day</strong></td>
<td>18.1±.4</td>
<td>14.9±.6</td>
<td>16.8±.6</td>
<td>21.7±.7</td>
</tr>
</tbody>
</table>

<sup>a</sup>Receptivity Quotient, a rating of degree of lordosis on a 0-3 scale, mean response to 10 vigorous mounts.
Figure 1. Effect of estradiol benzoate (EB, 2 µg/day) and MER-25 (20 mg/day) alone or in combination on body weight and food intake of ovariectomized female rats. Arrow indicates beginning of treatment.
cornified smears within 3 days of treatment but the smears contained only diestrous leucocytes after 5-6 days of treatment. Similar delayed inhibition has been reported for another antagonist, ICI 46,474 (Emmens, 1971). Smears of animals receiving only MER-25 has predominantly leucocytes and occasional scattered nucleated and cornified cells. Sexual behavior was also inhibited by this dose of MER-25, whether induced by EB alone, t(13)=1.88, p<.05, one-tailed, or EB facilitated by progesterone, t(13)=2.92, p<.05.
EXPERIMENT 2. TRANSIENT REDUCTION OF FOOD INTAKE AND PERMANENT REDUCTION OF BODY WEIGHT BY MER-25

Part 1 of this experiment was designed to demonstrate that MER-25 does depress food intake and body weight in OVX rats and to determine whether the reduction of food intake by MER-25 is transient, as estradiol-induced hypophagia is. Part 2 determined the minimum effective dose of MER-25 required to decrease food intake and body weight.

Procedure

Part 1. Twelve female rats weighing approximately 250 g were ovariectomized and allowed 42 days for food intake to restabilize. They were then divided into two groups (n=6) balanced according to baseline food intake and body weight and injected daily with 5 mg MER-25 in 0.1 cc sesame oil or with the vehicle alone. Food intake was measured daily and body weight was measured every third day. Injections were continued for 32 days.

Part 2. Thirty-eight female rats were ovariectomized 18 days prior to the beginning of treatment. Groups (n=7 or n=8) received either MER-25 (5 mg, 1 mg, 500 ug or 100 ug) or 101 vehicle daily for 20 days.

Results

Part 1. Food intake was significantly depressed by MER-25 during days 5-21, \( t(10)=3.25, p<.01 \), (Figure 2). Over the course of treatment eating returned to control levels. During the final 10 days MER-25 treated animals
Figure 2. Effect of the antiestrogen MER-25 (5 mg/day) and oil vehicle on body weight and food intake of ovariectomized female rats. Arrow indicates beginning of treatment.
ate an average of 26.6 g/day and controls ate 27.3 g/day, $t(10)=0.97, p < .35$. All MER-25 treated animals increased their food intake from the first 16 days to the last 10 days, $t(5)=3.09, p < .05$, and during the last 10 days did not differ significantly from their own baselines (27.5 g/day compared to 26.6 g/day, $t(5)=0.73, p < .45$). However, the body weight of MER-25 animals remained constant and at the end of treatment they had gained significantly less than controls, $t(10)=4.48, p < .01$.

Part 2. Food intake during days 6-20, computed as a percentage of baseline food intake, was significantly lower for the 5 mg MER-25 group compared to the oil-injected controls, $t(13)=2.63, p < .05$. The final body weight, computed as a percentage of baseline, was also lower for the 5 mg MER-25 group compared to oil controls, $t(13)=4.21, p < .01$. One mg/day of MER-25 was totally ineffective in decreasing eating and body weight, as were the 500 ug and 100 ug doses. The 5 mg MER-25 group received injections for an additional 16 days, and its mean food intake returned to approximately that of the control group by the 31st day.
EXPERIMENT 3. ATTENUATION OF MER-25-INDUCED REDUCTION OF FOOD INTAKE AND BODY WEIGHT BY CONCURRENT PROGESTERONE INJECTIONS

Since estradiol-induced suppression of food intake is inhibited by progesterone, the effects of progesterone on MER-25-induced changes in food intake were investigated. Both long-term ovariectomized rats whose weight had plateaued and recently ovariectomized rats that were still hyperphagic were tested.

Procedure

Part 1. Eighteen female rats were ovariectomized and allowed 42 days for food intake and body weight stabilization. Daily injections of oil, 5 mg MER-25, or 5 mg MER-25 plus 5 mg progesterone (n's = 6) were administered for 32 days.

Part 2. Seventeen female rats weighing an average of 250 g were ovariectomized. The following day injections were begun of oil (n=5), 5 mg MER-25 (n=6) or 5 mg MER-25 plus 5 mg progesterone (n=6). The oil controls were concurrently controls for Experiment 1, Part 1. Injections were continued for 22 days.

Results

Part 1. Over the 32 day period, progesterone caused a reversal of the effects of MER-25 on body weight since the progesterone plus MER-25 group gained significantly more than the MER-25 group (Table 3), t(10)=2.83, p<.05.
The effects of progesterone on MER-25-induced reduction in food intake were suggestive but not statistically reliable. During the third week of treatment when MER-25 suppression of food intake was maximal, \( t(10) = 3.91, p < .01 \), progesterone failed to clearly reverse the effect of MER-25, \( t(10) = 1.55, p < .05 \).

Part 2. In recently ovariectomized females progesterone countered the effects of MER-25 on both food intake and body weight (Table 3). During the third week of treatment, the MER-25 plus progesterone group ate more than the MER-25 group, \( t(10) = 1.97, p < .05 \), one-tailed. The effects of MER-25 on body weight were again attenuated by progesterone. Over the course of treatment the MER-25 group gained less than the MER-25 plus progesterone group, \( t(10) = 1.82, p < .05 \), one-tailed.
Table 3. Effects of MER-25 (5mg/day), MER-25 (5 mg/day) plus progesterone (5 mg/day), and oil vehicle on daily food intake during the third week of treatment and body weight gain during treatment. In Part 1 females were ovariectomized 42 days prior to treatment and injected for 32 days. In Part 2 females were ovariectomized one day prior to treatment and injected for 22 days. (Mean ± standard error of the mean)
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<td>+ Progesterone</td>
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<td>Men-25</td>
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<th>64 +</th>
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<td>37 +</td>
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<td>22 +</td>
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<th>74 +</th>
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<tr>
<td>18 +</td>
<td>6</td>
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<td>0 +</td>
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Body weight gain, lbs

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<th>0.0</th>
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Food intake, lbs

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</tr>
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<td>20.7 +</td>
<td>0.4</td>
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EXPERIMENT 4. SEX DIFFERENCE IN RESPONSIVENESS TO MER-25

Since estradiol is more effective in suppressing food intake and body weight in females than in males (Gentry and Wade, 1976b), this experiment examined the possibility of a sex difference in responsiveness to MER-25. To avoid confounding weight differences with sex differences, two groups of females were used, one of comparable age to the males and one of comparable body weight.

Procedure

Eight male rats castrated 3-4 weeks prior to the beginning of treatment averaged 338 g in body weight. The ten light females of comparable age were ovariectomized 15 days before treatment and averaged 291 g when injections began. The eight heavy females had been ovariectomized 3 months earlier and weighed an average of 352 g. Half of the animals in each group were injected daily with oil and the other half with 10 mg MER-25 for 28 days.

Results

A comparison of same age castrated males and females indicated that MER-25 affected the body weight of females more than males (Figure 3; an overall analysis of variance was performed on percentage weight change during 28 days of treatment and subsequent two-way analysis of males and light females resulted in a significant Sex X Drug interaction, $F(1,20)=4.53$, $p < .05)$. Males and females of the
Figure 3. Effects of MER-25 (10 mg/day) and oil vehicle on body weight of castrated male rats, ovariectomized female rats of comparable age (light females), and ovariectomized female rats of comparable weight (heavy females).
same initial weight appeared to be comparably affected; the initial weight appeared to be comparably affected; the females showed a decrease in weight whereas the males' weight gain only slowed, but in comparison with their own controls there was no sex difference. The sex difference in the effects of MER-25 on food intake was less ambiguous. Females were more strongly affected than males, regardless of initial body weight (Figure 4). Analysis was performed on mean food intake during the first 10 days of treatment as a percentage of baseline. Both light and heavy females showed greater inhibition of food intake than males (interaction term for males vs. light females, $F(1,20) = 11.40, p < .01$; interaction for males vs. heavy females, $F(1,20) = 8.85, p < .01$). During the first 10 days of injections the MER-25 treated males ate 6% less than their baseline, MER-25 treated light females ate 17% less than their baseline, and MER-25 treated heavy females ate 36% less than their baseline.
Figure 4. Effect of MER-25 (10 mg/day) and oil vehicle on food intake of castrated male rats, ovariectomized female rats of comparable age (light females) and ovariectomized female rats of comparable weight (heavy females). Lower panel is the difference for each condition from its respective control. Arrow indicates beginning of treatment.
EXPERIMENT 5. CONTROL FOR ILLNESS: A LEARNED AVERSION PARADIGM

An experiment was performed to ascertain whether the food intake reductions could be accounted for by a general illness possibly produced by MER-25 rather than a true estrogenic effect.

**Procedure**

A learned aversion paradigm (Nachman, 1970) was employed to investigate the possibility of the induction of gastrointestinal discomfort. Twenty OVX females were adapted to water's being available 30 min./day, beginning 2 hours after the onset of dark. Water intake was measured to the nearest ml in calibrated Richter tubes, and food intake was measured daily. Saccharin flavored (0.1%) water was presented as a novel taste, and followed at the end of the 30 min. period by a subcutaneous injection of lithium chloride (3.0 mEq/kg, i.e., 7.5 ml/kg of .4 M LiCl, n=7), MER-25 (125 mg/kg in oil, n=7), or oil (equi-volume to the MER-25 injections, n=6). On the following two days water was presented, and on the third day a second saccharin-injection pairing was made. Following the second pairing, 9 days were allowed for the disappearance of the direct effects of the MER-25 (a food intake decrease), so that any learned effects would be measured rather than direct drug effects. The LiCl group controlled for the
possibility that a learned aversion would fade during this time.

Results

MER-25 failed to produce a learned aversion to the taste of saccharin after one or two pairings (Figure 5); the oil and MER-25 groups drank virtually identical amounts in the two tests (21.0 vs. 19.8 ml on the first test and 21.0 vs. 21.1 ml on the second test. LiCl in the same paradigm produced a highly significant reduction in saccharin-water intake on both test, $t(11)=6.99$, $p<.001$ and $t(11)=12.50$, $p<.001$. The dose of MER-25 was approximately 10 times the daily dose required to reduce food intake, and caused a significant reduction in food intake after both injections. The change of food intake of oil and MER-25 treated rats for the two days pre- and post-injections differed significantly, $t(11)4.64$, $p<.001$. After the second injections, food intake remained depressed for 6 days, $t(11)2.11$, $p<.05$, one-tailed. Body weight was also clearly affected after the second injection; final body weight of oil and MER-25 injected rats differed significantly, $t(11)=3.59$, $p<.01$. Water intake was not reliably affected.
Figure 5. Effects of two injections (Inj) of oil, MER-25 (125 mg/kg), or lithium chloride (LiCl, 2.0 mEq/kg) following 30 min. presentations of saccharin-flavored water (S, 0.1% saccharin). Upper panel is food intake. Lower panel is consumption during daily 30 minute periods of unflavored water (connected by solid lines) or saccharin-flavored water (indicated by arrows and connected by dotted lines).
EXPERIMENT 6. CENTRAL EFFECTS OF MER-25 ON EATING

This experiment determined whether an antiestrogen implanted in the hypothalamus would produce effects on eating similar to those produced by estradiol.

Procedure

Female rats were ovariectomized and later stereotaxically implanted with bilateral, concentric double-walled stainless steel cannulas (27 inner gauge, and 22 outer gauge). Operations were performed under sodium pentobarbital anesthesia. Cannulas were aimed just dorsal to the ventromedial hypothalamus, anterior hypothalamus (AH), or preoptic area (PCA). After recovery and the recording of a stable baseline of food intake, the inner cannulas were removed and crystalline MER-25, cholesterol, estradiol or EB was tamped into the lumen. The chemical was left in place for three days, and then the inner cannulas were removed and cleared by combustion. After at least a week of restabilized baseline, another chemical was implanted for an additional three days. All rats received at least MER-25 and cholesterol in random order. Food intake was measured daily to the nearest .1 g.

Results

An animal was considered to have responded to a treatment if its mean food intake during the three days following implantation of the chemical was at least 10% less than its food intake during the three days prior to implantation. Four animals were eliminated from analysis
because they responded to cholesterol (two VMH and two AH placements); a fifth animal was eliminated because of hyperphagia apparently due to mechanical damage of the VMH. A total of 10 animals responded to MER-25 but not to cholesterol. Histological examination indicated that in 6 of these the tips of the cannulas had been located in or dorsal to the VMH (Figure 6); a seventh effective placement was at the same rostral-caudal plane but more lateral. Place-ments of the other 3 effective cannulas were in the POA and the bed nucleus of the stria terminalis (POA-ST). There were 5 ineffective POA-ST placements and 6 ineffective anterior hypothalamic placements. One animal had a placement close to the arcuate nuclei and failed to respond to MER-25. Statistically, responses to MER-25 implanted in the ventral hypothalamus differed from those in the AH-POA-ST (Fisher test, p < .001).

Four of the animals with VMH placements effective for MER-25 were implanted with EB or estradiol; all four decreased their food intake. EB was also effective in the arcuate placement which did not respond to MER-25. Of the remaining non-responders to MER-25, 6 were implanted with EB or estradiol, and none responded by decreasing food intake. The animals with effective placements in the ventral hypothalamus decreased their food intake an average of 22% to MER-25 and the average response to estradiol or EB was a 19% reduction in food intake. The three effective place-ments in the POA-ST caused greater reductions, averaging 50%.
Figure 6. Location of implants of antiestrogen which affected food intake in female rats. Squares indicate locations of bilateral implants of MER-25 which caused a reduction of food intake of at least 10% for the three days following implantation and showed no response to cholesterol implantation. Circles indicate location of implants which did not alter food intake. Coronal planes of the rat diencephalon are adapted from Pellegrino and Cushman.
On response to MER-25

☐ RESPONDED TO MER-25
☐ NO RESPONSE TO MER-25
EXPERIMENT 7. BODY WEIGHT EFFECTS OF MER-25 INDEPENDENT OF FOOD INTAKE CHANGES: A PAIR-FEEDING STUDY

This experiment determined whether some of the body weight reduction caused by EB or MER-25 treatment is mediated by factors other than reduction of food intake.

**Procedure**

Twenty-five female rats were ovariectomized 15 days prior to treatment. Twenty females were divided into 10 pairs matched for body weight, and these 10 pairs were divided into 2 groups balanced for body weight (mean body weight 281 g for all 4 groups). One member of each pair in group 1 received 5 ug of EB and was fed ad lib; the other member received oil injections and was allocated the amount of food consumed on the previous day by its matched pair. Similarly, five animals received 10 mg MER-25 daily and food ad lib while a pair-fed group received oil injections and individually tailored food allotments. The five remaining rats (average body weight 270 g) received oil injections and ad lib food. Body weight was measured every third day and injections were continued for 33 days.

**Results**

Both EB and MER-25 caused weight reductions which could not be simulated simply by food restriction in controls (Figure 7). Whereas animals injected with EB increased body weight by 4%, animals which ate the same amount but were injected with oil increased body weight by 18%, paired
Figure 7. Percentage body weight changes of ovariectomized female rats treated with 5 ug estradiol benzoate (EB)/day, 10 mg MER-25/day or with the sesame oil vehicle. Two additional groups were treated with sesame oil and pair-fed with either the EB- or MER-25-treated groups.
$t(4)=9.35, \ p < .01$. Neither of the pair-fed groups differed significantly from the ad lib oil controls.

It could be argued that a metabolic derangement or some other consequences of pair-feeding, per se, prevented the expected weight losses of the pair-fed rats. However, it has been shown that a pair-feeding regimen similar to ours produces weight losses, not gains, in rats (Rider and Chow, 1971). Thus, the body weight differences are not likely artifacts of a pair-feeding procedure.
EXPERIMENT 8. EFFECTS OF MER-25 IN GERBILS

Female Mongolian gerbils are unusual in that they respond to EB injections by increasing eating and body weight (Maass and Wade, unpublished); they therefore provide a particularly good opportunity to test the generality of the estrogenic effect of MER-25 on eating. If gerbils responded to MER-25 by increasing their food intake, it would demonstrate that MER-25 can produce estrogenic effects on food intake which cannot be confounded by hypothetical toxic effects.

Procedure

Five weeks after ovariectomy 30 adult gerbils were divided into four groups. Daily subcutaneous injections of the following compounds were administered for four weeks: 4 mg MER-25 (M; n=7), 4 mg MER-25 plus 1 mg progesterone (MP; n=8), 4 mg MER-25 plus 2 ug EB plus 1 mg P (MEP; n=7), or the sesame oil vehicle (0; n=8). All injections were in 0.1 ml. The effects of MER-25 in combination with P and EP were of interest, since in OVX gerbils progesterone augments the effects of EB on eating while having little or no effect when given alone (Maass and Wade, unpublished). The MP group might facilitate detection of an estrogenic effect of MER-25, whereas the MEP group would optimize the chances of observing an antagonistic action of MER-25. Analyses were performed on the difference between individuals' mean food intake or body
weight during the second 2-week period of treatment and a 2-week pretreatment baseline. Data from Maass and Wade (unpublished) of additional control animals (n=6) and animals given progesterone (1 mg; n=6) were added to the analysis to allow two-way analysis of variance of the O, M, MP, and P groups. Data of groups receiving E and EP are presented for comparison.

Results

The results indicate that MER-25 (M and MP) causes increasing eating and body weight in OVX gerbils, $F(1,31)=4.778$, $p<.05$, and $F(1,31)=4.944$, $p<.05$ (Table 4). This is presumably an estrogenic effect, since estradiol benzoate also increases eating and body weight in gerbils. Progesterone did not have any effect alone and did not interact with the effect of MER-25 on either measure. The three compounds together (MEP) markedly increased eating and body weight, $t(19)=5.227$, $p<.001$ and $t(19)=6.525$, $p<.001$. There was no evidence of an estrogen-antagonizing effect on these measures from a comparison of the MEP and EP groups. The effects of the ovarian hormones and the antiestrogen on body weight continued as long as treatment continued, and after treatment was discontinued the animals' body weights returned to control levels within seven weeks.
TABLE 4. FOOD INTAKE AND BODY WEIGHT OF MONGOLIAN GERBILS TREATED WITH THE ANTIESTROGEN MER-25 AND OVARIAN HORMONES

Ovariectomized gerbils were given daily injections of MER-25 (M, 4 mg), MER-25 plus progesterone (MP, 4 mg plus 1 mg), MER-25 plus estradiol benzoate plus progesterone (MEP, 4 mg plus 2 ug plus 1 mg), or sesame oil vehicle. Data are computed as change from 2 weeks of baseline to weeks 2-4 of treatment.

*Data of similarly treated animals from Maass and Wade (unpublished).
<table>
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DISCUSSION

Estradiol and the antiestrogen MER-25 have similar effects on eating and body weight in rats and gerbils:
(1) The food intake reduction during EB or MER-25 treatment is transient; eating remains depressed for a period of days and then gradually returns to control levels, but body weight remains lower than controls (Experiment 2). The time-course of the reinstatement of normal food intake levels varies, depending on the dose of MER-25 and pre-treatment body weight, but is approximately 30 days, similar to that found for EB treatment (Wade, 1975).

(2) Progesterone inhibited the effects of MER-25 as it does those of EB (Experiment 3). In recently ovariectomized females 5 mg of progesterone caused a 50% inhibition of the food intake suppression by MER-25, comparable to the effects of 5 mg progesterone counteracting 2 ug of EB (Gentry and Wade, 1976a). In heavier long-term ovariectomized females progesterone produced a similar, but not statistically significant, effect on food intake, and the effect of MER-25 on body weight was significantly attenuated by progesterone.

(3) MER-25 is more effective in female rats than in male rats in causing food intake reduction (Experiment 4). There are similar sex differences in the effectiveness of EB for a variety of behaviors including eating (Gentry and Wade, 1976b; Pfaff, 1970). The sex difference in food
intake effects was clear for either light or heavy females compared to males, but the sex difference for body weight effects was apparent only for the light females compared to males.

(4) When rats were implanted intracerebrally with the antiestrogen, the medial basal hypothalamus was an area in which MER-25 produced estrogenic actions on eating. For all 11 animals implanted with the antiestrogen and with estradiol at another time, there was a high correlation between responses to the antiestrogen and to the estrogen. Although the POA-ST placements were not tested with estradiol, it is less likely that the 3 responsive sites were actually estrogenic sites, since there were 5 ineffective implants in similar sites. The finding that the most consistently effective placements were in the medial basal hypothalamus confirms earlier reports on the localization of estradiol's actions on food intake (Beatty et al., 1971; Jankowiak and Stern, 1974; Wade and Zucker, 1970).

(5) Both EB and MER-25 produce some body weight reductions by means independent of their alterations in food intake (Experiment 7).

(6) The results of Experiment 8 with gerbils extend across species the parallel between the effects of EB and MER-25 on food intake. In rats estrogens and MER-25 decrease food intake and body weight, whereas in gerbils the estrogenic effect is indicated by the increases in food intake and body weight during treatment with MER-25.
For a number of reasons it is highly unlikely that MER-25 is having its effects on eating and body weight by inducing a nonspecific malaise:

(1) An aversion to the novel flavor of saccharin was not induced by twice pairing it with a very high dose of MER-25 (Experiment 5). The effectiveness of the LiCl treatment suggests that if any aversion had been created by MER-25 it would still have been evident at the final testing.

(2) MER-25 does not alter spontaneous running wheel activity levels of nonhormone-treated rats. Although MER-25 reduces estradiol- or testosterone-induced activity in male rats, it has no effect on the spontaneous activity of castrated males (Roy and Wade, 1975). In that experiment MER-25 did reduce the males' food intake; during the first 4 days of treatment MER-25 treated rats averaged 84% of their baseline, t(8)=2.40, p<.05 (unpublished data).

(3) The food intake reduction is transient while the body weight reduction is permanent.

(4) Within each experiment there was a tendency for MER-25 to have greater effects in heavier rats; for example, in Experiment 4 there was a high correlation between baseline weight and weight loss (r=.70 for long-term OVX females and r=.82 for recently OVX females). This suggests a weight regulatory effect rather than an illness effect, since the latter explanation would predict greater effects in lighter rats because they receive relatively higher doses.
(5) The increases in eating and body weight produced by MER-25 in gerbils (Experiment 8) also suggest that the effects in rats are specifically estrogenic.

(6) Finally, MER-25 can reduce food intake when implanted directly into the hypothalamus (Experiment 6). These data support the hypothesis that MER-25 is acting as an estrogen to lower food intake and body weight rather than by causing general illness. The results of hypothalamic implants indicate that the effects on eating of either MER-25 or EB are centrally mediated, but pair-feeding studies indicate that there are additional metabolic effects of both agents which cause further loss of weight (Experiment 7).

MER-25 is totally ineffective in antagonizing the effects of estradiol on eating and body weight (Experiment 1). Neither 5 mg nor 20 mg of MER-25 attenuated the effects of 2 ug of EB on eating and body weight at any time, yet it inhibited Eb's effects on sexual behavior, uterine weight and vaginal smears. The inhibition by MER-25 of these responses is well-established (Lerner et al., 1958; Meyerson and Lindstrom, 1968; Sodersten, 1974). Furthermore, MER-25 alone had no agonistic effects on these noneating parameters. This is consistent with other reports of the absence of or minimal agonistic effects on these measures (Komisaruk and Beyer, 1972; Lerner, 1964; Lerner et al., 1958; Torenius, 1971). There have been no reports of any induction of
female sexual behavior by MER-25, despite a wide variety of doses and timing routines tested (Meyerson and Lindstrom, 1968; Sodersten, 1974; Roy, unpublished data) and no evidence of an estrogenic effect on spontaneous activity (Roy and Wade, 1975). In gerbils, the similar increases in eating and body weight of the MEP and EP groups support the idea that MER-25 does not have any estrogen-antagonizing effects on these measures.

Thus, the estrogen-sensitive systems affecting eating and weight regulation differ biochemically from most other behavioral and peripheral systems, as reflected by the difference in responses to antiestrogens. The distinction is not simply that the antiestrogen is estrogenic, since other systems may sometimes exhibit some agonistic response under the appropriate conditions. The distinction is that the eating system shows a fully estrogenic response to the antiestrogen, so that no inhibition of estradiol's action can be demonstrated. Preliminary evidence indicates that the other triphenyl antiestrogens are similarly estrogenic with respect to eating, including CI-628 (Powers, personal communication) and nafoxidine (Roy, unpublished data). Recent reports (Baum and Vreeburg, 1976; Sodersten, 1974) indicate that MER-25 may also be solely "estrogenic" with respect to masculine sexual behavior. Mounting behavior is induced by EB or MER-25 in castrated rats of either sex, and the drug fails to inhibit EB-induced mounting in
females or testosterone-induced mounting in males. Thus, estrogens are able to influence behavior by at least two different mechanisms as indicated by the responses of various behaviors to antiestrogens. The manner in which estradiol’s actions on the eating system differ from its actions on other behavioral systems is unknown, but would presumably be related to the mechanism of action of the antiestrogens.
PART II. THE EFFECTS OF ANTIESTROGENS ON THE UPTAKE OF 

3H-ESTRADIOL IN THE CENTRAL NERVOUS SYSTEM AND 

FEMALE SEXUAL BEHAVIOR

Antiestrogens may produce their estrogen-antagonizing effects by forming relatively ineffective complexes with estrogen receptors, thereby lowering the estradiol uptake into target tissues. Their estrogenic actions could also be mediated by interactions with estrogen receptors. It is appropriate to begin an investigation of either the estrogen-antagonizing actions or estrogenic actions of antiestrogens by determining whether or not they interact with estrogen receptors, as indicated by competition with estradiol for binding sites. Competition for estrogen receptors of peripheral tissues has been clearly demonstrated (Black and Kraay, 1973; Jensen et al., 1966; Stone, 1964; Terenius, 1971; Whalen and Gorzalka, 1973), but reports concerning the effects of antiestrogens on brain estradiol uptake have been less consistent. Hypothalamic uptake of estradiol was reportedly not reduced by the antiestrogen CI-628 (Perry et al., 1973), even in a paradigm similar to one in which estrous behavior was inhibited (Whalen and Gorzalka, 1973). However, there have been recent reports of in vivo competition by CI-628 in the cytosol fraction (Whalen et al., 1975) and nuclear fraction (Chazal et al., 1975; Luttge et al., 1976) of the hypothalamus. Inhibition in the nuclear fraction may be particularly important if
estradiol acts by similar mechanisms in the brain and uterus. Nafoxidine is an antiestrogen widely used to study mechanisms of estrogen antagonism in the uterus (Caponi and Rochefort, 1975; Clark et al., 1974; Jensen et al., 1966; Katzenellenbogen and Ferguson, 1975). Its behavioral effects have not been studied, and there have been positive (Eisenfeld and Axelrod, 1967) and negative (Jensen et al., 1966) reports on its inhibition of hypothalamic estradiol uptake. The antiestrogen clomiphene competes with estradiol in the hypothalamus in vivo (Eisenfeld and Axelrod, 1967; Kato et al., 1968; Maurer and Woolley, 1971), but conclusions from these data regarding the mechanisms of estrogen-antagonism must be limited, since these studies have employed racemic clomiphene, one isomer of which is a full estrogen (Ross et al., 1973). MER-25 is a commonly used antiestrogen in studies of sexual behavior (Baum and Vreeburg, 1976; Komisaruk and Beyer, 1972; Meyerson and Lindstrom, 1968; Sodersten, 1974). In vitro MER-25 has a low affinity for hypothalamic cytosol estrogen receptors (Barley et al., 1974), but it has not been tested for its effects on hypothalamic estradiol uptake in vivo.

In order to clarify the relationship between estradiol binding in the brain and its behavioral actions, the effects of three antiestrogens (MER-25, CI-628, and nafoxidine) on the in vivo $^3$H-estradiol uptake and retention were determined in cell nuclear fractions of regions of the brain and
pituitary. In parallel studies their ability to block estradiol-induced female sexual behavior was examined.

Method

\( ^3\text{H}-\text{estradiol} \) Uptake. Mature female Sprague-Dawley rats (Charles River Breeding Laboratories), housed in group cages under a 12:12 lighting cycle, were ovariectomized at least four days prior to uptake studies. Groups were matched for body weights. Shortly before injections, \([2, 4, 6, 7]\) \(^3\text{H}-\text{estradiol-17B} \) (specific activity: 91.3 Ci/mM; New England Nuclear) was dissolved in 20% ethanol. Injections of 100 uCi (0.3 ug) in 0.2 ml were administered in the femoral vein under light methoxyflurane (Metofane) anesthesia. MER-25 (40 mg, in sesame oil), CI-628 (4 mg, in saline), nofoxidine (4 mg, in sesame oil), or sesame oil was injected intraperitoneally at various times before or after the \(^3\text{H}-\text{estradiol} \) injection. Doses of antiestrogens were based on the finding that 40 mg of MER-25 are required to inhibit mating in intact female rats, (Shirley, Folinsky, and Schwartz, 1968) and CI-628 and nafoxidine have approximately 10 times greater affinity than MER-25 for uterine estradiol receptors (Black and Kraay, 1973; Terenius, 1971). At various times after the estradiol injection, rats were anesthetized with sodium pentobarbital (Nembutal), a blood sample was taken, and the animals were perfused with cold saline.
Cell nuclei were isolated by the method of Zigmond and McEwen (1970) with modifications (Gentry, Wade, and Roy, 1976). All samples were homogenized in cold buffer containing .32 M sucrose, .25% Triton X-100, 3 mM MgCl₂, 1mM K₃PO₄, pH 6.5. An aliquot of this was taken as the whole homogenate. The remainder was centrifuged at 850 g for 10 min., and washed once in the same buffer without Triton. After purification of the nuclei by centrifugation through buffer containing 2 M sucrose, radioactivity was extracted with 3 x 4 ml toluene-based scintillation cocktail. Protein was precipitated with ethanol and estimated by the method of Lowry et al. (1951). Results are expressed as the ratio of tissue to plasma, i.e., disint./min./mg. protein divided by blood plasma disint./min./ul. All differences reported are significant at the .05 level or beyond using the Student's t-test. The results were the same whether or not the data were standardized by dividing by plasma levels of radioactivity.

In vitro Dissociation. For this experiment animals were treated as above with sesame oil or 40 mg MER-25 2 hours prior to ³H-estradiol and were killed 2 hours after the estradiol injection. The nuclei were isolated as above, but POA and hypothalamic sections were pooled. Isolated nuclei from 8 animals per group were resuspended in the .32 M sucrose buffer without Triton and incubated at 27°C. Aliquots were taken at various times, washed 3
times in cold buffer, and the radioactivity extracted. The supernatant from the washes (radioactivity not bound to nuclei) was counted in toluene scintillation cocktail containing 33% Triton X-100. Results are expressed as the percentage of the total radioactivity (supernatant disint./min. plus pellet disint./min.) found in the pelleted nuclei (percent bound).

**Behavioral Testing.** Three ug of unesterified estradiol-17B in 20% ethanol were injected intravenously 24 or 36 hours before testing. Progesterone (1 mg) was injected subcutaneously 5 hours prior to testing. Groups were matched for behavioral sensitivity to estradiol. Antiestrogens (MER-25, 40 mg; CI-628, 4 mg; nafoxidine, 4 mg) or vehicle were injected intraperitoneally 2 hours prior to the estradiol injection, except for one study in which MER-25 (40 mg) was injected 12 hours prior to the estradiol injection. The female was placed into a circular arena, and then two sexually experienced males were placed in the arena and allowed 10 vigorous mounts. Lordosis responding was rated on a 3-point scale after Powers and Valenstein (1972), 3 being the highest score and indicating extreme dorsoflexion of the head and rump. Observations were made without knowledge of the treatment received by particular animals. Animals used in the MER-25 studies were tested repeatedly with at least one week intervening between sessions and alternating control and experimental treatments, without detriment to the lordosis responding of control groups.
Student's $t$-test was used to determine statistical significance. The results were identical when the data were expressed as lordosis quotients.
EXPERIMENT 9. EFFECTS OF ANTIESTROGENS INJECTED 2 HOURS PRIOR TO $^3$H-ESTRADIOL ON UPTAKE IN WHOLE HOMOGENATES AND CELL NUCLEI OF THE BRAIN

Two-hour uptake.

When uptake was examined 2 hours after the injection of $^3$H-estradiol, the antiestrogen CI-628 most effectively reduced levels of radioactivity (Figure 8). Uptake was reduced in the whole homogenates of pituitary and all three brain areas. Nuclear $^3$H-estradiol binding was reduced even more than whole homogenate binding, with 99% inhibition in the pituitary, 79% inhibition in the hypothalamus, 87% inhibition in the POA, and 59% inhibition in the cerebral cortex.

Nafoxidine also effectively reduced $^3$H-estradiol uptake in the pituitary and brain (Figure 8). It was somewhat less effective than CI-628. Nafoxidine inhibited $^3$H-estradiol uptake in the nuclear fraction by 88% in the pituitary, 42% in the hypothalamus, and 45% in the POA. MER-25 on the other hand, failed to inhibit the 2-hour uptake of $^3$H-estradiol into any of the brain areas, even while inhibiting pituitary uptake by 63% in the whole homogenate and 70% in the nuclear fraction (Figure 8). There was also no inhibition of $^3$H-estradiol levels in hypothalamic cytosol from similarly treated animals.

Six-hour and twelve-hour retention.

It has been suggested that for uterine tissue the low levels of estradiol which are bound in cell nuclei for long
Figure 8. Effects of antiestrogens injected 2 hours prior to $^3$H-estradiol on levels of radioactivity in subcellular fractions of brain tissues and pituitary 2 or 12 hours after the $^3$H-estradiol injection. Mean values ± S.E. of the concentration of tissue radioactivity relative to plasma radioactivity are presented for whole homogenate and cell nuclear fractions of cerebral cortex (CTX), hypothalamus (HTH), preoptic area-septum (POA), and pituitary (PIT). Intraperitoneal injections of sesame oil vehicle ($n=4$ for 2-hour and $n=6$ for 12-hour), MER-25 (40 mg in oil, $n=4$ for 2-hour and $n=6$ for 12-hour), CI-628 (4 mg in saline, $n=4$ for 2-hour and $n=3$ for 12-hour), or nafoxidine (4 mg in oil, $n=4$ for 2-hour and $n=4$ for 12-hour) were administered 2 hours prior to an intravenous injection of 100 uCi (0.3 ug) of $^3$H-estradiol.

* $p < .05$, ** $p < .01$
ANTAGONISM OF UPTAKE AND RETENTION OF \( ^{3}H \)-ESTRADIOL
time intervals may be functionally significant (Clark and Peck, 1976). We investigated levels of bound radioactivity at later time intervals after the injection of $^3$H-estradiol, without changing the 2-hour interval between the antiestrogen and $^3$H-estradiol injections. At 6 hours after the injection of estradiol, MER-25 still showed no evidence of inhibiting brain $^3$H-estradiol binding in either whole homogenate or nuclear fractions, even through pituitary nuclear radioactivity was reduced by 81%.

Interestingly, by 12 hours after the $^3$H-estradiol injection MER-25 had reduced radioactivity found in hypothalamic nuclei by 26% and lowered POA nuclear radioactivity by 55% (Figure 8). Whole homogenate levels in brain tissues, which were quite low at this time, were not affected by MER-25. Pituitary binding of estradiol at 12 hours was reduced by MER-25 by 94% in whole homogenate and by 96% in nuclei.

Comparison of 2-hour uptake and 12-hour retention.

In addition to the appearance of significant inhibition of brain nuclear $^3$H-estradiol binding by MER-25 after a long time interval, CI-628 and nafoxidine showed a greater inhibition of nuclear binding at 12 hours than at 2 hours after the injection of estradiol. Figure 9 indicates that when expressed as a percentage of control values, 12-hour retention of $^3$H-estradiol was affected more than 2-hour uptake for all three antiestrogens. Percentages at 12 hours and 2 hours differed significantly for all samples except
Figure 9. Relative inhibition of $^3$H-estradiol retention in cell nuclei at 12 hours vs. at 2 hours. Data of nuclear concentration of radioactivity from Figure 8 are expressed as percentage of control values at the same time point. Twelve-hour percentages are significantly lower than 2-hour percentages for all samples except CI-628 pituitary and hypothalamus.
NUCLEAR ESTRADIOL
(Tissue/Plasma)

% CONTROL

2 HOURS  12 HOURS
CI-628 HTH and CI-628 PIT. CI-628 had already virtually eliminated $^3$H-estradiol from pituitary nuclei at 2 hours.
EXPERIMENT 10. **IN VITRO DISSOCIATION OF $^3$H-ESTRADIOL FROM NUCLEI AFTER MER-25 TREATMENT**

There are at least two possible explanations of the greater inhibition of 12-hour retention than 2-hour uptake. The first is that the antiestrogens are somehow altering nuclear binding sites so that estradiol is less firmly bound, causing the hormone to leave the nuclei faster in antiestrogen-pretreated tissues. The second is that continuing competition of the estrogen and antiestrogen occurs, and the ratio of antiestrogen to estrogen might be greater at later times. The first possibility was assessed by examining the *in vitro* dissociation of $^3$H-estradiol from nuclei of animals pretreated with MER-25 or oil and injected with $^3$H-estradiol in the same paradigm as the above uptake studies. If MER-25 were altering the ability of the nuclei to retain the $^3$H-estradiol, $^3$H-estradiol should dissociate from nuclei of MER-25 pretreated animals faster than from control nuclei. At sacrifice 2 hours after the $^3$H-estradiol injection there was again no inhibition of nuclear uptake by MER-25 in the hypothalamus-POA. During 10 further hours of *in vitro* incubation $^3$H-estradiol dissociated from nuclei of the hypothalamus-POA at the same rate whether nuclei were obtained from MER-25 treated animals or controls (Figure 10). Pituitary nuclei also showed similar rates of *in vitro* dissociation of $^3$H-estradiol when isolated from MER-25 pretreated or control nuclei.
Figure 10. In vitro dissociation of $^3$H-estradiol from brain cell nuclei. Animals were treated as in Figure 8 (oil vehicle or 40 mg MER-25 injected 2 hours prior to .3 ug $^3$H-estradiol) and nuclei prepared in the same manner except hypothalamus and preoptic area were pooled; tissues from 8 animals were prepared together. The isolated nuclei were resuspended in the .32 M sucrose buffer and incubated at $27^\circ$ for specified times; at these times aliquots of nuclei were removed and washed three times. Radioactivity was determined in the supernatants and final pellet of the washes, and results are expressed as percentage of total radioactivity bound to the pelleted nuclei.
IN VITRO DISSOCIATION OF $^3$H-ESTRADIOL FROM BRAIN CELL NUCLEI

% BOUND TO NUCLEI

INCUBATION TIME, Hours

○ CONTROL
● MER-25
These results make the second possibility more likely: competition for nuclear binding is still occurring long after the initial peak of binding. This would suggest that the binding of estradiol to nuclei is a dynamic process, with estradiol dissociating and reassociating with the nuclei. This interpretation was encouraged by examination of a logarithmic plot of the uptake and retention of \(^{3}H\)-estradiol in control and MER-25 treated animals (Figure 11). The control groups deviate from the linearity which characterizes simple second-order dissociation. While the statistical interaction of time and treatment was significant for all three tissues, a statistical analysis of the nonlinear contribution to the control levels of estradiol over time would require additional time points. MER-25 may have replaced dissociating estradiol complexes rather than allowing the possibility of reassociation.
Figure 11. Effect of MER-25 on nuclear retention of $^3$H-estradiol. Data of animals given oil or 40 mg MER-25 two hours prior to $^3$H-estradiol and killed 2 hours (n's=4) or 12 hours (n's=6) after the estradiol, and animals given the same treatments and killed at 6 hours (n's=3) are plotted in semilogarithmic coordinates. The interaction of time and treatment is significant for all three tissues.
EFFECT OF MER-25 ON NUCLEAR RETENTION OF $^3$H-ESTRADIOL

TIME AFTER $^3$H-ESTRADIOL INJECTION, Hours

LOG$_{10}$ TISSUE/PLASMA

HTH

POA

PIT

CONTROL

MER-25
EXPERIMENT 11. MER-25 ADMINISTERED 12 HOURS PRIOR TO $^{3}_\text{H}$-ESTRADIOL.

It is possible that MER-25 failed to inhibit 2-hour uptake simply because it was not in the brain soon after its injection and that the inhibition seen at 12 hours was due to delayed competition with estradiol. To test this hypothesis, MER-25 was injected 12 hours prior to an intravenous injection of $^{3}_\text{H}$-estradiol, and 2-hour uptake was examined again. In this paradigm uptake of $^{3}_\text{H}$-estradiol at 2 hours was significantly inhibited by MER-25 in nuclei of all brain samples and whole homogenates of the hypothalamus and POA (Figure 12). Pituitary nuclear uptake was reduced by 83%, hypothalamic nuclear uptake by 41%, POA nuclear uptake by 40%, and cortical nuclear uptake by 31%. 
Figure 12. Effect of 12-hour MER-25 pretreatment on 2-hour uptake of $^3$H-estradiol. MER-25 (40 mg; n=6) or oil vehicle (n=6) was injected intraperitoneally 12 hours prior to an intravenous injection of .3 ug of $^3$H-estradiol. Animals were killed 2 hours later and radioactivity measured in whole homogenate and nuclear fractions of cortex, hypothalamus, preoptic area-septum, and pituitary.
EFFECT OF 12 HOUR MER-25 PRETREATMENT ON 2 HOUR ³H-ESTRADIOL UPTAKE

WHOLE HOMOGENATE

NUCLEAR

BRAIN, TISSUE/PLASMA

PITUITARY, TISSUE/PLASMA

CONTROL
MER-25

CTX  HTH  POA  PIT

0  10  20  30  40  50

0  100  200  300  400  500

0  500  1000  1500  2000  2500

*  **  ***
EXPERIMENT 12. DISPLACEMENT BY AN ANTIESTROGEN OF 

$^{3}$H-ESTRADIOL BOUND TO NUCLEI

A more direct test of the possibility that antiestrogens can compete with normally exchanging estradiol was to administer the antiestrogen subsequent to the peak of nuclear estradiol binding and look for "displacement" of estradiol at a later time. Peak nuclear concentration of $^{3}$H-estradiol occurs 1-2 hours after a .3 ug intravenous injection (Mowles, Ashkanazy, Mix, and Sheppard, 1971). CI-628 was injected intraperitoneally 2 hours following an intravenous injection of $^{3}$H-estradiol, and the animals were sacrificed 12 hours after the estradiol. Radioactivity retained in the brain at this time was greatly reduced by CI-628 (Figure 13). CI-628 subsequent to estradiol reduced the binding of estradiol in whole homogenate of the hypothalamus-POA by 55% and reduced hypothalamic-POA nuclear binding by 83%. 
Figure 13. Displacement of $^3$H-estradiol from the hypothalamus by a subsequent injection of an antiestrogen.

CI-628 (4 mg; n=4) or saline vehicle (n=4) was injected intraperitoneally 2 hours after an intravenous injection of .3 ug $^3$H-estradiol. Animals were killed 12 hours after the estradiol injection, and radioactivity measured in whole homogenate and nuclear fractions of cerebral cortex (CTX) and pooled hypothalamus-preoptic area-septum (HTH-POA).

* p .05, ** p .01
DISPLACEMENT OF $^{3}$H-ESTRADIOL BY A SUBSEQUENT INJECTION OF ANTIESTROGEN

![Graph showing the displacement of $^{3}$H-ESTRADIOL by a subsequent injection of antiestrogen.](image)

- **Whole Homogenate**
  - Control
  - C-1 628

- **Nuclear**
  - CTX
  - HTH-POA

![Bar graph with error bars](image)
EXPERIMENT 13. INHIBITION OF ESTRADIOL-INDUCED LORDOSIS BY ANTIESTROGENS

The inhibition of behavior paralleled the inhibition of nuclear binding of estradiol. Two-hour pretreatment with CI-628 or nafoxidine, which produced substantial reductions of nuclear estradiol levels, totally eliminated female sexual behavior induced by an intravenous injection of unesterified estradiol (Figure 14). The females were active and readily mounted by the males, but they did not display the lordosis reflex. On the other hand, MER-25 when injected 2 hours prior to the estradiol injection, failed to inhibit early uptake of estradiol and also completely failed to inhibit sexual behavior tested at 24 hours (Figure 15). There was also no significant inhibition of sexual behavior when additional groups were tested at 36 hours after the estradiol injection. When tested at 48 hours, none of the 10 MER-25 treated animals was in heat, but only 4 of 8 control animals were even marginally in heat. However, when MER-25 was injected 12 hours prior to the intravenous estradiol injection, a paradigm in which brain uptake was reduced, clear inhibition of estradiol-induced sexual receptivity was observed at 24 hours (Figure 15).
Figure 14. Inhibition of estradiol-induced female sexual behavior by antiestrogens. Ovariectomized-hysterectomized rats were given intraperitoneal injections of sesame oil (n=4), CI-628 (4 mg in saline; n=4), or nafoxidine (4 mg in oil, n=4) 2 hours prior to an intravenous injection of 3 ug of unesterified estradiol. At 19 hours after the estradiol, they were injected subcutaneously with 1 mg progesterone, and at 24 hours tested for sexual receptivity with a vigorous male. The rating system is after Powers and Valenstein (1972).
INHIBITION OF ESTRADIOL-INDUCED SEXUAL BEHAVIOR BY ANTIESTROGENS

![Graph showing inhibition of estradiol-induced sexual behavior by antiestrogens.](image)

- Mean lordosis rating
- Control, CI-628, Nafoxidine
- ** indicates significant difference
Figure 15. Effect of antiestrogen MER-25 on estradiol-induced female sexual receptivity. Intraperitoneal injections of oil vehicle or MER-25 (40 mg in oil) were administered either 2 hours or 12 hours prior to an intravenous injection of 3 μg of unesterified estradiol. Females were tested with males at either 24 hours or 36 hours after the estradiol injection; 1 mg progesterone was injected subcutaneously 5 hours prior to testing. Oil or MER-25 injected 2 hours prior to estradiol, for 24-hour test n's=3 and 4; for 36-hour test, n's=13 and 14; oil or MER-25 injected 12 hours prior to estradiol, n's=7 and 7. **p < .01
EFFECT OF ANTIESTROGEN ON ESTRADIOL-INDUCED RECEPTIVITY

2.0

MER-25 2 HOURS BEFORE ESTRADIOL

OIL

MER-25

1.0

24 HOUR TEST 36 HOUR TEST

0

24 HOUR TEST

* *
DISCUSSION

These results clearly establish that antiestrogens are capable of inhibiting $^3$H-estradiol uptake in the hypothalamus and preoptic area of the rat brain. With CI-628, inhibition was most dramatic in the tissues with the highest uptake: pituitary POA hypothalamus cortex. Competition by unlabelled estradiol is also most evident in tissues with highest uptake (McEwen and Pfaff, 1970). The inhibition of $^3$H-estradiol uptake by a prior injection of CI-628 corroborates the finding of Chazal et al. (1975). In the present study inhibition of the low levels of uptake in the cerebral cortex was also found. This finding is consistent with the previously demonstrated reduction of cortical nuclear $^3$H-estradiol binding by a prior injection of unlabelled estradiol (Zigmond and McEwen, 1970; Roy and Wade, unpublished) as well as autoradiographic evidence that some $^3$H-estradiol binding occurs in this "non-target" tissue (Pfaff and Keiner, 1973).

Nafoxidine and MER-25 are also capable of inhibiting the uptake of $^3$H-estradiol into the hypothalamus and preoptic area. Nafoxidine is less effective than CI-628, but this may reflect the difference in injection vehicle (sesame oil vs. saline) rather than a substantial difference in affinity for neural estrogen receptors. The failure of MER-25 to inhibit 2-hour uptake when given 2 hours before $^3$H-estradiol is probably due to solubility characteristics.
of MER-25. MER-25 is not soluble in physiological saline, whereas CI-628 and nafoxidine are. However, when MER-25 (40 mg) is given adequate time to reach the brain (12 hours), it inhibits 2-hour uptake of $^3$H-estradiol to the same degree as nafoxidine (4 mg) (Figure 1 cf. Figure 4). This delay in the inhibition of brain uptake of $^3$H-estradiol by MER-25 is relevant to the interpretation of experiments intended to establish the time-course of the effects of estradiol by using this antiestrogen (Shirley, Wolinsky, and Schwartz, 1968).

The finding that an injection of CI-628 2 hours after an injection of $^3$H-estradiol reduces the content of radioactivity in the hypothalamus-POA 10 hours later is at variance with an earlier report by Whalen and Gorzalka (1973). These authors demonstrated that CI-628 subsequent to an intravenous estradiol injection is capable of inhibiting female sexual behavior, but they did not find significant inhibition of hypothalamic $^3$H-estradiol uptake. We find a 55% reduction of whole homogenate radioactivity and a 83% reduction of nuclear radioactivity in the hypothalamus-POA. This discrepancy is probably due to differences in dosage: Whalen and Gorzalka used a lower dose of CI-628 for their uptake study (100 ug) than for their behavioral study (1 mg.). This was rationalized on the grounds that the estradiol dose was also lowered, from 4 ug to .3 ug. However, an intravenous dose of .3 ug of estradiol or a subcutaneous injection of .5 ug of estradiol more than half-
saturates nuclear binding in the hypothalamus (Anderson, Peck, and Clark, 1973; Roy and Wade, unpublished), so lowering the estradiol dose from 4 ug to .3 ug probably does not lower the estradiol-receptor complex concentration by 10-fold. We used the same dose for behavioral and uptake studies, 4 mg, which was higher than the doses used by Whalen and Borzalka.

The greater inhibition of 12 hour retention than 2 hour uptake by both MER-25 and nafoxidine probably also reflects displacement of estradiol by the antiestrogens. Therefore, it is likely that the brain cells which concentrate estradiol do not differ in this respect from cells of the anterior pituitary and uterus in which antiestrogens are capable of displacing bound estradiol (Callantine, Clemens, and Shih, 1968; Whalen and Gorzalka, 1973). It is also likely that unlabelled estradiol would be capable of displacing bound \(^3\text{H}\)-estradiol in the brain as it does in the uterus (DeHertogh, Ekka, Vanderheyden, and Hoet, 1971).

The functional significance of the low levels of bound \(^3\text{H}\)-estradiol at long time intervals after injection has not been clearly established or discounted. The RNA synthesis inhibitor, actinomycin D, has been used to suggest a role of genetic transcription in estradiol induction of sexual behavior and positive feedback on LH release, and it effectively inhibits these responses when given 12-13 hours after estradiol (Jackson, 1973; Whalen, Gorzalka, DeBold, Quadagno, Ho, and Hough, 1974). CI-628 given up to 6 hours after
estradiol completely blocks the induction of lordosis, and some inhibition of lordosis is produced by injections as late as 18 hours after estradiol, or 5 hours before testing (Whalen and Gorzalka, 1973). Blaustein and Wade (unpublished) have recently found that the period of estrous conditioning produced by estradiol benzoate (EB) can be shortened by CI-628 administered after the onset of sexual receptivity. Thus, while the interval from the initial uptake of estradiol to the appearance of its behavioral effects has been reasonably well characterized (Green, Luttge, and Whalen, 1970), the duration of estradiol's effects after it is removed from brain cell nuclei needs further clarification. It is possible that the low levels which are retained in nuclei for at least 24 hours after estradiol injections (McEwen, Pfaff, Chaptal, and Luine, 1975) are exerting significant effects for most of that time.

A comparison of the behavioral data with the uptake studies shows that procedures which inhibit behavior also inhibit brain uptake of $^3$H-estradiol. Both CI-628 and nafoxidine produce inhibition of sexual receptivity as well as substantial reduction of brain $^3$H-estradiol when injected 2 hours prior to estradiol. CI-628 injected subsequent to an $^3$H-estradiol injection reduces brain $^3$H-estradiol retention, and others have demonstrated that CI-628 can inhibit sexual behavior when injected subsequent to estradiol (Whalen and Gorzalka, 1973) or EB (Blaustein and Wade, unpublished). MER-25 injected 12 hours prior to estradiol
inhibits both 2-hour uptake and sexual behavior. MER-25 injected 2 hours prior to estradiol inhibits neither the early uptake of $^3$H-estradiol nor estradiol-induced sexual behavior. Although the 12-hour retention of $^3$H-estradiol was inhibited by MER-25, this delayed inhibitory action was apparently not sufficient to antagonize the higher dose of estradiol used in the behavioral studies (3 ug cf. .3 ug).

The behavioral actions of the antiestrogens are probably due to specific interactions with estrogen-sensitive tissues rather than toxic effects of the drugs. The dose of MER-25 used in this study fails to produce a learned aversion (Experiment 5), an objective index of toxicity.

The demonstration that antiestrogens are capable of competing with estradiol for brain estradiol receptors is only a first step in determining the mechanism by which they exert their inhibition of estradiol-induced sexual behavior. The reasons that this presumed antiestrogen-receptor complex is less effective than the estradiol-receptor complex remain to be determined. One hypothesis (Capony, and Rochefort, 1975; Clark and Peck, 1976) of the inhibitory action of antiestrogens in the uterus suggests that antiestrogens are capable of binding to cytosol receptors and are fully capable of translocating them to the nucleus in order to produce an initial estrogenic response. However, the antiestrogen-receptor complex fails to cause the replenishment of the cytosol receptors which is required for a full
estrogenic response, or causes only a delayed replenishment. A delay of replenishment has also been suggested as a mechanism of antagonism for behavioral responses (Whalen, Martin, and Olsen, 1975). This hypothesis as well as others (Katzenellenbogen, and Ferguson, 1975; Ruh and Ruh, 1974) presupposes competition of the antiestrogen and estrogen for the estrogen receptors. Beyond this competition, demonstrated here and elsewhere, there is at present little evidence to support any particular hypothesis of antiestrogens' actions in the central nervous system.

Some experiments which have provided evidence that a failure of replenishment is a mechanism of antagonism in the uterus have not produced analogous results when transferred to behavioral paradigms. For example, when an antiestrogen and estrogen are given concurrently, no inhibition of the estrogen's effect on uterine weight is seen at 24 hours (Clark, Peck, and Anderson, 1974; Lerner, Holthaus, and Thompson, 1958), but in a similar paradigm sexual behavior can be totally inhibited. Also CI-628 and nafoxididine by themselves produce an initial estrogenic response on uterine weight and morphology (Clark, Peck, and Anderson, 1974; Kang, Anderson, and DeSombre, 1975), whereas there is no evidence of an estrogenic effect of these antiestrogens for normal female sexual behavior in rats (Arai and Gorski, 1968; Meyerson and Lindstrom, 1968; Powers, 1975; Ross, Shryne, Gorski, and Marshall, 1973; Sodersten, 1974). It should be noted that some estrogenic
central responses to antiestrogens have been reported: MER-25 and CI-628 have estrogenic central effects on the regulation of eating behavior (PART I); MER-25 acts as an estrogen on male copulatory behavior (Baum and Vreeburg, 1976); CI-628 produces estrogenic responses on certain hypothalamic enzyme activities (Luine, Wallach, and McEwen, 1975); and cis-clomiphene can facilitate or inhibit sexual behavior in female guinea pigs depending on the experimental paradigm (Walker and Feder, in preparation). Therefore, the subcellular actions of antiestrogens that allow them to inhibit estradiol-induced female sexual behavior are likely to be partially estrogenic actions. Characterization of the partial actions of the antiestrogens may suggest which aspects of natural estrogen action are important in the induction of sexual behavior, as well as which aspects are important in the effects of estrogens on eating. The results presented here are not inconsistent with a receptor-mediated nuclear action of estradiol to induce sexual receptivity, but actual support for this notion will require assays of receptor dynamics and other responses with and without antiestrogen involvement.

Antiestrogens may thus be useful in two ways: First, for sexual behavior and other responses which antiestrogens inhibit, an analysis of subcellular responses which antiestrogens prevent may tentatively link the estrogenic induction of a behavior to certain subcellular responses.
For example, if antiestrogens prevented the nuclear localization of receptors, it would suggest, though not establish, that nuclear localization of the receptors which antiestrogens do elicit might be involved in the behavioral effects that antiestrogens induce. Thus the antiestrogens are actually partial estrogens and partial antiestrogens, depending on the behavior observed, and these estrogenic and estrogen-antagonizing behavioral effects may be correlated with subcellular responses.
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