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THE ROLE OF HYPOTHALAMIC PROGESTIN RECEPTORS IN THE
ACTIVATION AND MAINTENANCE OF SEXUAL RECEPTIVITY IN FEMALE
RATS AND GUINEA PIGS

Iowa State University

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The role of hypothalamic progestin receptors in the activation and maintenance of sexual receptivity in female rats and guinea pigs

by

Theodore J. Brown

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of the Requirements for the Degree of DOCTOR OF PHILOSOPHY

Major: Zoology

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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>GENERAL INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>SECTION I. INHIBITION OF SEXUAL BEHAVIOR IN FEMALE GUINEA PIGS BY A PROGESTIN RECEPTOR ANTAGONIST</td>
<td>27</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>29</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>30</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>32</td>
</tr>
<tr>
<td>RESULTS</td>
<td>37</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>48</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>50</td>
</tr>
<tr>
<td>SECTION II. 1-(o-CHLOROPHENYL)-1(o-CHLOROPHENYL) 2,2,2-TRICHLOROETHANE INDUCES FUNCTIONAL PROGESTIN RECEPTORS IN THE RAT HYPOTHALAMUS AND PITUITARY GLAND</td>
<td>52</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>54</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>55</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>57</td>
</tr>
<tr>
<td>RESULTS</td>
<td>63</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>79</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>83</td>
</tr>
<tr>
<td>SECTION III. SUPPLEMENTAL PROGESTERONE DELAYS HEAT TERMINATION AND THE LOSS OF PROGESTIN RECEPTORS FROM HYPOTHALAMIC CELL NUCLEI IN FEMALE GUINEA PIGS</td>
<td>87</td>
</tr>
</tbody>
</table>
GENERAL INTRODUCTION

During the rodent estrous cycle, a period of sexual behavior of limited duration is exhibited by the female around the time of ovulation (12, 93, 182-184). The occurrence of this period of sexual behavior is most often characterized by the presence of the lordosis posture in response to genital stimulation by the male. Although this posture varies slightly among species, it consists of a general immobilization and a pronounced dorsiflexion of the spine. By assuming this posture, the female greatly increases the chance of successful copulation occurring. In addition to lordosis, or receptive behavior, proceptive behaviors are displayed during this period. These behaviors, in the rat, include darting and hopping, rapid ear vibration, and approach to and withdrawal from the male (48, 97). The exhibition of these behaviors is thought to increase the attractiveness of the female to the male, thereby increasing the likelihood that mounting will be attempted (7, 89).

Hormonal Control of Sexual Behavior

The occurrence of the period of sexual behavior (heat) in many rodent species, including rats and guinea pigs, is regulated by the sequential secretion of the ovarian hormones, estradiol and progesterone. Surgical removal of the ovaries prevents the appearance of the behavior while treatment with estradiol and progesterone restores it (24, 34, 38, 45, 47). Although estradiol treatment alone can
activate sexual receptivity in ovariectomized (OVX) animals, progesterone given 18 - 60 hours after estradiol has a facilitory effect (63, 143). This facilitation may be manifest as a decrease in latency from estradiol administration to the onset of lordosis, as an increase in the intensity of lordosis, as an increase in the appearance of proceptive behaviors, and as a reduction in the amount of estradiol required to induce lordosis (24, 63, 143, 175). Progesterone administered in the absence of prior estrogen treatment has no effect on sexual behavior (11, 63).

The importance of progesterone in the regulation of sexual behavior is well-established. Myers et al. (125) first demonstrated in the mid-1930s that the onset of sexual behavior in estrous-cycling female guinea pigs coincided with the beginning of the preovulatory growth phase of the ripening ovarian follicle. Prior to this study, it was thought that sexual behavior resulted from the actions of estrogens alone (3); however, because preovulatory growth of the ovarian follicle was known to be triggered by luteinizing hormone (LH) (66), this study raised the possibility that a second factor was involved.

Dempsey et al. (45), were the first to suggest that this second factor was progesterone. This was based on their finding that LH activated lordosis only when injected during the stages of the cycle when ripening ovarian follicles capable of ovulation were present. In comparison, progesterone was effective even when injected at earlier stages of the cycle. In addition, progesterone administered to estrogen-pretreated OVX guinea pigs facilitated lordosis, whereas no effect was observed with LH injection. Dempsey and coworkers concluded
that sexual behavior required the action of progesterone which is released from the preovulatory follicle following stimulation by LH. However, because progesterone was thought at that time to be released only by the corpus luteum, this conclusion was at first largely ignored by some workers in the field (8). Later studies in which progesterone concentrations were measured in serum obtained from general circulation (30, 39, 62, 64, 163) and in serum obtained from the ovarian vein (81), confirmed the preovulatory release of progesterone. Furthermore, this release of progesterone has been shown to just precede the onset of sexual receptivity in both rats (62, 163) and guinea pigs (64). The source of this preovulatory progesterone is most likely not corpora lutea from preceding ovulations. Feder and Goy (61) have recently reported that in prepubertal guinea pigs, administration of estradiol followed by injection of either LH or follicle-stimulating hormone resulted in the activation of lordosis. This treatment also resulted in increased circulating progesterone levels just prior to lordosis onset, with no luteinization of unruptured follicles.

Further evidence that progesterone is involved in the activation of sexual behavior is provided by the finding that female guinea pigs (83) and rats (5, 140) do not become sexually receptive when deprived of preovulatory progesterone by ovariectomy just prior to the onset of sexual receptivity. Progesterone, exogenously administered to these animals, restored the period of receptivity. Injection of supplemental estradiol, however, had no effect; therefore, acute ovariectomy in these studies prevented the onset of sexual receptivity by acting to limit progesterone and not estradiol.
In addition to its facilitory effects, progesterone has also been shown to have an inhibitory effect on sexual receptivity (63, 71, 99, 114). Following the termination of the period of sexual receptivity in intact estrous-cycling guinea pigs, injection of progesterone or sequential injection of estradiol and progesterone does not result in the activation of a second receptive period (45, 185, 187). Soy et al. (71) were the first to suggest that this refractoriness was the result of progesterone action. They found that sequential injection of estradiol and progesterone in guinea pigs during various stages of the estrous cycle was less effective in activating sexual receptivity when a functional corpus luteum was present.

Similar inhibitory effects have been obtained in OVX animals. Following the termination of sexual receptivity in hormonally-treated OVX guinea pigs, a second injection of progesterone does not result in the activation of a second receptive period (63, 114). Similar findings have been reported for mice (47), hamsters (34), and rats (22, 92, 99, 126, 186). This inhibition has been termed sequential inhibition (142), because it occurs subsequent to the period of estrogen priming. In a similar fashion, progesterone treatment initiated at the time of estradiol treatment inhibits the ability of a subsequent progesterone stimulus to facilitate a period of sexual behavior (11, 21, 75, 174). This type of inhibition has been termed concurrent inhibition because the inhibitory effects occur during the estrogen priming period (142).

The sequential inhibitory effects of progesterone are largely determined by the initial dose of progesterone (26, 27). In rats, a greater dose of progesterone is required to cause sequential inhibition
than is required to facilitate sexual receptivity (49). The opposite relationship occurs in guinea pigs (119). In both species, sequential inhibition, as well as concurrent inhibition, can be overcome with a greater dose of progesterone (14, 75). In addition, supplemental injection of estradiol at the time of the initial injection of progesterone restores the ability of a subsequent progesterone injection to facilitate sexual behavior (22, 82, 126, 160, 185). These findings suggest that the inhibition is actually the result of a decreased sensitivity to progesterone.

Neural Site of Hormonal Control of Sexual Receptivity

The identification of the principal neural sites for the activation of lordosis by estradiol and progesterone was highly controversial until fairly recently. The methods used to elucidate these sites were primarily steroid autoradiography, used to identify regions capable of sequestering hormone, and hormone implantation, used to identify groups of cells capable of responding to the hormone (6).

Results of estradiol benzoate implantation into rat brain implicate both the anterior hypothalamus-preoptic area (AH-POA) and ventromedial nucleus of the hypothalamus (VMN) as the primary sites for estrogens effects on lordosis (91, 181). A similar finding has been reported in guinea pigs (118).

Davis et al. (41, 42), and Rubin and Barfield (155), reported that estradiol implants are most effective in priming OVX rats to exhibit lordosis when placed into the VMN. These studies were conducted
with estradiol diluted 1:250 with cholesterol in order to minimize the spread of estradiol, and thereby achieve a more concise localization. In addition, this manipulation allowed for the specific identification of sites where estradiol had a priming effect on lordosis (i.e., required subsequent progesterone treatment), rather than an activational effect. The results obtained in these studies indicated that such implants placed in the VMN, but not the AH-POA, permitted a sufficient priming effect to allow progesterone-facilitated lordosis to occur.

In support of the VMN as the site of estrogen action, steroid autoradiography (136, 168) and immunohistochemistry (121) have shown that the VMN contains neurons capable of accumulating estradiol. In addition, biochemical assay of microdissected VMN has confirmed that the VMN contains high-affinity estrogen binding sites (147).

A variety of neural sites for progesterone actions on lordosis has been implicated by progesterone implant studies in the rat and guinea pig brain. These include the mediobasal hypothalamus (116, 141), caudate putamen (181), interpeduncular nucleus (94), ventral tegmental area (94), arcuate nucleus (116), and the midbrain reticular formation (181). The discrepancy among these studies may be due to the use of high priming doses of estradiol, or to diffusion of progesterone to sites distant from that of the implant. In a carefully controlled study, Rubin and Barfield (156) found that only implants placed bilaterally into the VMN of estradiol-primed OVX rats were able to facilitate lordosis. Cannulae placements in the preoptic area, midbrain reticular formation-interpeduncular nucleus, or midbrain central gray were ineffective. In addition, the facilitation was independent of the
adrenal gland, and therefore, not the result of an effect on the adrenal-pituitary axis to increase adrenal secretion of progesterone.

Autoradiography of progestin uptake in estrogen-pretreated rat (177) and hamster brain (124) demonstrates that the VMN contains cells capable of accumulating progestins. In addition, biochemical assay of microdissected VMN has confirmed that the VMN contains a high concentration of high-affinity estrogen-dependent progestin binding sites (132).

In further support of the importance of the VMN in the hormonal activation of sexual receptivity, Mathews and Edwards (102, 103) have shown that electrolytic lesions of the VMN block the hormonal activation of lordosis. In addition, Pfeifle et al. (138) reported that bilateral parasagittal knife cuts lateral to the VMN prevent progesterone-facilitated sexual receptivity. Furthermore, electrical stimulation of the VMN facilitates lordosis in estrogen-pretreated animals (137). Nevertheless, the VMN may not be the only site where progesterone’s effects on receptivity are manifest, although it appears to be the principal site.

Neural sites within the MBH have also been identified as the site for the inhibitory effect of progesterone on sexual behavior (19, 100, 157). Rubin and Barfield (157) reported that bilateral progesterone implants placed in the rat VMN first facilitate sexual receptivity and subsequently result in the animal becoming refractory to a systemic injection of progesterone. Progesterone implants placed in the preoptic area or in various parts of the midbrain neither facilitated lordosis nor caused refractoriness to further progesterone stimulation,
indicating that VMN stimulation is necessary for the inhibitory effect of progesterone to occur. Furthermore, these studies demonstrate a common neural site in the rat for progesterone's dual effects on lordosis.

In contrast, the neural site for the inhibitory effect of progesterone in guinea pigs is thought to be neuroanatomically distinct from the facilitory site (116, 117). Morin and Feder (117) reported that progesterone implants placed bilaterally near the zona compacta of the midbrain substantia nigra failed to facilitate sexual receptivity in estradiol-pretreated OVX guinea pigs, yet prevented subsequent systemically-injected progesterone from facilitating the behavior. Furthermore, implants in the MBH which facilitated lordosis failed to inhibit the actions of subsequent progesterone (116). However, closer examination of the data presented in this study has yielded opposite conclusions (116).

In their study, Morin and Feder (116) injected progesterone systemically 8 hours after intrahypothalamic placement of progesterone implants. No difference in heat duration was noted as compared to animals that did not receive the progesterone injection. This was taken as evidence that the progesterone implant did not inhibit the effect of the systemically-injected progesterone. However, in the absence of the progesterone implant, the progesterone injection would have been expected to result in prolonged heat duration. Because the full expected effects of the systemic progesterone injection did not occur, these results could be interpreted as evidence that progesterone placed in the MBH exerts an inhibitory influence on lordosis.
In a second experiment, Morin and Feder (116) injected progesterone into animals that had not responded by 8 hours after intrahypothalamic placement of progesterone or cholesterol. Although 91% of the cholesterol-implanted animals responded to this injection, only 65% of the progesterone-implanted animals responded. The progesterone-implanted animals also showed a trend toward shorter heat durations as compared to cholesterol-implanted animals. When the scores of the non-responsive animals (heat duration = 0 hours) were factored into the analysis, a statistically significant decrease in heat duration by progesterone implants was found (16).

From a third experiment, Morin and Feder (116) concluded that progesterone implants placed in the MBH failed to inhibit responsiveness to a progesterone injection administered 24 hours after implantation. This was based on the finding that a similar percentage of cholesterol-implanted and progesterone-implanted guinea pigs responded to the progesterone injection (33% and 38% respectively). However, when compared with 100% of non-implanted animals that responded to the injection, the inhibitory effect of the cannulation is apparent. The problem lies in the fact that either cannulation alone or cholesterol exposure had an inhibitory effect which may have masked the effect of the implanted progesterone. Therefore, based on this experiment, it cannot be concluded that implants placed in the MBH fail to inhibit responsiveness to additional progesterone.

Taken together, the reanalysis of the work of Morin and Feder (116) agrees with work done in the rat, showing that both the facilitory and inhibitory effects of progesterone occur within the MBH. It has not
yet been possible to determine if these effects are due to actions within the same cell.

Cellular Mechanisms of Hormonal Control of Sexual Receptivity

The activation of sexual receptivity by estradiol and progesterone is thought to be mediated by intracellular, steroid-specific hormone receptors. High-affinity, estrogen-specific intracellular estrogen binding sites have been demonstrated in several brain regions, including the MBH (68, 121, 147). Following injection of estradiol, the concentration of these binding sites, or receptors, decreases as the concentration in the cell nuclear compartment increases (90, 154). Based primarily on studies of estrogen action in peripheral tissues, it is thought that as a result of increased nuclear binding of estradiol-estrogen receptor complexes, specific RNA-dependent alterations in protein synthesis occurs (73, 104). In support of this, Rainbow et al. (144) reported that administration of a protein synthesis inhibitor, anisomycin, near the time of estradiol treatment, inhibited the activation of sexual receptivity in response to a progesterone injection 24 hours later. In addition, antiestrogens, which act to decrease nuclear estrogen accumulation and retention (55, 87, 170), block estradiol's effects on sexual receptivity (55, 87, 88, 153, 170, 171).

One result of the action of estradiol in the brain is the induction of intracellular progestin binding sites. These binding sites are of high affinity (Kd = 0.3 nM) and high specificity, and are
susceptible to degradation by pronase (19, 95, 96, 111, 132).
Estradiol-induced increases in these binding sites, or receptors, have been demonstrated in the hypothalamus, pituitary gland, preoptic area, and midbrain, but not in the cortical amygdala, hippocampus, or cerebral cortex (19, 95, 111, 132). This increase in progestin receptor levels can be blocked by antiestrogen treatment (55, 153), or by administration of the protein synthesis inhibitor, anisomycin (144), suggesting the involvement of a genomic mechanism.

The induction of progestin receptors by estradiol is dose dependent. Doses of estradiol that are sufficient to prime OVX rats (112, 133) or guinea pigs (19) to respond to progesterone are sufficient to increase hypothalamic intracellular progestin receptor levels. Furthermore, progestin receptor induction is temporally linked to the estrogen priming period (19, 60, 112, 131, 133). Depending on the dose of estradiol, an interval of 18 to 60 hours between estradiol and progesterone treatment results in maximal activation of lordosis (63, 143). An interval of less than 18 h is insufficient to allow progesterone facilitation to occur. In agreement with this, Blaustein and Feder (19), and Parsons et al. (131, 133), have shown that estradiol induction of progestin receptors is not detectable before 12 to 18 h after estradiol injection.

Blaustein and Feder (20), found that subcutaneous injection of progesterone in OVX estradiol-pretreated guinea pigs, resulted in a transient increase in progestin receptor levels in hypothalamic nuclear fraction as measured by an in vitro exchange assay. Concurrent with this increase, cytosol progestin receptor levels decreased. The
transient increase in nuclear progestin receptor levels paralleled the concentration of progesterone measured in serum, and temporally correlated with the display of sexual receptivity. Nuclear receptor levels increased prior to the initiation of sexual receptivity, and decreased as the behavior dissipated (12 - 24 hours after progesterone injection). A similar finding has been reported by McGinnis et al. (106), following intravenous injection of progesterone to estradiol-pretreated rats.

Hypothalamic estrogen and progestin receptor levels have been measured during the estrous cycle of the rat (107, 146). Changes in nuclear estrogen receptor concentrations during the cycle parallel changes in serum estradiol levels; receptor concentrations are low during estrus and diestrus I, but begin to increase on diestrus II (metestrus). Peak levels of nuclear estrogen receptors are observed at proestrus. In a similar pattern, the concentration of cytosol progestin receptors are at their lowest level during diestrus I. These levels begin to increase on diestrus II, and attain peak levels on the morning of proestrus, just prior to the preovulatory release of progesterone (107). Nuclear progestin receptor concentrations, which are low during other stages of the cycle, increase dramatically on the afternoon of proestrus, just prior to the onset of sexual behavior (146). These patterns of receptor concentration fluctuations provide further support that intracellular steroid hormone receptors are involved in the activation of sexual behavior.

In support of the contention that progesterone acts through a genomic mechanism to facilitate the onset of sexual receptivity, Rainbow
et al. (144) reported that injection of anisomycin around the time of progesterone injection prevented the initiation of sexual receptivity. This effect was most likely not due to debilitation of the animals since injection of anisomycin to animals in estrogen-induced heat had no effect on lordosis. In a later study, Rainbow et al. (145) found that anisomycin applied directly to the VMN prevented progesterone's facilitory effects on lordosis. A similar finding was reported by Glaser and Barfield (69), who also demonstrated that this effect was specific for the VMN. Anisomycin placed in the preoptic area or in the midbrain in the region of the interpeduncular nucleus had no effect on sexual behavior. In addition to demonstrating that progesterone facilitation of lordosis is protein synthesis-dependent, these studies provide further evidence that the VMN is the principal site for progesterone's effects on lordosis.

Following the injection of progesterone, cytosol progestin receptor levels decrease and are not replenished as the concentration of nuclear progestin receptors decreases (13, 19, 112). This has led to the hypothesis that progesterone inhibition is, in effect, a hyposensitivity to progesterone caused by a progesterone-induced decrease in the availability of progestin receptors (19, 112, 159). In support of this hypothesis, Moguilewsky and Raynaud (112), and Blaustein and Feder (19), have reported that progesterone administered at the time of estradiol (concurrent inhibition), almost completely blocked the induction of cytosol progestin receptors by estradiol. In addition, in the sequential inhibition paradigm, a second behaviorally-ineffective injection of progesterone 24 hours after the initial injection only
slightly increased the concentration of nuclear progestin receptors (14, 20). A larger dose of progesterone, one that is capable of overcoming the inhibition, increased nuclear progestin receptor levels by 41% over what was measured following the lower progesterone dose (14). Likewise, injection of supplemental estradiol at the time of the initial progesterone injection, a treatment that restores sensitivity to subsequent progesterone, allowed a 107% greater increase in nuclear progestin receptor accumulation in response to a second injection of progesterone 24 hours later (13). These studies suggest that progesterone decreases sensitivity to itself by decreasing the availability of progestin receptors, and that supplemental estradiol restores sensitivity by increasing intracellular progestin receptor levels.

Although the studies discussed above support the hypothesis that a downregulation in the concentration of intracellular progestin receptors is involved in mediating the effects of progesterone on sexual behavior, a report by Parsons and McEwen (129), contradicts this evidence. In this study, Parsons and McEwen extended the findings of an earlier report by Wallen et al. (176), showing that a protein synthesis inhibitor can prevent the occurrence of the refractory period in guinea pigs. Parsons and McEwen (129) reported that injection of anisomycin, 3 to 9 hours after injection of progesterone blocked the occurrence of a refractory period to progesterone in OVX estradiol-pretreated rats. Twenty-four hours after the initial injection of progesterone, cytosol progestin receptor levels were decreased; however, a second injection of progesterone at this time appeared to have facilitated sexual
receptivity. Unfortunately, animals were not tested prior to the second injection of progesterone to verify that they were not still responsive from the initial progesterone exposure. Blaustein et al. (17) found no effect of protein synthesis inhibition on the refractory period to progesterone; however, with a procedure similar to that used by Parsons and McEwen (129), they found that anisomycin delayed the termination of the period of sexual receptivity induced by the first progesterone injection. It is possible that in the study by Parsons and McEwen, rats were still receptive at the time of behavioral testing from the first progesterone treatment, rather than receptive from the second progesterone treatment. Therefore, the results obtained by Parsons and McEwen (129), do not necessarily contradict the hypothesis that intracellular progestin receptors are involved in mediating progesterone action on sexual behavior.

It should be noted that in the earlier study by Wallen et al. (176), a slightly different treatment sequence was used. OVX estradiol-pretreated guinea pigs were treated with cycloheximide, a protein synthesis inhibitor, one hour before or one hour after receiving progesterone. Animals were tested for lordosis, and 8 hours after progesterone injection, a second sequence of estradiol followed 36 hours later by progesterone was initiated. Animals were tested again for lordosis following the second progesterone injection. Based on the finding that more animals that had received cycloheximide were receptive during the second test, as compared to non-cycloheximide treated animals, the authors concluded that progesterone-induced inhibition was protein synthesis-dependent. However, the results of this experiment
are difficult to interpret because latency to lordosis following the second injection of progesterone was not reported. It is possible that prior cycloheximide treatment increased the sensitivity to estradiol, or in some other progesterone-independent manner activated sexual receptivity. As in the case of the study by Parsons and McEwen (129), animals must be tested prior to progesterone in order to verify that they are, in fact, responding to that injection of progesterone. Furthermore, Shivers et al. (160) reported that in rats, the RNA synthesis inhibitor, actinomycin D, infused into the third ventricle had no effect on the refractoriness to progesterone induced by the sequential inhibition paradigm.

The mechanism by which progesterone induces down regulation of its own receptors is not fully understood. Zucker (185, 187) first hypothesized, based on behavioral data alone, that progesterone acted to prevent estradiol "from acting at the neural sites that mediate receptivity." It is conceivable that by preventing estradiol action in the hypothalamus, progesterone could prevent further induction of intracellular progestin receptors. However, a number of studies have shown that progesterone does not affect accumulation or retention of \(^{3}H\)estradiol (21, 43, 98, 172), depletion or repletion of cytosol estrogen receptors following estradiol treatment (43, 134), or the binding of estradiol to hypothalamic estrogen receptors (50). A recent report, however, indicates that progesterone under certain conditions, has an inhibitory influence on hypothalamic estrogen receptor levels (15). In OVX rats treated chronically with estradiol, progesterone administration resulted in a decreased level of nuclear estrogen
receptors as measured 24 hours after progesterone injection. At an earlier time after injection (6 hours), a decrease in cytosol estrogen receptors was observed; therefore, this decrease in nuclear receptor levels was most likely the result of a decreased availability of cytosol receptors. It seems unlikely that such a mechanism is responsible for progesterone's inhibitory effect, since a high dose of progesterone overcomes the inhibition rather than exacerbates it. Furthermore, it is uncertain if nuclear estrogen receptor occupation at the time of progesterone injection is required for sexual behavior to occur.

In a study examining the time course of retention of \((^3\text{H})\text{estradiol}\) in hypothalamic cell nuclei following subcutaneous estradiol injection, Walker and Feder (172), found that nuclear accumulation of estradiol peaked at 11 hours following injection, and remained elevated at this level until 43 hours after injection. Progesterone injection 39 hours after estradiol injection did not alter this retention. In a later study, Walker and Feder (173) found that injection of an antiestrogen 40 or 47 hours after estradiol treatment inhibited sexual behavior in response to progesterone injected 60 hours after estradiol treatment. These experiments raised the possibility that estradiol had a maintenance effect on the activation of sexual behavior. That is, estradiol had to be present in hypothalamic cell nuclei at the time of onset of sexual receptivity.

Blaustein et al. (18) measured the retention of \((^3\text{H})\text{estradiol}\) in rat hypothalamic cell nuclei following a single intravenous injection of 0.3 µg of estradiol. This dose of estradiol can provide sufficient priming action to allow an injection of progesterone 18 hours later to
facilitate sexual receptivity. Twenty-four hours after the injection, a small but statistically significant amount of (\(^{3}H\))estradiol was still present in hypothalamic cell nuclei. This small amount could be displaced by antiestrogen treatment, indicating that it was specifically-bound estradiol. Furthermore, injection of either CI-628 or nafoxidine, along with progesterone, inhibited the onset of sexual receptivity. Taken together, these studies provide good evidence that estradiol must be present in the cell nucleus at the time of onset of sexual behavior.

In contrast, McEwen et al. (105) reported no reliably detectable amount of (\(^{3}H\))estradiol present in hypothalamic cell nuclei 24 hours following intraperitoneal injection of 10 \(\mu\)g estradiol to OVX rats. In their study, nuclear-bound radioactivity declined substantially between 12 and 24 hours after injection. At 24 hours after injection, levels of radioactivity that were only twice background levels were present. Because, by definition, these levels are at the limit of the sensitivity of their assay, the authors concluded that little, if any, estradiol is retained by cell nuclei, and that the behavioral effects of estradiol on sexual receptivity do not require occupation of nuclear binding sites by estradiol. In support of their conclusions, Morin et al. (120) reported that the antiestrogen MER-25 injected 2 to 12 hours before progesterone did not prevent the onset of sexual receptivity.

Recent studies indicating that short, discrete exposure to estradiol is sufficient to provide sufficient priming action for the activation of lordosis by progesterone (35, 112, 130, 164, 180), and to increase the concentration of cytosol progestin receptors (84, 169), may
also suggest that long-term estradiol retention may not be necessary for the activation of lordosis. However, the question of nuclear estradiol retention following these treatments has not yet been addressed. Unfortunately, the question of the role of long-term retention of nuclear estradiol on sexual receptivity, and on the refractory period to progesterone, remains unresolved.

In addition to inducing the formation of intracellular progestin receptors, estrogen action in the hypothalamus also results in a number of changes in neuronal ultrastructure. Carrer and Aoki (33) found that following ovariectomy in the rat, many cells of the VMN contained a large, globular, oval nucleus, and a scarcity of cytoplasm. Other cells appeared unaffected by hormonal depletion. Following estrogen treatment, significant changes were noted in many of the cells affected by ovariectomy. Cell nuclei had become larger, more irregular in contour, and had acquired indentations in the nuclear envelope. The endoplasmic reticulum had greatly hypertrophied in these cells, with an increase in the number of attached ribosomes and polysomes. Other changes were also noted in the Golgi apparatus and mitochondria. Similar findings have also been reported by Cohen and Pfaff (37). It is possible that these described changes in ultrastructure, along with an increased level of intracellular progestin receptor levels, are necessary during the estrogen priming period for sexual receptivity to occur.

In the absence of estrogen stimulation, a low, but significant, concentration of estrogen-independent progestin receptors is present in the hypothalamus (19, 95, 111). Etgen (56, 57), has proposed that
estradiol-induced increases in intracellular progestin receptor levels are not a necessary part of the estrogen priming process for sexual receptivity to occur. This is based on the finding that when rats were primed with o,p'-DDT, a polychlorinated pesticide with estrogenic properties, rather than estradiol, progesterone was able to activate sexual behavior despite there being no apparent induction of hypothalamic progestin receptors. These data were interpreted to indicate that the low level of estrogen-independent progestin receptors could mediate progesterone's effects, or that progesterone was acting through a membrane-bound receptor system. Because these findings question the hypothesis that estrogen-induced progestin receptors are necessary for the activation of sexual receptivity by progesterone, they were reexamined in the second study presented in this dissertation.

Other researchers have also questioned the role of intracellular progestin receptors in mediating progesterone's effect on sexual behavior. This is based partly on reports that progesterone administered intravenously can facilitate lordosis by 30 minutes after injection (86, 109). This is considered by some to be too short a time period to allow for a genomic mechanism; however, steroid hormones have been shown to exert genomic effects within 20 minutes in peripheral tissues (122). Furthermore, as mentioned earlier, McGinnis et al. (98) have demonstrated that nuclear progestin receptor levels following intravenous progesterone injection increase prior to the onset of sexual receptivity. In addition, protein synthesis inhibition blocks progesterone's facilitory effect on sexual behavior (69, 144, 145).

Beyer and coworkers (9, 10, 65) have proposed that progesterone
acts at the membrane level to facilitate sexual receptivity. According to their hypothesis, progesterone acts to increase the concentration of cyclic nucleotides, thereby resulting in the activation of previously inactive, lordosis-relevant, cellular proteins. As a result of the activation and subsequent actions of these proteins, a period of sexual behavior occurs. These proteins, they suggest, may be synthesized in an inactive form in response to estrogen treatment. The evidence to support this hypothesis is scant. Dibutyryl cAMP administered to estrogen-primed OVX rats facilitated the display of sexual receptivity (10). In the absence of estrogen priming, cAMP had no effect. The degree and duration of facilitation by cAMP could be increased by administration of theophylline, a phosphodiesterase inhibitor. In addition, theophylline had the same effect on progesterone-facilitated sexual receptivity (9). In further support of this hypothesis, Towle and Sze (169) have recently demonstrated the existence of steroid-specific progestin binding sites in synaptosomes isolated from whole rat brain. However, the findings reported by Beyer and coworkers merely suggest that cyclic nucleotides play a role in the activation of sexual receptivity. They do not provide evidence that progesterone acts directly to affect these levels. It seems more likely that as a result of progesterone action, neurotransmitters are released, which in turn act through a cyclic nucleotide mechanism to activate lordosis. In support of this, catecholamines, thought to be involved in the activation of lordosis, have been shown to act by increasing cAMP levels (128). Furthermore, more research is required on the membrane-bound steroid hormone binding sites described by Towle and Sze before
In the first study of this dissertation, evidence is provided to support the hypothesis that intracellular progestin receptors are involved in the activation of sexual receptivity by estradiol and progesterone. The use of a recently developed antiprogesteron, RU 486, is employed. This synthetic steroid has been found to antagonize progesterone's action on the maintenance of the uterine endometrium (72, 78, 80), on the maintenance of pregnancy (80, 85), on the growth of progesterone-sensitive breast cancer cells (4), and on release of pituitary hormones (79). These effects are thought to be the result of RU 486 acting to prevent progesterone interaction with the intracellular progestin receptor (4, 78, 80, 139).

**Mechanism Responsible for the Termination of Sexual Receptivity**

Little is known of the mechanism by which the period of sexual receptivity terminates; however, in OVX animals, both estradiol and progesterone have been shown to influence the length of the receptive period. Moreines and Feder (113), found that shortening the exposure time of OVX guinea pigs to estradiol capsules decreased the duration of the receptive period induced by a progesterone injection. Similarly, administration of supplemental estradiol at the time of progesterone injection has been shown to result in increased heat duration (13, 38, 82). Morin and Feder (115) have shown that injections of progesterone every three hours to OVX hormonally-treated guinea pigs prolonged the period of sexual receptivity by more than two hours. Interestingly,
heat termination occurred despite the continued administration of progesterone. Similarly, Hansen and Sodersten (74) reported that heat duration in rats is dependent upon the dose of both estradiol and progesterone, but heat termination occurred despite the continued administration of both hormones.

As discussed earlier, the transient increase in hypothalamic nuclear progesterin receptor concentration following the injection of progesterone to OVX, estradiol-pretreated guinea pigs correlates temporally with the expression of sexual receptivity (20). This correlation suggests that heat termination may result from the loss of progesterin receptors from the cell nucleus. Perhaps as the concentration of progesterone-progesterin receptor complexes in cell nuclei decreases, the cellular effects of progesterone diminish, resulting in the loss of sexual responsiveness. Based on this hypothesis, hormonal manipulations that delay the loss of progesterin receptors from hypothalamic cell nuclei should also delay the termination of the period of sexual receptivity.

In support of this hypothesis, Blaustein (13) has reported that injection of supplemental estradiol at the time of progesterone injection to estradiol-pretreated OVX guinea pigs delayed heat termination and the loss of hypothalamic nuclear progesterin receptors. In the last three studies included in this dissertation, further hormonal manipulations were conducted in order to test the relationship between heat termination and the loss of progesterin receptors from hypothalamic cell nuclei.

In the studies included in this dissertation, progesterin receptor levels were measured in MBH-PDA or in MBH, rather than in the VMN alone.
This is because assay techniques to measure nuclear progestin receptor levels exclusively in the VMN have not yet been perfected. In addition, it was not until the studies of Rubin and Barfield (155 - 157) that it was demonstrated that the VMN was the principal site for the hormonal activation of sexual behavior. Prior to these studies, it was thought that the POA, which contains estrogen-dependent progestin binding sites, also is involved in the hormonal regulation of sexual behavior. In light of the studies by Rubin and Barfield, receptor levels measured in the last study were measured in the MBH rather than the MBH-POA.

It should be noted that recent studies have questioned the intracellular location of unoccupied steroid hormone receptors. Studies by King and Greene (84), and Welshons et al. (178) suggest that steroid hormone receptors, both occupied and unoccupied, reside primarily in the cell nucleus. According to the proposed model of steroid action resulting from these studies (36, 70), steroid hormone receptors are in loose association with nuclear components in the absence of bound ligand. Upon binding steroid hormone, the receptor complex becomes tightly associated with specific nuclear components. An alternative model has been proposed from the findings of Martin and Sheridan (101). They propose that unoccupied intracellular steroid hormone receptors are present in both the cytoplasm and the nucleus of the cell in a state of equilibrium. As receptors bind ligand, they become associated with chromatin, and equilibrium between the remaining unoccupied receptors is reestablished. These models suggest that all or much of the apparent localization of unoccupied receptors in the cytoplasm is an artifact of tissue homogenization. However, consistent with the classical model of
steroid hormone action, these models hold that hormone binding of the receptor results in a greater or tighter association with cell nuclear components. Therefore, receptors measured in the cytosol fraction by routine techniques such as those used in the following studies, may represent loosely associated, unoccupied receptors released from cell nuclei during tissue homogenization, whereas receptors measured in the nuclear fraction may represent primarily tightly associated, occupied receptors. In the following studies, the classical terminology (cytosol and nuclear receptors) was used as a matter of convenience rather than as an indication of where receptors are located in vivo.
Explanation of Dissertation Format

The dissertation is composed of five sections corresponding to five papers that have been published, submitted for publication, or are currently in press (25-29). All of the research presented in these studies was conducted by the degree candidate, with guidance provided by the major professor, Dr. Jeffrey D. Blaustein. Because some of the research presented in this dissertation was conducted following the relocation of Dr. Blaustein's laboratory from the Department of Zoology at Iowa State University, to the Division of Neuroscience and Behavior, Department of Psychology, University of Massachusetts, Amherst, MA, the name of that institution appears on the title page of some of the papers. Each section or paper is in a form similar to that as submitted for publication, and as such includes its own set of references. A general discussion of these five papers is included at the end of the dissertation. The references for the General Introduction and Summary and Discussion are located at the end of the dissertation under the heading Literature Cited.
SECTION I. INHIBITION OF SEXUAL BEHAVIOR IN FEMALE GUINEA PIGS BY A PROGESTIN RECEPTOR ANTAGONIST
Inhibition of sexual behavior in female guinea pigs by a progestin receptor antagonist

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ABSTRACT

The new steroidal progestin receptor antagonist, RU 38486, was used to determine if progesterone-facilitation of sexual behavior in female guinea pigs requires interaction of the hormone with neural progestin receptors. Five mg but not 0.5 mg RU 38486 inhibits the expression of sexual behavior in ovariectomized, estrogen-primed guinea pigs treated with 0.1 mg progesterone. This inhibition can be overcome by administration of a large dose of progesterone, suggesting that the drug effect is specific to the progestin receptor system. RU 38486 binds, in vitro, to progestin receptors and decreases the availability of hypothalamic progestin receptors in estrogen-treated guinea pigs. These studies provide strong evidence that progesterone interaction with intracellular neural progestin receptors mediates the facilitation of sexual behavior by progesterone in female guinea pigs.
INTRODUCTION

During the estrous cycle of some female rodents, including guinea pigs, the sequential secretion of estradiol and progesterone from the ovaries activates a period of sexual behavior (heat) which is characterized, in part, by the elicitation of the lordosis posture in response to mating stimulation (20). Progesterone has a dual effect on female sexual behavior in guinea pigs. In estrogen-pretreated animals, it first facilitates the expression of the behavior, and then causes a hyposensitivity to itself (1, 2, 22). The result is that guinea pigs are refractory to additional progesterone a day after receiving progesterone.

Results from a variety of studies suggest that some of progesterone’s effects in the brain may be mediated by interaction with intracellular progestin receptors, as are some of its effects in peripheral reproductive tissues (4, 6, 10, 13, 16). Evidence in support of this hypothesis comes from a variety of studies. Estradiol treatment in ovariectomized (OVX) guinea pigs increases the level of cytosol progestin receptors in some regions of the guinea pig brain, primarily the mediobasal hypothalamus and preoptic area (4, 19). These regions are thought to be the primary site for the hormonal induction of female sexual behavior by estradiol and progesterone (15). Administration of progesterone causes a transient increase in the level of progestin receptors in the cell nuclear fraction, while concurrently decreasing the level of progestin receptors in the cytosol (5). This accumulation
of nuclear progestin receptors precedes the initiation of sexual behavior. As the period of sexual behavior terminates, by about 14 h after progesterone injection, the level of nuclear progestin receptors returns to baseline, while cytosol progestin receptor levels remain depressed (5, unpublished observations). This down-regulation by progesterone of its own cytosol receptors may explain why animals are refractory to a second injection of progesterone one day after the first injection.

A recently developed steroidal progesterone antagonist, RU 38486, has been shown to antagonize a variety of progesterone's effects in the mammalian uterus (11, 17). This drug also binds, in vitro, with high affinity to uterine cytosol progestin receptors, suggesting that it may antagonize progesterone's action by inhibiting binding of progesterone to intracellular progestin receptors (17). In the present study, we provide evidence, using RU 38486, that at least one of progesterone's behavioral effects is mediated by intracellular neural progestin receptors.
MATERIALS AND METHODS

Animals

Adult, Hartley strain female guinea pigs (Camm Research Institute, Wayne, NJ) were group housed in 38 X 48 X 20 cm cages with lights on from 0600 - 2000 h. Guinea pig chow (Teklad Mills) and water were freely available. All animals were treated with tetracycline-HCl (American Cyanamid, Wayne, NJ) in their drinking water (6 g/l) for 2 days prior to and 5 days following ovariectomy. Ovariectomies were performed three weeks prior to use under a combination of Innovar-Vet (Pitman-Moore, Washington Crossing, NJ, 0.04 ml/animal, i.m.) and Chloropent (Fort Dodge Laboratories, Fort Dodge, IA, 1.7 ml/kg, i.p.) anesthesia. All hormonal injections were subcutaneous. Estradiol benzoate (EB) was dissolved in sesame oil; progesterone and RU 38486 (17β-hydroxy-11β-(4-dimethylaminophenyl)-17α-(1-propynyl)-estra-4,9,11-androster-3-one; Roussel-Uclaf, Romainville, France) were dissolved in sesame oil containing 15% benzyl benzoate and 5% benzyl alcohol. Injection volumes were 0.1 ml for EB and progesterone, and 0.2 or 0.4 ml for RU 38486 (0.5 mg and 5.0 mg, respectively). In all experiments, 10 µg EB was injected 40 h prior to progesterone.

Behavioral Testing

Animals were tested hourly for lordosis by manually stimulating the flanks and perineal area and measuring the length of time the lordosis posture was held (21). An animal was considered sexually
receptive if she held the lordosis posture for 1 sec or longer during two consecutive tests. Testing continued until all animals were nonresponsive or for a minimum of 14 h. An animal was considered no longer sexually receptive when she failed to respond during two consecutive tests. Latency to lordosis, heat duration, and maximum lordosis duration were calculated as described previously (3, 9).

Brain Dissection

Guinea pigs were decapitated and their brains rapidly removed. The mediobasal hypothalamus (MBH) and preoptic area (POA) were dissected on a chilled block. The wedge-shaped MBH sample was bounded rostrally by the caudal edge of the optic chiasm and caudally by the caudal edge of the mammillary bodies. Cuts were made extending from the lateral hypothalamic fissure to the midpoint of the corpus callosum to form the lateral boundaries, and a cut through the level of the fornix formed the dorsal boundary. The POA sample extended 3 mm rostrally from the hypothalamic section and included the anterior hypothalamus and preoptic area. A line continuous with the lateral ventricles formed the lateral boundaries and the anterior commissure formed the dorsal boundary. The MBH and POA from each animal were pooled.

Combined Cytosol and Nuclear Progestin Receptor Assay

Cytosol and nuclear progestin receptor levels were measured as described previously (1, 2) with minor modifications. All procedures were at 0-4 °C. Tissues were placed in a glass tissue grinder containing 1.0 ml buffer A (0.32 M sucrose, 1 mM KH₂PO₄, 1 mM MgCl₂,
10% glycerol, 12 mM monothioglycerol, pH 7.2) and were homogenized with a Teflon pestle with appropriate clearance for cell nuclear isolation. Homogenates were then transferred to 12 X 75 polycarbonate tubes and the tissue grinders rinsed with 0.5 ml buffer A. The homogenates were centrifuged at 1000 X g for 5 min. Cytosol extract was obtained by centrifuging the supernatant at 48,000 X g for 30 min. The pellet from the first centrifugation was washed twice with 2.0 ml buffer A, and the nuclei sedimented in 0.3 ml buffer A + 1.0 ml buffer B (2.15 M sucrose, 1 mM KH2PO4, 1 mM MgCl2, 10% glycerol, pH 7.2) by centrifuging at 25,000 X g for 20 min. The pellicle and supernatant were removed and the walls of the tube dried, leaving only purified cell nuclei.

For the cytosol receptor assay, 300 µl samples of cytosol were applied to 4.5 X 68 mm columns of Sephadex LH-20 (equilibrated with buffer A) to remove potentially competing steroids. Immediately after running into the column, the sample was washed in with 100 µl buffer A and the macromolecular fraction eluted with 700 µl buffer A. Two-hundred-fifty µl aliquots of filtered cytosol were incubated with 50 µl buffer A containing 1% ethanol (final concentration) and (17α-methyl-3H)R 5020 (New England Nuclear, Boston, MA; Specific activity = 87 Ci/mmol) +/- 250-fold concentration of unlabeled progesterone. A final concentration (in 300 µl) of 0.4 nM (3H)R 5020 was used because this concentration results in binding almost exclusively to the progestin receptor with almost no specific binding to blood contaminants or low affinity binders (4).

Following a 16-20 h incubation at 0-4 °C, bound (3H)R 5020 was separated from free by gel filtration using Sephadex LH-20 columns.
(Pharmacia Fine Chemicals, Piscataway, NJ) equilibrated with buffer AT. Two-hundred-fifty μl samples of incubate were applied to the column and washed in with 150 μl buffer AT. Thirty min after applying the sample, the protein peak was eluted into scintillation vials with 700 μl buffer AT. Five ml scintillation fluid (0.17 g Bis MSB + 5.5 g PPO / liter Scintillation grade toluene (Fisher Scientific) containing 33% Triton X-100) were added and radioactivity was counted on a Packard Tri-Carb Model 300C spectrophotometer at an efficiency of 41%. Specific binding was calculated by subtracting nonspecific binding (with unlabeled progesterone) from total binding (without unlabeled progesterone). Protein concentration was determined using the dye-binding method of Bradford (7). Results are expressed as femtomoles (${}^{3}$H)R 5020 specifically bound per mg cytosol protein.

For the nuclear receptor assay, the cell nuclear pellet was dispersed with 280 μl buffer TEST (10 mM Tris-HCl, 1.5 mM Na₂EDTA, 10% glycerol, 12 mM monothioglycerol, pH 7.4). After 15 min. an equal volume of buffer TESTK 1.0 (TEST + 1.0 mM KCl, pH 7.4) was added. The tube contents were vortexed and incubated for 15 min. The nuclei were centrifuged at 48,000 X g for 5 min. Two-hundred-fifty μl samples of the supernatant (nuclear extract) were incubated with 50 μl buffer TESTK 0.5 (TEST + 0.5 mM KCl, pH 7.4) containing 1% ethanol (final concentration) and (${}^{3}$H)R 5020 +/- 250-fold concentration of unlabeled progesterone. A final concentration of 4.0 nM (${}^{3}$H)R 5020 was used (5). Following a 16-20 h incubation, bound (${}^{3}$H)R 5020 was separated from free as described above except that buffer TESTK 0.5 was used. The DNA content of the nuclear pellet was determined by the method of Burton.
Results are expressed as femtomoles (³H)R 5020 specifically bound per mg DNA.

Competition Assay

For cytosol progestin receptor competition, the MBH-PQA from 8 OVX, estrogen-treated guinea pigs were pooled and homogenized in 10 ml buffer TEGT using a Polytron Tissue Homogenizer at a setting of 5 for 6 sec. Homogenate was centrifuged at 48,000 X g for 30 min.

Two-hundred-fifty µl aliquots of the supernatant (cytosol) were incubated with 50 µl buffer TEGT containing 1.0% ethanol (final concentration) and 0.4 nM (³H)R 5020 +/- increasing concentrations of progesterone or RU 38486. Following a 6 h incubation, bound (³H)R 5020 was separated from free as described above.

For cytosol estrogen receptor competition, the procedure was similar to that for progestin receptor competition. Aliquots of MBH-PQA cytosol obtained from 8 OVX guinea pigs were incubated with 1.0 nM tetralabeled (³H)estradiol (Amersham, Arlington Heights, IL; SA = 114 Ci/m mole) +/- increasing concentrations of unlabeled estradiol or RU 38486. Relative binding affinities were calculated as (the concentration of RU 38486 required to displace specific binding of the labeled ligand by 50% / the concentration of unlabeled progesterone (for progestin receptors) or estradiol (for estrogen receptors) required to displace specific binding of the labeled ligand by 50%) X 100.
RESULTS

RU 38486 Inhibition of Progesterone-Facilitated Sexual Behavior

In the first experiment, the efficacy of RU 38486 in blocking progesterone facilitation of sexual behavior was tested. OVX guinea pigs pretreated with 10 µg EB received either vehicle, 0.5 mg, or 5.0 mg RU 38486 one h prior to receiving 0.1 mg progesterone. Animals were tested hourly for lordosis until they no longer responded. Although the lower dose of RU 38486 was ineffective in blocking the response to progesterone (Table 1), the higher dose inhibited lordosis in nearly all of the animals (91% vs. 11%; p = 0.002). Twenty-four h after the progesterone injection, animals were treated with 0.5 mg progesterone and tested hourly for lordosis to determine if RU 38486 blocks the desensitization effect of progesterone. As has been reported previously (1, 2), animals which had received an injection of progesterone 24 h earlier failed to respond to the second progesterone injection, contrasted with responding by 90% of the control animals that had received the oil vehicle 24 h earlier. Both groups of animals receiving RU 38486 failed to respond to the second progesterone injection.
Table 1. RU 38486 inhibition of progesterone-facilitated sexual behavior

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>Test 1</th>
<th>Percent Responding*</th>
<th>Heat Duration (h)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group</td>
<td>-1 h</td>
<td>0 h</td>
<td>n</td>
</tr>
<tr>
<td>I. Veh</td>
<td>Po.1</td>
<td>10</td>
<td>90%</td>
</tr>
<tr>
<td>II. RU0.5</td>
<td>Po.1</td>
<td>9</td>
<td>100%</td>
</tr>
<tr>
<td>III. RU1</td>
<td>Po.1</td>
<td>9</td>
<td>11%</td>
</tr>
<tr>
<td>IV. Veh</td>
<td>Veh</td>
<td>10</td>
<td>0%</td>
</tr>
</tbody>
</table>

*All animals received 10 μg estradiol benzoate at -40 h. Veh = vehicle, P = progesterone, RU = RU 38486, subscripts are doses in mg. Hourly tests for lordosis began just prior to injection at -1 h, and continued until all animals were nonresponsive (Test 1). At 24 h, all animals received 0.5 mg progesterone and were tested hourly (Test 2). Values reported are means +/- S.E.M.

*Includes responding animals only.

•I vs. III: P = 0.002, Fisher's Exact Probability Test.

••I vs. III: P < 0.002, Mann-Whitney U-test.

•••III vs. IV: P = 0.002, Fisher's Exact Probability Test.
<table>
<thead>
<tr>
<th>Heat Duration (h)</th>
<th>Latency to Lordosis (h)</th>
<th>Maximum Lordosis Duration (sec)</th>
<th>Test 2 Percent Responding***</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.11 +/-0.42</td>
<td>4.44 +/-0.63</td>
<td>12.33 +/-1.08</td>
<td>0%</td>
</tr>
<tr>
<td>8.00 +/-0.99</td>
<td>4.56 +/-0.34</td>
<td>14.89 +/-1.66</td>
<td>0%</td>
</tr>
<tr>
<td>5.00</td>
<td>7.00</td>
<td>10.00</td>
<td>11%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>90%</td>
</tr>
</tbody>
</table>
Ability of Increased Progesterone to Overcome RU 38486-Induced Inhibition

Because RU 38486 is also a potent glucocorticoid receptor antagonist (14), it is possible that in high doses the drug renders the animals incapable of displaying lordosis because of debilitation caused by impaired glucocorticoid function. This is unlikely, because no debilitating effects of RU 38486 were observed in the previous experiment. Nevertheless, to test for this and other nonspecific effects of the drug, OVX, EB-injected guinea pigs were treated with either 0.1 mg or 5.0 mg progesterone one h after receiving 5.0 mg RU 38486. If the RU 38486-induced inhibition of progesterone-facilitated lordosis is due to a debilitating effect, then it would be expected to inhibit lordosis regardless of the dose of progesterone. However, a greater percentage of animals responded to the larger dose of progesterone than the smaller dose (100% vs. 40%; Table 2). Furthermore, animals receiving 5 mg progesterone had longer heat durations (9.3 vs. 2.0 h, nonresponding animals included) than animals receiving 0.1 mg progesterone. These results suggest that the inhibition by RU 38486 is specific to progesterone.

RU 38486 Interaction with Neural Progestin Receptors

In the next experiment, the ability of RU 38486 to interact with MBH-POA cytosol progestin and estrogen receptors, in vitro, was tested in competition studies. RU 38486 has a high affinity for guinea pig MBH-POA cytosol progestin receptors (relative binding affinity = 50
Table 2. Ability of a large dose of progesterone to overcome RU 38486-induced inhibition of progesterone-facilitated sexual behavior

<table>
<thead>
<tr>
<th>Treatment *</th>
<th>-1 h</th>
<th>0 h</th>
<th>n</th>
<th>Percent Responding</th>
<th>Heat Duration** (h)</th>
<th>Heat Duration b (h)</th>
<th>Latency to Lordosis b (h)</th>
<th>Maximum Lordosis Duration b (sec)***</th>
</tr>
</thead>
<tbody>
<tr>
<td>RUa P0.1</td>
<td>10</td>
<td>40%</td>
<td>1</td>
<td>2.00 +/− 1.19</td>
<td>5.00 +/- 2.38</td>
<td>5.75 +/- 1.75</td>
<td>10.67</td>
<td></td>
</tr>
<tr>
<td>RUa P1</td>
<td>9</td>
<td>100%</td>
<td>1</td>
<td>9.33 +/- 0.77</td>
<td>9.33 +/- 0.77</td>
<td>3.67 +/- 0.50</td>
<td>10.67</td>
<td></td>
</tr>
</tbody>
</table>

*A11 animals received 10 μg estradiol benzoate at -40 h. Abbreviations are the same as in Table 1. Subscripts are doses in mg. Values reported are means +/- S.E.M.

*Includes Responding animals only.

**P = 0.02, Fisher's Exact Probability Test.

***P < 0.02, Mann-Whitney U-test.
compared with progesterone) but not estrogen receptors (relative binding affinity < 0.0001 compared with estradiol, Figure 1). This suggests that the inhibitory effects of RU 38486 are due to an interaction with progestin receptors and not estrogen receptors.

To verify that RU 38486 also interacts in vivo with intracellular progestin receptors, MBH-POA cytosol and nuclear progestin receptors were assayed in OVX, estrogen-treated guinea pigs two h after the injection of 5.0 mg RU 38486 or vehicle. Eight OVX guinea pigs were treated with 10 μg EB. Forty h later, they received either RU 38486 or the oil vehicle. Two h later, animals were decapitated, and MBH-POA cytosol and nuclear progestin receptor levels were measured.

RU 38486 decreased the concentration of available cytosol progestin receptors in MBH-POA by 35% compared with vehicle-injected controls (15.2 femtomoles/mg protein for drug-treated vs. 23.4 femtomoles/mg protein for controls; p < 0.0025, Student's t test). Although we did not observe a statistically significant difference in nuclear receptor levels, recent evidence suggests that receptor-bound RU 38486 may not exchange with the (3H)progestin used in the nuclear progestin receptor assay (17). Further study using radioactively labeled RU 38486 is required to determine if the drug causes the translocation of progestin receptors to cell nuclei.

Scatchard Analysis

In order to determine if the observed decrease in MBH-POA cytosol progestin binding was due to a decrease in receptor concentration or to a decreased affinity of the receptor for the (3H)progestin, cytoplasmic
Figure 1. Competition of RU 38486 for MBH-PDA cytosol progestin and estrogen receptor binding
MBH-POA progestin receptors were assayed and the results analyzed by the method of Scatchard (18). EB-treated, OVX guinea pigs were injected with 5 mg RU 38486 or vehicle. Two h later, animals were killed, and MBH-POA cytosol was obtained as described for the competition assay. Cytosol was pooled for each group and passed through an LH-20 column to remove potentially competing steroids. Aliquots of filtered cytosol were incubated with various concentrations of (³H)R 5020 (0.1 - 8 nM) +/- 250-fold excess of progesterone. Following a 16-20 h incubation, bound (³H)R 5020 was separated from free as described. When analyzed by this method, RU 38486 resulted in a 44% decrease in the concentration of available progestin binding sites (28.3 fmoles/mg protein for the vehicle-injected group vs. 15.9 fmoles/mg protein for the RU 38486-injected group) with no alteration in the binding affinity (Figure 2).
Figure 2. Scatchard representation of specific (\(^{3}\text{H}\))R 5020 binding in MBH-POA cytosol obtained from guinea pigs injected with 10 \(\mu\)g EB and either 5 mg RU 38486 or vehicle.

Two h after the injection of RU 38486 or vehicle, animals were decapitated and MBH-POA cytosol progestin receptors were assayed in pooled MBH-POA cytosol.
VEHICLE

Kd = 0.19 nM
NS = 29 fmoles/mg protein

RU 38486

Kd = 0.16 nM
NS = 16 fmoles/mg protein
DISCUSSION

These findings support earlier studies on the mechanisms of progesterone's effects on sexual behavior in guinea pigs, which suggested that interaction of progesterone with intracellular progestin receptors is a prerequisite for progesterone to facilitate sexual behavior. In the present experiments, the progestin receptor antagonist, RU 38486, was found to inhibit progesterone-facilitated sexual receptivity, presumably by blocking the ability of progesterone to bind to its neural receptors. The RU 38486-induced inhibition of progesterone-facilitated lordosis is specific to progesterone, because a large dose of progesterone is able to overcome the inhibition. RU 38486 interacts with neural progestin receptors both in vitro and in vivo. This interaction, in vivo, leads to a decreased number of cytosol progestin binding sites available and not to an alteration in binding affinity.

The failure of RU 38486 to block progesterone's desensitization effect should not be interpreted as evidence that progestin receptors are not involved in this effect. Decreasing the availability of cytoplasmic progestin receptors at the time of the initial progesterone injection by administering RU 38486 does not interfere with progesterone's desensitization effect. However, these results do not conflict with the interpretation that desensitization by progesterone is caused by subsequent down-regulation of cytosol progestin receptors (1, 2, 4, 5). Since both RU 38486 and progesterone decrease the
concentration of cytosol progestin receptors in the MBH-POA, all animals except the vehicle-injected animals would be expected to be hyposensitive to the second progesterone injection.

These experiments provide direct evidence that progesterone's facilitation of sexual behavior in female guinea pigs requires interaction of the hormone with neural progestin receptors. Furthermore, these experiments establish RU 38486 as a useful probe in elucidating the mechanisms by which progesterone acts in the brain to influence neuroendocrine function.
REFERENCES


SECTION II. 1-(o-CHLOROPHENYL)-1-(p-CHLOROPHENYL)2,2,2-TRICHLOROETHANE INDUCES FUNCTIONAL PROGESTIN RECEPTORS IN THE RAT HYPOTHALAMUS AND PITUITARY GLAND
1-(o-Chlorophenyl)-1ig-chlorophenyl)2,2,2-
trichloroethane induces functional progestin receptors
in the rat hypothalamus and pituitary gland

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ABSTRACT

A recent report suggests that estrogen induction of hypothalamic progestin receptors is not a prerequisite for the facilitation by progesterone of female sexual behavior in rats. Progesterone was found to facilitate sexual behavior despite no apparent induction of progestin receptors by g,g'-DDT. To investigate these findings further, ovariectomized rats were treated with daily injections of g,g'-DDT for 3 d followed by progesterone on day 4. Daily injections of 400 mg g,g'-DDT/kg resulted in the activation of lordosis in 50% of the animals; injections of 200 mg/kg were ineffective. Cytoplasmic and nuclear progestin receptor levels in the mediobasal hypothalamus-preoptic area and pituitary gland were then determined in similarly treated animals. Because competition assays revealed that g,g'-DDT interacts with progestin binding sites in vitro, residual g,g'-DDT was removed from the cytosol fraction prior to assay. g,g'-DDT treatment increased the level of cytoplasmic progestin receptors by 43% compared with oil-injected controls. Progesterone administration to g,g'-DDT-treated rats resulted in a 137% increase in the level of nuclear progestin receptors relative to levels observed in animals receiving no progesterone. These findings contradict a previous report using g,g'-DDT, and support the hypothesis that estrogen-induced progestin receptors are required for the facilitation of sexual receptivity by progesterone.
INTRODUCTION

Sexual behavior during the estrous cycle of several rodent species is activated by the sequential presence of estradiol and progesterone secreted from the ovaries (13). In ovariectomized, estrogen-pretreated animals, administration of progesterone activates a similar period of sexual responsiveness (7, 13).

Several studies indicate that estrogen-induced intracellular progestin receptors mediate progesterone-facilitated sexual receptivity in female rats and guinea pigs. Estradiol treatment increases the concentration of cytoplasmic progestin receptors in several brain regions, including the mediobasal hypothalamus and preoptic area (4, 21, 25, 29, 34). These regions are thought to contain the primary sites for the activation of sexual receptivity by progesterone (22, 26, 30, 33). Progesterone administration to estrogen-pretreated animals results in an accumulation of these receptors in cell nuclei (2, 3, 5, 6, 24). This accumulation and retention of nuclear progestin receptors in the hypothalamus correlates closely with the initiation and duration of the receptive period (2, 5, 10, 24). In addition, we have recently shown that the progestin receptor antagonist RU 486 can block progesterone-facilitated lordosis in female guinea pigs (9), providing strong evidence that progestin receptors are involved in mediating progesterone's facilitory effect on sexual behavior.

A recent study by Etgen (12) has questioned the role of cytoplasmic progestin receptors in the activation of sexual receptivity
in female rats by progesterone. It was reported that when rats were primed with \( g,g' \)-DDT, a polychlorinated pesticide with estrogenic properties (14, 15, 27, 32), rather than estradiol, progesterone-activated sexual receptivity occurred despite no apparent induction of cytoplasmic progestin receptors in the hypothalamus. These findings seemingly suggest that estrogen-induced progestin receptors are not critical for the activation of sexual receptivity by progesterone. Because these findings conflict with a variety of previous studies that suggest estrogen induction of intracellular progestin receptors is necessary for progesterone's facilitory effects on sexual receptivity, we attempted to replicate and extend Etgen's findings.
MATERIALS AND METHODS

Animals

Female Sprague-Dawley rats (Charles River Breeding Laboratories; Wilmington, MA) weighing 175-225 g were ovariectomized (31) 1-2 weeks prior to experimental treatment. Animals were housed separately in 17 X 24 X 18 cm wire mesh cages or in groups of 4 in 41 X 24 X 18 cm wire mesh cages. Food (Purina Laboratory Chow, St. Louis, MO) and water were freely available, and a 14:10 light/dark cycle was maintained with lights on from 2300-1300 h.

Injections

All injections were administered subcutaneously. Estradiol benzoate (EB) was dissolved in sesame oil and injected in a 0.1 ml volume. Progesterone was dissolved in peanut oil and injected in a 0.1 ml volume. \( \text{\textit{g},\textit{g}'-DDT}, (1-(\text{g-}\text{chlorophenyl})-1-(\text{g-}\text{chlorophenyl})2,2,2-\text{trichloroethane}; \text{Aldrich Chemical Co., Milwaukee, WI}) \) was dissolved in a 1:1 mixture of peanut oil and benzyl benzoate, 250 mg/ml.

Behavioral Testing

Tests for lordosis were conducted by placing the experimental female in a round plexiglass arena (53 cm diameter X 41 cm high) with 2 stimulus males until 10 mounts were observed. The quality of each lordosis response was rated on a scale of 0-3 (no - full dorsiflexion) (17) with the experimenter blind to the assigned treatment group.
Results are expressed as mean lordosis quotient (percentage of mounts resulting in a lordosis response) and mean lordosis rating (sum of quality scores/10). With the exception of the pretest in experiment 1, all tests occurred 2-3 h after the onset of darkness.

Brain Dissection

Rats were decapitated and their brains and pituitary glands rapidly removed. The mediobasal hypothalamus (MBH) and preoptic area (POA) were dissected on a chilled block. The wedge-shaped MBH sample was bounded rostrally by the caudal edge of the optic chiasm and caudally by the caudal edge of the mammillary bodies. Cuts were made extending from the lateral hypothalamic fissure to the midpoint of the corpus callosum to form the lateral boundaries, and a cut through the level of the fornix formed the dorsal boundary. The POA sample extended 2 mm rostrally from the hypothalamic section and included the anterior hypothalamus and preoptic area. A line continuous with the lateral ventricles formed the lateral boundaries and the anterior commissure formed the dorsal boundary. The MBH and POA were pooled for each animal.

Combined Cytosol and Nuclear Progestin Receptor Assay

Cytosol and nuclear progestin receptor levels were measured as described previously (2, 3). All procedures were conducted at 0-4 °C. Tissues were pooled for 2 similarly-treated rats in 1.0 ml buffer A (0.32 M sucrose, 1 mM KH₂PO₄, 1 mM MgCl₂, 10% glycerol, 12 mM monothioglycerol, pH 7.2) and homogenized in glass tissue grinders with
a teflon pestle. The homogenates were transferred to 12 x 75 mm polycarbonate tubes and the tissue grinders were rinsed with 250 µl A T. Following centrifugation at 1,000 x g for 5 min, the supernatant was transferred to polycarbonate tubes and centrifuged at 48,000 x g for 30 min to obtain the cytosol fraction.

The pellet from the first centrifugation was washed twice by dispersing in 2.0 ml A T and centrifuging at 1,000 x g for 5 min. Following the second wash, the crude nuclear pellet was resuspended in 300 µl A T, and 1.0 ml buffer B (2.15 M sucrose, 1 mM KH₂PO₄, 1 mM MgCl₂, 10% glycerol, pH 7.2) was added. The tube contents were then vortex-mixed. Following centrifugation at 27,500 x g for 20 min, the pellicle and supernatant were discarded and the walls of the tube dried. The resulting purified nuclei were resuspended in 285 µl buffer TEGT (10 mM Tris-HCl, 1.5 mM Na EDTA, 10% glycerol, 12 mM monothioglycerol, pH 7.4). Following a 15 min incubation, 285 µl buffer TEGTK-1.0 (TEGT + 1.0 M KCl) were added and the contents vortex-mixed periodically during a 15 min incubation. The nuclei were then centrifuged at 48,000 x g for 5 min. The supernatant (nuclear extract) was decanted and 250 µl samples were incubated at 0-4 °C with 50 µl buffer TEGTK-0.5 (TEGT + 0.5 M KCl) containing ethanol (1% final concentration) and (³H)R 5020 (SA = 87 Ci/mmol; New England Nuclear) with or without a 250-fold concentration of unlabeled progesterone. A final concentration of 1.5 nM (³H)R 5020 was used because this concentration saturates the progestin receptor pool while minimizing binding to nonspecific binders (31).

Bound (³H)R 5020 was separated from free by gel filtration using
4.5 X 68 mm Sephadex LH-20 columns (Pharmacia Fine Chemicals, Piscataway, NJ). Aliquots of incubate (250 µl) were loaded onto the columns and washed in with 150 µl TEGTK-0.5 (TEGT + 0.5 M KCl). Thirty min after sample loading, the macromolecular fraction (containing the bound ($^3$H)R 5020) was eluted into scintillation vials with 700 µl TEGTK-0.5. Five ml scintillation fluid (0.17 g Bis-MSB + 5.5 g PPO/liter scintillation grade toluene containing 33% scintillation surfactant (Fisher Scientific) were added and radioactivity counted on a Packard Tricarb model 300C spectrophotometer at an efficiency of 41%.

The DNA concentration of the nuclear pellet was determined by the method of Burton (11). Results are expressed as femtomoles ($^3$H)R 5020 specifically bound/mg DNA.

To remove potentially competing molecules from the cytosol fraction, 300 µl samples of cytosol were loaded onto Sephadex LH-20 columns (3). The samples were washed in with 100 µl A+ and eluted with 700 µl A+. Aliquots (250 µl) of filtered cytosol were incubated overnight with 50 µl A+ containing ($^3$H)R 5020 +/- 250-fold concentration of progesterone. A 0.4 nM concentration of ($^3$H)R 5020 was used to minimize binding to blood contaminants and low-affinity binders (4).

Following an overnight incubation at 0-4 °C, bound ($^3$H)R 5020 was separated from free as described for the nuclear assay except that buffer A+ was used. Cytosol protein concentration was determined by the dye-binding method of Bradford (8) with bovine serum albumin (Sigma Chemical Co., St. Louis, MO) used as a standard. Results are expressed as femtomoles ($^3$H)R 5020 specifically bound/mg cytosol protein.
Cytosol Estrogen Receptor Assay

Cytoplasmic estrogen receptor levels were measured by a modification of the method by Ginsburg et al. (16). Animals were decapitated and MBH-POA and pituitary dissected and homogenized as described above in 1.0 ml buffer TEGT. Uterine horns were also removed from the animal, minced, placed in 12 X 75 mm polycarbonate tubes containing 2.0 ml TEBT, and homogenized with a Polytron PT-7 tissue homogenizer. MBH-POA and pituitary homogenates were transferred to 12 X 75 mm polycarbonate tubes and the tissue grinders were rinsed with 250 µl TEGT. Homogenates were centrifuged at 48,000 x g for 30 min and the supernatant (cytosol) was decanted and filtered as described above. Aliquots (250 µl) of cytosol were incubated at 0-4 °C with 50 µl TEGT containing 1% ethanol and 1.0 nM (³H)estradiol (New England Nuclear, SA = 95 Ci/mmol) with or without 100 nM unlabeled estradiol (final concentrations in 300 µl). Following an overnight incubation, bound (³H)estradiol was separated from free as described for the progestin receptor assays except that buffer TEBT was used.

Competition Assays

For estrogen receptor competition assays, MBH-POA and pituitary cytosol obtained from 16 OVX rats was pooled. Aliquots (250 µl) of the pooled cytosol were incubated with 50 µl TEGT containing 1% ethanol and 1.0 nM (³H)estradiol (final concentration in 300 µl) with or without increasing concentrations of unlabeled estradiol (0.02 nM - 0.1 µM) or g,δ′-DDT (0.02 µM - 0.4 mM). Following a 5 h incubation at 0-4 °C,
bound (\(^{3}H\))estradiol was separated from free as described.

For progestin receptor competition, MBH-POA and pituitary cytosol was obtained and pooled from 12 OVX rats treated with 10 µg EB 2 days prior to assay. Aliquots (250 µl) of cytosol were incubated with 50 µl TEGT containing 2% ethanol and 0.4 nM (\(^{3}H\))R 5020 with or without increasing concentrations of progesterone (0.017 nM - 1.0 µM) or \(\text{g}, \text{g}^{\prime}-\text{DDT}\) (1.5 µM - 0.35 mM).

Relative binding affinities (RBAs) were calculated as (the concentration of \(\text{g}, \text{g}^{\prime}-\text{DDT}\) required to displace specific binding of the labeled ligand by 50% / the concentration of unlabeled estradiol (for estrogen receptors) or progesterone (for progestin receptors) required to displace specific binding of the labeled ligand by 50%) \times 100.
RESULTS

Experiment 1

To confirm the finding that g,g'-DDT can substitute for estradiol in the activation of progesterone-facilitated sexual receptivity, we treated OVX female rats as in the previous study by Etgen (12). In addition, we tested the animals prior to progesterone treatment to ensure they were actually responding to the progesterone injection. Animals were injected daily with 200 mg g,g'-DDT/kg body weight or 1 µg EB for 3 days. On the fourth day, animals were tested for sexual receptivity (pre-test), injected with 0.5 mg progesterone, and tested again 4 h later (post-test). Because cervico-vaginal stimulation has been shown to influence subsequent behavior tests (1, 20), vaginal masks (31) were used to prevent intromission.

Animals treated with daily injections of estradiol exhibited a low level of sexual receptivity in the absence of progesterone (pre-test, Table 1). Following progesterone treatment, these animals became highly receptive (post-test). In contrast to the study by Etgen, DDT-treated animals did not respond to progesterone, nor were they responsive prior to the progesterone injection.

Experiment 2

In the study by Etgen (12), a single injection of 500 mg g,g'-DDT/kg resulted in maximal depletion of cytoplasmic estrogen receptors in the hypothalamus 8 h after injection. Because in
Table 1. Failure of 200 mg g,g'-DDT/kg to mimic the action of estradiol on lordosis

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Pre-test</th>
<th>Post-test</th>
<th>Pre-test</th>
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<tr>
<td>EB</td>
<td>16</td>
<td>28.75</td>
<td>100*</td>
<td>0.59</td>
<td>2.35*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+/-0.70</td>
<td>-</td>
<td>+/-0.21</td>
<td>+/-0.08</td>
</tr>
<tr>
<td>DDT</td>
<td>16</td>
<td>0.63</td>
<td>0.63</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+/-0.63</td>
<td>+/-0.63</td>
<td>+/-0.01</td>
<td>+/-0.01</td>
</tr>
</tbody>
</table>

*Animals received daily injections of 1 μg estradiol benzoate (EB) or 200 mg g,g'-DDT/kg for three days. On day 4, animals were tested for lordosis (pre-test), injected with 0.5 mg progesterone, and tested for lordosis 4 h later (post-test). Results are expressed as means +/- S.E.M.

*Pre-test vs. Post-test: p < 0.001, Mann-Whitney U-test.
Experiment 1 we failed to replicate the previously reported behavioral results obtained by Etgen, we examined the amount of cytoplasmic estrogen receptor depletion in our rats under similar conditions.

A single injection of 500 mg g,g'-DDT resulted in no detectable depletion of cytoplasmic estrogen receptors at 8 h in our rats (Figure 1). Uterine estrogen receptors, however, were decreased by 50% by g,g'-DDT injection.

Experiment 3

The binding of g,g'-DDT to cytosol uterine estrogen receptors is well-established (14, 19, 27, 28). Because we failed to detect depletion of MBH-POA and pituitary cytoplasmic estrogen receptors by g,g'-DDT, we conducted binding competition assays to verify that g,g'-DDT can interact with estrogen receptors from these tissues. Binding competition assays for progestin receptors were also conducted to determine if g,g'-DDT interacts with progestin receptors.

For estrogen receptor binding competition, MBH-POA and pituitary cytosol obtained from OVX rats was incubated with 1.0 nM \(^{3}H\)estradiol in the presence or absence of various concentrations of unlabeled estradiol or g,g'-DDT. For progestin receptor binding competition, cytosol obtained from estrogen-treated OVX rats was incubated with 0.4 nM \(^{3}H\)R 5020 in the presence or absence of various concentrations of unlabeled progesterone or g,g'-DDT.

As expected, g,g'-DDT competed for both MBH-POA and pituitary estrogen binding sites (RBA compared to estradiol = 0.01 and 0.02 respectively; Figure 2). Surprisingly, in contrast to the previous
Figure 1. Cytoplasmic estrogen receptor levels 8 h following the injection of 500 mg \( \text{g,0}^\prime \text{-DDT/kg (DDT; n=6)} \) or oil vehicle (VEH; n=6).

Bars represent the group mean + S.E.M.
SPECIFICALLY BOUND [3H]ESTRADIOL
(fmoles per mg protein)

MBH-POA

PITUITARY

UTERUS

DDT

VEH
Figure 2. Competition of o,p'-DDT for MBH-POA and pituitary cytosol estrogen binding sites

Aliquots of cytosol were incubated with 1.0 nM (³H)estradiol +/- various concentrations of unlabeled estradiol or o,p'-DDT.
report by Etgen (12), g,g'-DDT was also found to compete for MBH-POA and pituitary progestin binding sites (RBA compared to progesterone = 0.02 and 0.03 respectively; Figure 3).

Experiment 4

Based on the results of Experiment 1 and 2, it is possible that our rats may be less sensitive to g,g'-DDT than were those used in the study by Etgen. Therefore, we tested the ability of a larger dose of g,g'-DDT to substitute for estradiol in the activation of progesterone-facilitated sexual receptivity. Animals were injected daily with 400 mg g,g'-DDT/kg for three days. On the fourth day, animals were injected with 0.5 mg progesterone or the oil vehicle, and tested 4 h later.

In the absence of progesterone treatment, none of the animals exhibited sexual behavior while 50% of the animals injected with progesterone responded (Table 2).

Experiment 5

In Experiment 4, we confirmed that g,g'-DDT can substitute for estradiol in providing responsiveness to progesterone in the activation of sexual receptivity. If intracellular progestin receptors are involved in mediating progesterone's effects on lordosis, then hypothalamic nuclear progestin receptor levels should be elevated following progesterone treatment. To test this as well as to determine if g,g'-DDT induces cytoplasmic progestin receptors, both cytoplasmic and nuclear progestin receptor levels were measured in animals treated
Figure 3. Competition of $o,p'$-DDT for MBH-PDA and pituitary cytosol progestin binding sites

Aliquots of cytosol obtained from estradiol-treated OVX rats were incubated with 0.4 nM ($^3$H)R 5020 +/- various concentrations of unlabeled progesterone or $o,p'$-DDT.
Table 2. Ability of 400 mg g,g'-DDT/kg to mimic the action of estradiol on lordosis

<table>
<thead>
<tr>
<th>Group*</th>
<th>n</th>
<th>Percent Responding*</th>
<th>Lordosis Quotient**</th>
<th>Lordosis Rating**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Veh</td>
<td>11</td>
<td>0%</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PROG</td>
<td>12</td>
<td>50%</td>
<td>45.83</td>
<td>1.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+/-13.40</td>
<td>+/-0.30</td>
</tr>
</tbody>
</table>

*Animals received daily injections of 400 mg g,g'-DDT/kg for three days. On day 4, animals received either 0.5 mg progesterone (PROG) or the oil vehicle (Veh), and were tested for lordosis 4 h later. An animal with a lordosis quotient of 20 or greater was considered responsive. Results are expressed as means +/- S.E.M.

*p < 0.013, Fisher's Exact Probability Test.

**p < 0.05, Mann-Whitney U-test.
as in Experiment 4.

Rats received daily injections of 400 mg g,g'-DDT/kg or the oil vehicle for three days. On the fourth day, animals received either 0.5 mg progesterone or the oil vehicle and were killed three hours later for determination of MBH-POA and pituitary cytoplasmic and nuclear progestin receptor levels. Because g,g'-DDT binds to progestin receptors in vitro (Experiment 3), cytosol was filtered through Sephadex LH-20 columns to remove any potentially competing g,g'-DDT.

In contrast to the previous report (12), g,g'-DDT treatment increased MBH-POA and pituitary progestin receptors by 43% and 42% respectively (p<0.01; Figure 4). Progesterone administration resulted in a depletion of pituitary cytoplasmic receptors (p<0.05, 1-tail test); however, the decrease seen in the MBH-POA was not statistically significant.

MBH-POA and pituitary nuclear progestin receptors were increased by 137% and 172% respectively by progesterone treatment (compared to DDT-VEH animals; p<0.01 and p<0.05; Figure 5). DDT-treated rats which received the vehicle rather than progesterone exhibited a slight elevation in MBH-POA nuclear progestin receptor levels (p<0.05, 1-tail) compared to animals treated with only vehicle injections.
Figure 4. Cytoplasmic progestin receptor levels

OVX rats were injected daily with 400 mg \text{5B'-DDT/kg} or the oil vehicle (VEH) for three days. On the fourth day, rats received 0.5 mg progesterone (PROG) and were killed 3 h later to determine progestin receptor levels. Bars represent means + S.E.M. (n=6).
Figure 5. Nuclear progestin receptor levels in animals described in Figure 4 (n=5 or 6)
DISCUSSION

Confirming a previous report by Etgen (12), \( \text{g},\text{g}'\text{-DDT} \) was found to have an estrogenic effect on female sexual behavior. Rats treated with daily injections of 400 mg \( \text{g},\text{g}'\text{-DDT/kg} \) became sexually receptive following the injection of progesterone. While the previous study reported that daily injections of 200 mg/kg are sufficient, this dose was without effect in our animals. This is most likely due to a lower sensitivity of our animals to \( \text{g},\text{g}'\text{-DDT} \). In support of this interpretation, no depletion of MBH-POA or pituitary cytoplasmic estrogen receptor levels was detected in our animals at 8 h following a single injection of 500 mg \( \text{g},\text{g}'\text{-DDT/kg} \). In the previous study, a 50% depletion was observed under similar conditions.

In contrast to the results reported by Etgen (12), \( \text{g},\text{g}'\text{-DDT} \) treatment was found to induce cytoplasmic progestin receptors in the MBH-POA and pituitary. Furthermore, these receptors appear to be functional since progesterone treatment results in accumulation of progestin receptors in the cell nucleus.

One reason for the discrepancy in results between our study and Etgen's could be that we used a higher dose of \( \text{g},\text{g}'\text{-DDT} \). Perhaps at lower doses, \( \text{g},\text{g}'\text{-DDT} \) does not induce progestin receptors but does produce other estrogenic effects which may be involved in lordosis activation. However, this argument is unlikely because the dose used in the present study was near the threshold dose required to elicit lordosis in our animals; only 50% of the animals treated with 400 mg
$g,g'$-DDT/kg injections were found to respond to progesterone. In addition, one-half the amount of this dose was ineffective in our animals.

A more likely explanation for the difference in results is that in the present study, cytosol was filtered prior to assaying progestin receptor levels in order to remove residual $g,g'$-DDT. Based on the finding that $g,g'$-DDT competes for progestin binding sites in vitro (Experiment 3), residual $g,g'$-DDT, if not removed, could compete with ($^3$H)R 5020 for progestin receptor binding in the binding assay. It is possible that in the previously reported study (12), residual $g,g'$-DDT masked the increase in cytoplasmic progestin receptor levels.

It should be pointed out that although the same strain of rats was used in both studies, the animals were obtained from different suppliers. The possibility that the difference in sensitivity to $g,g'$-DDT observed between the previous and present studies is due to subtle animal differences cannot be ruled out. It is also possible that different preparations of $g,g'$-DDT used in the two studies contained different amounts of the two enantiomers of $g,g'$-DDT. The levo form has been shown to have more estrogenic activity than the dextro form in the immature rat uterus (23). It is interesting to note that MBH-POA nuclear progestin receptors are elevated in $g,g'$-DDT-treated rats in the absence of progesterone. It is conceivable that $g,g'$-DDT not only increases their availability, but also binds to progestin receptors in vivo and causes their translocation to cell nuclei; however, more evidence is required to determine if this is the case.
In Experiment 2, depletion of cytosol estrogen receptor levels by \( g,g'-\text{DDT} \) was observed in the uterus but not in the MBH-POA or pituitary. The fact that depletion was observed in the uterus indicates that \( g,g'-\text{DDT} \) was capable of interacting with estrogen receptors in the present study. However, further conclusions based on this experiment are limited because receptor levels were measured at only one time point following \( g,g'-\text{DDT} \) injection. Perhaps estrogen receptor depletion in the MBH-POA and pituitary would have been observed if receptor levels were measured at an earlier or later time. The time point we chose was based on work by Etgen (12) showing that maximal depletion of hypothalamic estrogen receptors occurred 8 h after \( g,g'-\text{DDT} \) injection.

To our knowledge, Experiment 3 provides the first evidence that \( g,g'-\text{DDT} \) interacts with progestin receptors. \( g,g'-\text{DDT} \) was found to compete for progestin binding sites with about the same affinity as it competes for estrogen binding sites. The level of estrogen receptor competition obtained in this study agrees with that reported for uterine estrogen receptors (14, 27, 28). An approximately 10-fold higher concentration of \( g,g'-\text{DDT} \) is required to obtain the same level of competition as is observed with unlabeled estradiol.

In light of recent studies (18, 35), it may be necessary to slightly modify our current concepts of steroid receptor action. According to these studies, steroid receptors may reside primarily in the cell nucleus. Upon binding to steroid hormone, the receptor protein may become tightly associated with nuclear components. Therefore, receptors measured in the cytosol fraction by present assaying techniques may represent loosely associated, unbound receptors released
from the cell nucleus during tissue homogenization, while those measured in the nuclear fraction may represent predominantly tightly-associated receptors. In any event, our results demonstrate that $g,g'$-DDT acts in the brain to increase the availability of intracellular progestin receptors.

In summary, in contrast to a previous report (12), we have found that a minimal dose of $g,g'$-DDT that provides neural responsiveness to progesterone for facilitation of sexual behavior, does indeed increase the concentration of cytoplasmic progestin receptors. Therefore, our results provide further support for the hypothesis that estrogen induction of intracellular progestin receptors is a prerequisite for progesterone to facilitate the expression of female sexual behavior.
REFERENCES


SECTION III. SUPPLEMENTAL PROGESTERONE DELAYS HEAT TERMINATION AND THE LOSS OF PROGESTIN RECEPTORS FROM HYPOTHALAMIC CELL NUCLEI IN FEMALE GUINEA PIGS
Supplemental progesterone delays heat termination and the loss of progestin receptors from hypothalamic cell nuclei in female guinea pigs

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ABSTRACT

Previous studies indicate that the retention of hypothalamic nuclear progestin receptors parallels the expression of sexual receptivity in ovariectomized, hormonally-treated guinea pigs. In this study, the effect of a supplemental progesterone injection on heat termination and retention of nuclear progestin receptors was examined. Ovariectomized guinea pigs were injected with 50 μg progesterone 48 h after receiving 4 μg estradiol benzoate and were tested hourly for lordosis. Eight h after progesterone treatment, animals received either 500 μg progesterone or the oil vehicle. The supplemental progesterone injection delayed the termination of heat by more than 2 h compared to oil-injected animals.

The effect of supplemental progesterone on the retention of nuclear progestin receptors was then determined. Animals were killed 8, 10, or 14 h after the initial injection of progesterone, and cytosol and nuclear progestin receptor concentrations in mediobasal hypothalamus-preoptic area were determined. Supplemental progesterone further increased the level of nuclear progestin receptors measured at 10 h, and at 14 h these levels were still elevated above baseline. In contrast, nuclear progestin receptor levels in animals which received only the initial progesterone injection had returned to baseline by 14 h, a time when similarly-treated animals were no longer receptive. These results suggest that the mechanism by which heat termination occurs may involve loss of nuclear progestin receptors.
INTRODUCTION

Estradiol and progesterone interact to activate a period of sexual behavior in intact, estrous-cycling as well as ovariectomized (OVX) guinea pigs (11, 15, 29). This period of sexual behavior (heat), which is characterized, in part, by the elicitation of the lordosis posture by mating stimulation, has a duration of about 8-10 hours (31). While the mechanisms regulating the onset of sexual behavior have been well studied, little is known of the mechanisms responsible for its termination.

It has been hypothesized that neural intracellular progestin receptors mediate the activation of sexual receptivity by estradiol and progesterone (3, 6, 8, 16). In OVX guinea pigs, estradiol treatment increases the level of cytoplasmic progestin receptors in certain regions of the brain, primarily the mediobasal hypothalamus (MBH) and preoptic area (POA) (3, 27). These regions are thought to contain the principal sites for the facilitation of female sexual receptivity by estradiol and progesterone in rodents (20, 22, 24). Administration of progesterone to OVX, estrogen-treated guinea pigs results in a transient increase of progestin receptors in the hypothalamic cell nuclear fraction while concurrently decreasing the level of receptors in the cytosol (4).

This transient accumulation of nuclear progestin receptors correlates with the expression of sexual receptivity; nuclear progestin receptor levels increase prior to the initiation of sexual receptivity.
and decline to baseline levels as the receptive period terminates (4). This correlation suggests that heat termination may result from the loss of progestin receptors from the cell nucleus. Based on this hypothesis, hormonal manipulations that delay heat termination should also delay the decline in nuclear progestin receptor levels. In support of this hypothesis, we recently confirmed previous studies (11, 14) showing that a supplemental injection of estradiol at the time of progesterone treatment increased the duration of heat (1). Furthermore, we found that 12 h after supplemental estradiol treatment, hypothalamic nuclear progestin receptor concentrations were 55% greater than in animals which received only progesterone. In the present study, we determine the effects of a supplemental progesterone injection on heat termination and the retention of nuclear progestin receptors.

In previous studies measuring nuclear progestin receptors by an in vitro exchange assay, we used the potent synthetic progestin, R 5020, as the labeled ligand (1, 2, 4). R 5020 is preferred over progesterone because it binds specifically to progestin receptors with a higher affinity than progesterone and dissociates more slowly from the receptor (3, 16). However, in order to increase the sensitivity of the assay, (³H)progesterone, which is available at a higher specific activity than (³H)R 5020, was used in this study to detect nuclear progestin receptor levels.
MATERIALS AND METHODS

Animals

Adult, Hartley strain female guinea pigs (Biolab Corp., St. Paul, MN) were group-housed in 38 X 48 X 20 cm cages with lights on from 0600 - 2000 h. Guinea pig chow (Teklad Mills, Madison, WI) and water were freely available. All animals were OVX as described previously (8) and had received estradiol and progesterone treatment in previous experiments. A 3 week interval from the last treatment was allowed before animals were used in this study. All hormone injections were subcutaneous in a 0.1 ml volume. Estradiol benzoate (EB) was dissolved in sesame oil, and progesterone was dissolved in sesame oil containing 15% benzyl benzoate and 5% benzyl alcohol. In experiments validating assay procedures, guinea pigs were injected with 1 mg progesterone 48 h after receiving 10 μg EB. Animals were killed 2 h after progesterone treatment.

Behavioral Testing

Guinea pigs were tested hourly for lordosis using manual stimulation with the experimenter blind to treatment groups (30). Testing began just prior to the initial progesterone injection and continued until all animals were nonresponsive. An animal was considered responsive if she held the lordosis posture for a minimum of 1 sec during two consecutive hourly tests. Heat was considered terminated in a responsive animal when she failed to respond during two
consecutive tests. Latency to lordosis, maximum lordosis duration, and heat duration were calculated as described previously (5, 13). Latency to lordosis is the time interval from progesterone administration to the first lordosis response. Heat duration is the time in hours from the first lordosis response to the last lordosis response with both the hour of first and last lordosis counted. Maximum lordosis duration is the maximum amount of time that an animal held the lordosis posture. Time of last lordosis is the hour after the initial progesterone injection in which the animal last responded.

Combined Cytosol and Nuclear Progestin Receptor Assay

Cytosol and nuclear progestin receptor levels were measured as described previously (1, 2). Guinea pigs were decapitated and their brains rapidly removed. The MBH, PDA (8), and cerebral cortex (CTX) (3) were dissected on a chilled block as described previously. The MBH and PDA were pooled for each animal. All subsequent procedures were at 0-4 °C. Tissues were placed in glass tissue grinders containing 1.0 ml Buffer TEGST (10 mM Tris-HCl, 1.5 mM Na₃EDTA, 0.32 M sucrose, 10% glycerol, 12 mM monothioglycerol, pH 7.2) and homogenized with a teflon pestle with appropriate clearance for cell nuclear isolation. Homogenates were transferred to 12 X 75 mm polycarbonate tubes and the tissue grinders were rinsed with 0.5 ml TEGST. The homogenates were then centrifuged at 1,000 g for 5 min. Cytosol extract was obtained by centrifuging the supernatant at 48,000 g for 30 min. The pellet from the first centrifugation was washed twice with 2.0 ml Buffer A (0.32 M sucrose, 1 mM KH₂PO₄, 1 mM MgCl₂, 10% glycerol, 12 mM monothioglycerol,
pH 7.2) by centrifuging at 1,000 g and decanting the supernatant. The crude nuclei were then purified by adding 0.3 ml A + 1.0 ml Buffer B (2.15 M sucrose, 1 mM KH$_2$PO$_4$, 1 mM MgCl$_2$, 10% glycerol, pH 7.2), vortexing, and centrifuging at 25,000 g for 20 min. The pellicle and supernatant were removed and the walls of the tube dried leaving the purified cell nuclei.

To remove potentially competing steroids from the cytosol (2), 400 μl samples of cytosol were applied to 4.5 × 68 mm Sephadex LH-20 columns (Pharmacia Fine Chemicals, Piscataway, NJ). Immediately after running into the column the macromolecular fraction was eluted with 700 μl TEGST. Two hundred and fifty μl aliquots of filtered cytosol were incubated with 50 μl TEGST containing 1% ethanol (final concentration) and (³H)R 5020 (New England Nuclear, Boston, MA; Specific activity = 87 Ci/mmol) +/- 100-fold excess of unlabeled R 5020. A final concentration of 0.4 nM (³H)R 5020 was used because this concentration results in binding almost exclusively to the progestin receptor with almost no specific binding to blood contaminants or low affinity binders (3).

Following a 12-16 h incubation at 0-4 °C, bound (³H)R 5020 was separated from free by gel filtration with Sephadex LH-20 columns equilibrated with TEGST. Two hundred and fifty μl samples of incubate were applied to the column and washed in with 150 μl TEGST. Thirty min after applying the sample the protein peak was eluted into scintillation vials with 700 μl TEGST. Five ml toluene-based scintillation fluid containing 33% Triton X-100 were added and radioactivity was counted on a Packard Tri-Carb Model 300C spectrophotometer at an efficiency of 41%.
Specific binding was calculated by subtracting non-specific binding (with unlabeled R 5020) from total binding (without unlabeled R 5020). Protein concentration of the filtered cytosol extract was determined by the protein dye-binding method of Bradford (7). Receptor concentrations are expressed as femtomoles ($^3$H)R 5020 specifically bound per mg protein.

For the nuclear progestin receptor assay, the cell nuclear pellet was dispersed in 280 µl Buffer TEBT (10 mM tris-HCl, 1.5 mM Na₂EDTA, 10% glycerol, 12 mM monothioglycerol, pH 7.4). After 15 min, an equal volume of Buffer TEGTK 1.0 (TEBT + 1.0 M KCl, pH 7.4) was added and the tube contents vortexed. Following a 15-30 min incubation, the nuclei were centrifuged at 48,000 g for 5 min. Two hundred and fifty µl samples of the supernatant (nuclear extract) were incubated with 50 µl Buffer TEGTK 0.5 (TEBT + 0.5 M KCl) containing 1% ethanol (final concentration) and ($^3$H)progesterone (New England Nuclear, SA = 165-179 Ci/m mole) +/- a 100-fold concentration of unlabeled R 5020. A final concentration of 5 nM ($^3$H)progesterone was used. Following a 12-16 h incubation at 0-4 °C, bound ($^3$H)progesterone was separated from free as described for the cytosol receptor assay except that Buffer TEGTK 0.5 was used and the macromolecular fraction was eluted 20 minutes after sample application. The DNA content of the nuclear pellet was determined by the method of Burton (10). Receptor concentrations are expressed as femtomoles ($^3$H)progesterone specifically bound per mg DNA.
Statistical Analysis

Results are presented as mean +/- S.E.M. Differences between group means were considered statistically significant if p<0.05 as determined by Student's t test. Probability levels given are for two-tailed comparisons unless stated otherwise.
RESULTS

Saturation Curve and Scatchard Analysis

A Scatchard analysis (25) of \( ^{3}H \)progesterone binding in MBH-POA nuclear extract was conducted to verify that binding is to a high affinity, saturable binder. Five OVX guinea pigs were injected with 1 mg progesterone 2 days after receiving 10 \( \mu \)g EB. Two h later, animals were killed and MBH-POA nuclear progestin receptors assayed as described except that nuclear extract was pooled and incubated with a range of \( ^{3}H \)progesterone concentrations (0.1-20.0 nM) with or without a 100-fold concentration of unlabeled R 5020.

Scatchard analysis revealed that \( ^{3}H \)progesterone binds with high affinity (apparent dissociation constant = 0.33 nM) to a saturable binder in MBH-POA nuclear extract (Figure 1a). Binding to a lower affinity binder (apparent dissociation constant = 8.0 nM) was also detected. Similar results were obtained in the CTX (data not shown). As determined by the saturation curve (Figure 1b), a concentration of 5 nM \( ^{3}H \)progesterone saturates the progestin receptor pool while minimizing binding to nonspecific and low-affinity binders.

Time Course of Binding

To determine the time course of the binding reaction, 6 OVX guinea pigs were treated with EB and progesterone. Two h after the injection of progesterone, MBH-POA nuclear extract was obtained, pooled, and incubated with \( ^{3}H \)progesterone with or without unlabeled R 5020 for
Figure 1. Scatchard Analysis of ($^3$H)progesterone binding

A: Scatchard representation of specific ($^3$H)progesterone binding in MBH-POA nuclear extract

Nuclear extract obtained from OVX guinea pigs treated with EB and progesterone was incubated with a range of ($^3$H)progesterone concentrations (0.1-20.0 nM) +/- 100-fold concentration of R 5020.

B: Saturation curves of data represented in Scatchard plot

(■) = total binding (●) = specific binding, (▲) = nonspecific binding
$K_d = 0.33 \text{nM}$

$K_d = 8.00 \text{nM}$
1, 2, 4, 8, or 16 h. Bound (³H)progesterone was separated from free as described.

The *in vitro* binding reaction is complete by 8 h with no loss of specific binding detected by 16 hours of incubation (Figure 2).

**Dissociation of Bound (³H)Progesterone in Contact with Sephadex LH-20**

The amount of dissociation of specifically bound (³H)progesterone and (³H)R 5020 in contact with Sephadex LH-20 was determined by applying 250 µl samples of MBH-POA nuclear extract incubate to Sephadex LH-20 columns. Immediately after running into the column, samples were washed in with 150 µl buffer. Macromolecular fractions were eluted with 700 µl buffer 15, 25, and 35 min after sample application.

The (³H)R 5020-progestin receptor complex is relatively stable during separation from free ligand (Figure 3). (³H)Progesterone-progestin receptor complexes are less stable with a 15% and 36% decrease in specific binding seen at 25 and 35 min respectively. Therefore, to minimize loss of binding, columns were eluted 20 min after sample application.

**Supplemental Progesterone Delays Heat Termination**

To test if supplemental progesterone can delay heat termination in estrogen-treated OVX guinea pigs, animals were treated with 50 µg progesterone (0800 h) 48 h after receiving 4 µg EB. Animals were tested hourly for lordosis. Eight h after the injection of progesterone,
Figure 2. Time course of (3H)progesterone binding

Nuclear extract obtained from OVX guinea pigs treated with EB and progesterone was incubated with 5 nM (3H)progesterone +/- 500 nM R 5020 for 1, 2, 4, 8, or 16 h. (■) = total binding, (●) = specific binding, (▲) = nonspecific binding.
Figure 3. Stability of specifically bound (³H)progesterone and (³H)R 5020 in contact with Sephadex LH-20

Aliquots of nuclear extract incubates were applied to columns of Sephadex LH-20. The macromolecular fraction was eluted 15, 25, or 35 min after sample application. 100% = levels detected at 15 min.
animals were matched for latency and divided into two groups. Animals in one group received an injection of 500 μg progesterone, ten times the initial dose, while animals in the other group received the oil vehicle. Testing continued until all animals were nonresponsive.

Heat termination was delayed by 2 h in animals treated with the supplemental progesterone injection compared with control animals (p<0.05, Table 1). No difference in maximum lordosis duration after the supplemental progesterone injection was observed between groups.

Supplemental Progesterone Delays the Loss of Nuclear Progestin Receptors

To determine if the supplemental progesterone injection delays the loss of nuclear progestin receptors, cytoplasmic and nuclear progestin receptor levels were determined in OVX guinea pigs treated as in the behavioral experiment. A third group which received EB and oil injections rather than progesterone was included to establish baseline levels of nuclear progestin receptors. Animals were sacrificed and MBH-POA and CTX progestin receptor levels were determined at 8, 10, and 14 h after the initial injection of progesterone (0, 2, and 6 h after the second progesterone injection).

MBH-POA nuclear progestin receptor levels were still elevated 8 h after the initial injection of progesterone (p<0.005, compared to baseline levels; Figure 4b). The injection of supplemental progesterone increased the level of nuclear progestin receptors measured at 10 h (p<0.005). At 14 h, a time when 80% of similarly treated animals were still in heat (Figure 4a), nuclear progestin receptor levels were still
<table>
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*Forty-eight hours prior to receiving the initial injection of progesterone, OVX guinea pigs were treated with 4 µg estradiol benzoate. At 0 h, animals were tested for lordosis and injected with 50 g progesterone (PROG). Eight h later, animals were matched for latency to lordosis, and injected with either 500 µg progesterone or the oil vehicle (Veh). Values reported are means +/- S.E.M.

*After injection at 8 h.

*p < 0.05, Student's t-test, one-tail.

**p < 0.05, Student's t-test.
Figure 4. MBH-POA progestin receptor levels

A: Percent of guinea pigs responding during tests for lordosis (data from Table 1)

Animals were treated with 50 µg progesterone 48 h after receiving 4 µg EB. Eight h later, animals received a supplemental injection of 500 µg progesterone or oil (At 14 h: p=0.02, two-tailed Fisher's Exact Probability Test).

B: MBH-POA nuclear progestin receptor levels in guinea pigs treated as in A

Animals received 50 µg progesterone or oil 48 h after receiving 4 µg EB. Eight h later, animals were sacrificed or received 500 µg progesterone or oil and were sacrificed 10 or 14 h after the initial progesterone injection (n = 5-9).

C: MBH-POA cytoplasmic progestin receptor levels measured in the animals described in B
Specifically bound \(^{3}H\)-R 5020, fmol/mg protein

Specifically bound \(^{3}H\)-progesterone, fmol/mg DNA

% responding

Time after initial progesterone injection, h

0 10 20 30 40 50 60 70 80 90 100

0 50 100

Graph showing changes in specifically bound \(^{3}H\)-R 5020, \(^{3}H\)-progesterone, and % responding over time after initial progesterone injection.
elevated in supplemental progesterone-treated guinea pigs (p<0.025). In contrast, receptor levels in animals treated with only the initial injection of progesterone have returned to baseline by 14 h, a time when 80% of similarly treated animals were no longer in heat.

As expected, supplemental progesterone further decreased the level of MBH-POA cytoplasmic progestin receptors (p<0.01; Figure 4c). In animals receiving only the initial injection of progesterone, cytoplasmic progestin receptors were indistinguishable from baseline levels at 10 and 14 h.

In CTX, supplemental progesterone further decreased the level of available cytoplasmic progestin receptors (p<0.01, 10 h; Figure 5). No other significant differences were detected in cytosol receptor levels after supplemental progesterone injection nor were any differences noted in nuclear receptor levels.
Figure 5. Nuclear (A) and cytoplasmic (B) progesterin receptor levels in CTX from the same animals described in Figure 4.
Specifically bound (\(^3\text{H}\))-R 5020, fmol/mg protein

Specifically bound (\(^3\text{H}\))-progesterone, fmol/mg DNA

Time after initial progesterone injection, h
DISCUSSION

The experiments validating the use of (3H)progesterone in the nuclear exchange assay indicate that (3H)progesterone binds with different affinity to two components in MBH-POA nuclear extract. A similar finding has been reported with (3H)R 5020 binding in hypothalamic nuclear extract (28). The dissociation constant obtained for the high-affinity binding component is similar to that obtained using (3H)R 5020 (4, 28). The binding capacity of this component has been shown to vary with behaviorally relevant hormonal treatments (4); however, the identity and function of the low-affinity component is unknown.

The period of sexual behavior in intact estrous-cycling guinea pigs is temporally linked with ovulation. The onset and termination of the receptive period parallels the rise and fall of the preovulatory surge of progesterone (12). However, in OVX estrogen-treated guinea pigs, heat termination occurs despite prolonged elevation of blood progesterone levels, indicating that the availability of progesterone is not the primary determinant of heat duration (19). We recently found that in OVX guinea pigs implanted with Silastic capsules containing 10% estradiol and capsules containing various lengths of crystalline progesterone, heat termination occurred despite the continued presence of estradiol and progesterone (9).

The results of the present study confirm the work of Morin and Feder (19) showing that progesterone can influence the time at which
heat termination occurs in OVX guinea pigs. In their study, multiple injections of 0.1 mg progesterone administered every 3 h prolonged the duration of heat by more than 2 h. We found that a supplemental progesterone injection, ten times the dose of the initial injection, delayed heat termination by more than 2 hours. In addition, supplemental progesterone was found to increase the cell nuclear retention of MBH-POA progestin receptors. In animals receiving only the initial progesterone injection, nuclear progestin receptor levels returned to baseline by 14 h, a time when similarly-treated animals were no longer receptive. In contrast, animals which received supplemental progesterone were still sexually receptive at 14 h and still had an elevated level of nuclear progestin receptors. These findings are consistent with the hypothesis that heat termination results from the loss of progestin receptors from the cell nucleus.

Six hours following the supplemental injection of progesterone, nuclear progestin receptor levels were only slightly elevated. However, if the initial progesterone injection was not given, nuclear progestin receptor levels would not be expected to return to baseline until 12-24 h after injection (4). This raises the question of what factors regulate the retention of progestin receptors in cell nuclei. One such factor may be circulating progesterone levels. It seems likely that as the circulating level of progesterone decreases, no further nuclear accumulation of progestin receptors would occur. Indeed, experiments by Blaustein and Feder (4), and McGinnis et al. (17), show an apparent correlation between blood progesterone levels and nuclear progestin receptor retention. However, in our experiments, blood progesterone
levels would not be expected to return to baseline until 12-24 h following the supplemental injection of progesterone (4). This suggests that circulating progesterone is not the only factor which regulates nuclear progestin receptor retention.

Another factor which may regulate nuclear progestin receptor retention is the concentration of cytoplasmic progestin receptors. As cytoplasmic progestin receptor levels decrease because of translocation to cell nuclei and declining estrogen-priming action, the level of nuclear progestin receptors decreases (1, 2). We found that the supplemental progesterone injection further decreased cytoplasmic progestin receptor levels and that as nuclear receptor levels declined, the level of cytoplasmic receptors was not replenished (4, 17). It is possible that the magnitude of this sustained decrease in cytoplasmic progestin receptors prevented further translocation of receptors to cell nuclei. This could then have resulted in the decline of nuclear progestin receptors even in the presence of elevated circulating progesterone levels. This may explain how termination of the receptive period can occur despite maintained high levels of progesterone.

Although no significant differences were noted in nuclear accumulation and retention of progestin receptors in CTX following supplemental progesterone treatment, the pattern observed is similar to that seen in the MBH-POA. Any comparison of the effects of supplemental progesterone on estrogen-induced vs. nonestrogen induced receptors would require further study.

While the results of the present study suggest that termination of heat may result from the loss of nuclear receptors from neurons
involved with activating lordosis, there are other mechanisms by which heat termination can occur. Reading and Blaustein (23) recently found that mating-induced heat termination in rats is independent of neural progestin receptors. Two h of mating stimulation which prematurely terminated heat had no apparent effect on the level of either cytoplasmic or nuclear progestin receptor concentrations. Heat termination occurred despite elevated nuclear progestin receptor levels. The mechanism by which mating-induced heat abbreviation occurs, therefore, may involve a more direct effect on neurons involved in lordosis and may be unrelated to the mechanism by which heat terminates in the non-mated animal.

Sodersten et al. (26) have demonstrated that vasopressin can terminate heat in OVX rats treated with estradiol and progesterone, and have suggested that a circadian increase in brain vasopressin may play a role in terminating heat in the intact rat. However, if a diurnal increase in vasopressin causes termination of receptivity, it follows that animals should fail to show or show diminished levels of lordosis during the light portion of their light-dark cycle. We have not observed such an effect in our animals (Brown and Blaustein, unpublished observations, Iowa State University). Therefore, further study is necessary to determine if vasopressin plays any role in the termination of heat.

The mechanisms by which a decline in nuclear progestin receptor concentration may lead to termination of sexual receptivity are unknown. Rainbow et al. (21, 22) have shown that progesterone's effects on lordosis are protein-synthesis dependent, suggesting that progesterone
may activate sexual receptivity via a genomic mechanism. Perhaps as nuclear progestin receptor concentrations decrease, the progesterone-induced protein changes dissipate leading ultimately to the termination of the receptive period.

The results of this study suggest that in intact, non-mated guinea pigs, heat termination may result from a decline in nuclear progestin receptor concentration. This decline in nuclear progestin receptors may result from either a decline in circulating progesterone levels, a decrease in cytoplasmic progestin receptor concentration in target neurons, or both. Further studies are planned to investigate progestin receptor dynamics and how they relate to heat termination in the intact animal.
REFERENCES


SECTION IV. LOSS OF HYPOTHALAMIC NUCLEAR-BOUND PROGESTIN RECEPTORS:
FACTORS INVOLVED AND THE RELATIONSHIP TO HEAT TERMINATION
IN FEMALE GUINEA PIGS
Loss of hypothalamic nuclear-bound progestin receptors:
Factors involved and the relationship to heat termination
in female guinea pigs

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ABSTRACT

The hypothesis that the termination of sexual receptivity (heat) in female guinea pigs results from loss of progestin receptors from hypothalamic cell nuclei was tested. First, we attempted to find an optimal dose of progesterone that would result in a prolonged period of sexual receptivity. Ovariectomized guinea pigs were implanted with 10% estradiol capsules. Forty h later, each received one of several sized progesterone capsules before being tested hourly for lordosis. Surprisingly, none of the progesterone doses resulted in delayed heat termination. In order to determine whether elevated levels of estradiol and progesterone maintain elevated levels of nuclear progestin receptors despite the lack of effect on heat duration, animals were treated as described above except that only one size progesterone capsule (3.0 cm) or an empty capsule was implanted. Despite elevated serum progesterone concentrations, nuclear progestin receptor levels decreased gradually and approached control levels at about the same time as heat termination had occurred in similarly-treated animals. Cytosol progestin receptor levels decreased following progesterone treatment and remained lowered at all times measured. In order to further investigate the relationship between blood progesterone concentrations and retention of nuclear progestin receptors we decreased blood progesterone concentrations by removing progesterone capsules 2 h following insertion. Nuclear progestin receptor levels declined gradually concurrent with a decline in serum progesterone levels in animals exposed to progesterone capsules.
for only 2 h. In animals exposed to capsules continuously, nuclear progestin receptor levels again decreased but at a slightly slower rate. In order to test the effect of progesterone capsule removal on female sexual behavior, ovariectomized guinea pigs were treated as described and tested hourly for lordosis. Fewer animals in the group exposed to progesterone capsules for 2 h became sexually receptive as compared to animals continuously-exposed to progesterone capsules. Of those animals that did respond, heat termination had occurred by the same time that nuclear progestin receptor levels had returned to control levels in similarly-treated animals. These experiments support the hypothesis that heat termination results from the loss of progestin receptors from hypothalamic cell nuclei. In addition, they demonstrate that circulating progesterone levels play a role in regulating nuclear progestin receptor retention.
INTRODUCTION

Substantial experimental evidence exists suggesting that the hormonal activation of female sexual behavior in several rodent species is mediated by hypothalamic intracellular progestin receptors. Estradiol treatment in ovariectomized (OVX) rats and guinea pigs increases the concentration of progestin receptors in cytosols obtained from several brain regions, including the mediobasal hypothalamus (MBH) and preoptic area (POA) (16, 17, 20, 22, 26). These regions have been shown to contain the primary site for the hormonal induction of female sexual behavior by estradiol and progesterone (17, 21, 23-25).

Administration of progesterone to estrogen-pretreated animals results in a transient elevation of progestin receptor levels measured in hypothalamic cell nuclei, and a sustained decrease in receptor levels measured in cytosol (6, 19). This sustained decrease in cytosol progestin receptor levels appears to cause refractoriness to further stimulation by progesterone (2, 3, 5).

The transient elevation of nuclear progestin receptors following progesterone injection has been shown to correlate with the expression of sexual receptivity in guinea pigs; nuclear progestin receptor levels increase prior to the initiation of sexual receptivity and return to baseline as the receptive period ends. This correlation has led to the hypothesis that the termination of sexual receptivity (heat) results from the loss of progestin receptors from hypothalamic cell nuclei (6, 9). Perhaps as the concentration of progesterone-progestin receptor
complexes in cell nuclei decreases, the cellular effects of progesterone diminish, resulting in the loss of sexual responsiveness.

Based on this hypothesis, hormonal manipulations that affect the duration of the period of sexual receptivity should also affect the retention of progestin receptors in hypothalamic cell nuclei. In support of this hypothesis, we have recently reported that a supplemental injection of progesterone, administered to hormonally-treated OVX guinea pigs, not only delays the termination of heat, but also delays the loss of progestin receptors from cell nuclei (9). Furthermore, we have shown that a similar delay results from supplemental injection of estradiol (2).

In some circumstances, estradiol and progesterone administered in a continuous fashion, can activate extended periods of sexual behavior in female hamsters (15). Lisk (15) infused progesterone at three different rates into OVX hamsters treated with chronically-placed estrogen pellets. Infusion of progesterone at the slow rate failed to activate sexual behavior, while infusion at the high rate activated periods of sexual behavior of normal duration. Infusion at the moderate rate, however, resulted in animals remaining sexually receptive for an extended period of time. Based on our hypothesis that heat duration is linked to the retention of nuclear progestin receptors, a possible explanation for these results is that at the optimum level of progesterone, nuclear progestin receptor levels may remain elevated for an extended period of time. This could occur by a balance being attained between receptor induction (by estradiol), receptor transformation (by progesterone), and receptor inactivation. As long as
such a balance is maintained, nuclear progestin receptor levels might remain elevated, and the period of sexual receptivity may be extended. Because this phenomenon provides a unique test of the hypothesis that heat termination results from the loss of progestin receptors from hypothalamic cell nuclei, we attempted to study it in OVX guinea pigs treated with estradiol and progesterone administered in a continuous fashion.
METHODS

Animals

Adult, Hartley strain female guinea pigs obtained from Biolab Corp. (St. Paul, MN; Experiment 1 and 2) or from Camm Research Inst. (Wayne, NJ; Experiment 3) were group housed in 51 X 56 X 23 cm cages with lights on from 0600 - 2000 h. Guinea pig chow (Experiment 1: Teklad, Madison, WI; Experiment 2 and 3: Ralston Purina, St. Louis, MO) and water were freely available. All animals were bilaterally ovariectomized 2-3 weeks prior to use under conditions described previously (8).

Hormones

Five mm capsules containing 10% crystalline estradiol-17β were constructed from Silastic Medical Grade tubing (id: 1.47 mm, od: 1.96 mm; Dow Corning, Midland, MI) as described previously (4). Capsules containing crystalline progesterone or progesterone mixed with cholesterol were constructed in a similar manner (id: 3.35 mm; od: 4.70 mm; length: 0.5, 1.0, 1.5, or 3.0 cm). To stabilize the diffusion of steroid, capsules were incubated in host female guinea pigs for 24 h just prior to their insertion into experimental animals. At the time of capsule insertion, animals were lightly anesthetized with methoxyflurane (Metofane; Pitman-Moore, Washington Crossing, NJ) and capsules were placed subcutaneously in the nape of the neck (estradiol) or in the axillary region (progesterone).
Estradiol benzoate was dissolved in sesame oil and was injected subcutaneously in a 0.1 ml volume.

Behavioral Testing

Guinea pigs were tested hourly for lordosis by manually stimulating the flanks and perineal area and measuring the length of time the lordosis posture was held (28). Testing began just prior to the insertion of progesterone capsules and continued until all animals were unresponsive. All tests were conducted with the experimenter blind to the assigned treatment group. An animal was considered responsive if the lordosis posture was held for at least 1 sec during two consecutive tests. Heat was considered terminated in a responsive animal when no response was noted during two consecutive tests. Latency to lordosis, maximum lordosis duration, heat duration, and time of last lordosis were calculated as described previously (9, 13).

Combined Cytosol and Nuclear Progestin Receptor Assay

Guinea pigs were decapitated and their brains rapidly removed. The mediobasal hypothalamus (MBH) and preoptic area (POA) were dissected on a chilled block and pooled for each animal (B). Cytosol and nuclear progestin receptor levels were measured as described previously with minor modifications (2, 3). All procedures were conducted at 0-4 °C. Tissues were placed in a glass tissue grinder containing 1.0 ml buffer A (0.32 M sucrose, 1 mM KH2PO4, 1 mM MgCl2, 10% glycerol, 12 mM monothioglycerol, pH 7.2) and were homogenized with a Teflon pestle with
appropriate clearance for cell nuclear isolation (18). Homogenates were transferred to 12 X 75 mm polycarbonate tubes and the tissue grinders were rinsed with 0.5 ml \( A_T \). The homogenates were centrifuged at 1000 X g for 5 min. Cytosol extract was obtained by centrifuging the resulting supernatant at 48,000 X g for 30 min. The pellet from the first centrifugation was washed twice by adding 2 ml \( A_T \) and centrifuging at 1000 X g for 5 min. The crude nuclear pellet was then purified by dispersing in 0.3 ml \( A_T \) + 1.0 ml buffer B (2.15 M sucrose, 1 mM \( K_2HPO_4 \), 1 mM \( MgCl_2 \), 10% glycerol, pH 7.2) and centrifuging at 25,000 X g for 20 min. The pellicle and supernatant were removed and the walls of the tube were dried.

For the cytosol assay, 300 µl samples of cytosol were applied to 4.5 X 68 mm columns of Sephadex LH-20 (equilibrated with buffer \( A_T \)) to remove potentially competing steroids (3). Immediately after running into the column, the sample was washed in with 100 µl \( A_T \) and the macromolecular fraction was eluted with 700 µl \( A_T \). Aliquots of 250 µl filtered cytosol were incubated with 50 µl \( A_T \) containing 1% ethanol (final concentration) and (17α-methyl-\( ^{3} \)H)R 5020 (New England Nuclear, Boston, MA; spec. act. = 87 Ci/mmol) +/- 250-fold concentration of unlabeled progesterone. A final concentration (in 300 µl) of 0.4 nM (\( ^{3} \)H)R 5020 was used because this concentration results in binding almost exclusively to the progestin receptor with almost no specific binding to blood contaminants or low affinity binders (5, 16).

Following an overnight incubation at 0-4 °C, bound (\( ^{3} \)H)R 5020 was separated from free by gel filtration on Sephadex LH-20 columns (Pharmacia Fine Chemicals, Piscataway, NJ). Samples of incubate
(250 μl) were applied to the column and washed in with 150 μl of buffer A7. Thirty min after applying the sample, the macromolecular fraction was eluted into scintillation vials with 700 μl of A7. Five ml of scintillation fluid (0.17 g Bis MSB + 5.5 g POP / liter scintillation grade toluene (Fisher Scientific) containing 33% Triton X-100) were added and radioactivity was counted on a Packard Tri-Carb Model 300C spectrophotometer at an efficiency of 40%. Specific binding was calculated by subtracting nonspecific binding (with unlabeled progesterone) from total binding (without unlabeled progesterone). Protein concentration was determined by the dye-binding method of Bradford (7), using bovine serum albumin as the standard. Results are expressed as femtomoles (3H)R 5020 specifically bound per mg cytosol protein.

For the nuclear receptor assay, the purified nuclear pellet was dispersed with 285 μl buffer TEGT (10 mM Tris-HCl, 1.5 mM Na2EDTA, 10% glycerol, 12 mM monothioglycerol, pH 7.4). After 15 min, an equal volume of buffer TEGT-K1.0 (TEGT + 1 M KCl, pH 7.4) was added. The tube contents were vortex-mixed and incubated for 15 min. The nuclei were then centrifuged at 48,000 X g for 5 min. Samples (250 μl) of the supernatant (nuclear extract) were incubated with 50 μl buffer TEGT-K0.5 (TEGT + 0.5 M KCl, pH 7.4) containing 1% ethanol (final concentration) and (3H)R 5020 +/- 250-fold concentration of unlabeled progesterone. A final concentration of 3.0 nM (3H)R 5020 was used (6). Following an overnight incubation at 0-4 °C, bound (3H)R 5020 was separated from free as described above except that buffer TEGT-K0.5 was used. The DNA content of the nuclear pellet was determined by the
method of Burton (11). Results are expressed as femtomoles \((^{3}H)R\,5020\) specifically bound per mg DNA.

Serum Progesterone Levels

At the time of decapitation, trunk blood was collected in 13 X 100 mm culture tubes and kept at 0-4°C overnight. Serum was obtained by centrifuging the blood at 1,000 X g for 20 min, and was stored at -20°C until time of assay. Serum progesterone was measured by a modification of the technique by Abraham et al. (1).

Extraction

Samples (200 µl) of serum were placed into 13 X 100 mm culture tubes immediately after thawing. Fifty µl of assay buffer (0.1% gelatin-phosphate buffered saline) were added to each tube. To a duplicate set of samples, 50 µl buffer containing 2,000 cpm \((^{3}H)\)progesterone (New England Nuclear, Boston, MA; spec. act. = 115 Ci/mmol) were added to determine extraction efficiencies. Each sample was extracted twice with 1.5 ml cyclohexane (Fisher Scientific) by vortex-mixing each tube for 30 sec and centrifuging at 1500 X g for 5 min. The organic phases were collected in 12 X 75 mm culture tubes and the cyclohexane was dried off under vacuum.

Radioimmunoassay

The dried residue was redissolved in 2.0 ml assay buffer. Triplicate 500 µl aliquots were taken from the duplicate tubes to calculate extraction efficiency. Triplicate 500 µl aliquots from the
remaining tubes were transferred to 12 X 75 mm tubes for radioimmunoassay. One-hundred µl of antiserum (no. 337, provided by G. Niswender; formed against progesterone-11- bovine serum albumin) at a dilution of 1:400 in assay buffer were added. Thirty min later, 50 µl buffer containing 30,000 cpm (³H)progesterone were added to each tube. A standard curve was constructed with 0, 25, 50, 100, 200, 400, 800, 1600, 3200, and 6400 pg progesterone per 2 ml buffer. Triplicate 500 µl aliquots were taken from the standard curve tubes and treated as described above.

Following an overnight incubation at 0-4 °C, bound (³H)progesterone was separated from free by gel filtration on 4.5 X 68 mm Sephadex LH-20 columns. Three-hundred µl samples from each tube were loaded onto the columns. Immediately after running into the column bed, the samples were washed in with 100 µl buffer. Thirty min after sample application, the bound fraction of (³H)progesterone was eluted into scintillation vials with 700 µl buffer. Five ml of scintillation fluid were added and radioactivity was determined. Results are expressed as nanograms of progesterone per ml serum.

**Specificity**

The antiserum used has less than 1% crossreactivity with other steroids except: 11β-hydroxyprogesterone (139%), 5α-pregnane-3,20-dione (44%), 17α-hydroxyprogesterone (2.9%), corticosterone (2.2%), 20α-hydroxypregn-4-en-3-one (1.7%), pregnenolone (1.1%), and deoxycorticosterone (1.0%) (12).
Sensitivity

The sensitivity of the assay was approximately 50 pg progesterone (0.25 ng/ml).

Extraction Efficiency

The mean efficiency of extraction was 85.9% +/- 0.7 (mean +/- S.E.M.). No differences in extraction efficiencies between treatment groups were noted.

Accuracy

The accuracy of the assay was assessed by adding 100, 1000, or 5000 pg progesterone to serum obtained from OVX guinea pigs. The mean recoveries were 96.4, 1124.9, and 5262.5 pg. The percent deviations were -3.6%, +12.5%, and +5.3% respectively.

Precision

Because progesterone levels were determined in more than one assay, the precision within and between assays (intraassay and interassay variability) was evaluated by measuring progesterone levels in the same samples twice. The within assay coefficient of variation (C.V., as computed by Snedecor’s formula (1) for the 17 duplicates considered was 14.16% and the between assay C.V. for the 10 duplicates considered was 10.92%.
RESULTS

Experiment 1

In an attempt to find the optimal dose of progesterone that would result in greatly extended heat durations, Silastic capsules containing 10% estradiol were implanted into OVX guinea pigs. Forty hours later, animals received one of the following progesterone capsule treatments: one 5% 0.5 cm capsule, one 10% 0.5 cm capsule, one 25% 0.5 cm capsule, one 50% 0.5 cm capsule, one 100% 0.5 cm capsule, one 1.5 cm capsule, one 3.0 cm capsule, one 1.5 and one 3.0 cm capsule (4.5 cm), or two 3.0 cm capsules (6.0 cm). Animals were tested hourly for lordosis until all animals were unresponsive.

The percentage of animals in each treatment group that became sexually receptive is presented in Table 1. Treatment with 5% or 10% 0.5 cm progesterone capsules resulted in less than 20% of the animals becoming sexually receptive; therefore, these treatments were considered subthreshold and were not included in the data analysis.

The time at which heat termination occurred did not differ among treatment groups (time of last lordosis; Figure 1c; F(6,110) = 1.222, p>0.25); heat termination occurred at about the same time regardless of progesterone dose and despite the continued administration of estradiol and progesterone. While some differences between groups were noted in heat duration (F(6,110) = 2.896, p<0.025; Figure 1b), these differences can be attributed to differences in latency to lordosis (F(6,110) = 3.838, p<0.005; Figure 1a).
Table 1: Percentage of animals that became sexually receptive in each treatment group

<table>
<thead>
<tr>
<th>Progesterone Content</th>
<th>Capsule Length</th>
<th>n</th>
<th>Percent Responding</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 %</td>
<td>0.5 cm</td>
<td>12</td>
<td>17 %</td>
</tr>
<tr>
<td>10</td>
<td>0.5</td>
<td>11</td>
<td>10</td>
</tr>
<tr>
<td>25</td>
<td>0.5</td>
<td>11</td>
<td>82</td>
</tr>
<tr>
<td>50</td>
<td>0.5</td>
<td>18</td>
<td>83</td>
</tr>
<tr>
<td>100</td>
<td>0.5</td>
<td>18</td>
<td>83</td>
</tr>
<tr>
<td>100</td>
<td>1.5</td>
<td>18</td>
<td>89</td>
</tr>
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<td>100</td>
<td>3.0</td>
<td>33</td>
<td>88</td>
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<tr>
<td>100</td>
<td>4.5</td>
<td>13</td>
<td>92</td>
</tr>
<tr>
<td>100</td>
<td>6.0</td>
<td>27</td>
<td>78</td>
</tr>
</tbody>
</table>
Figure 1. Effect of estradiol and various doses of progesterone, administered via capsules, on sexual behavior in female guinea pigs

OVX guinea pigs were implanted with an estradiol capsule. Forty h later, animals received a progesterone capsule, one of several lengths and concentrations. Animals were tested hourly for lordosis until heat terminated. Bars represent group means ± S.E.M. Groups with different letters are significantly different from one another as determined by a protected Duncan’s Multiple Range Test (alpha = 0.05).
Experiment 2

The finding that heat termination occurs despite continued administration of estradiol and progesterone introduced the possibility of a dissociation between nuclear progestin receptor retention and heat duration. Perhaps elevated blood levels of these hormones maintained elevated nuclear progestin receptor levels, even though they did not prolong the duration of the period of sexual receptivity. Such a dissociation might suggest that some process other than loss of nuclear-bound progestin receptors is responsible for heat termination. Therefore, we measured the retention of hypothalamic nuclear progestin receptors following progesterone capsule insertion.

Guinea pigs were treated as in the previous experiment except that only one size progesterone capsule (3.0 cm) or an empty capsule was implanted. Animals were killed 4, 14, 18, or 20 h after capsule insertion, and progestin receptor levels in the cytosol and nuclear fractions of the MBH-POA were measured. Because this experiment was conducted at a site different from the first, a group of 8 OVX guinea pigs were treated as described and tested hourly for lordosis. Progestin receptor levels were compared to the level of sexual responsiveness in these animals.

By 4 h after capsule insertion, the concentration of nuclear progestin receptors was increased by approximately 200% ($t_{10} = 8.861$, $p<0.001$; Figure 2a) and the concentration of cytosol progestin receptors was decreased by 50% ($t_{10} = 8.768$, $p<0.001$; Figure 2b) as compared to control levels. The level of cytosol progestin receptors remained
Figure 2. Progestin receptor levels following 3.0 cm progesterone capsule insertion

A: Nuclear progestin receptor levels measured in MBH-POA

OVX guinea pigs were treated with a Silastic capsule containing 10% estradiol 40 h prior to receiving either a 3.0 cm progesterone capsule or an empty capsule. Receptor levels were measured 4, 14, 18, and 20 h after progesterone capsule insertion (n = 6 except at 18 h, n = 3).

B: Cytosol progestin receptor levels measured in the same animals described in A

C: Percent of guinea pigs, treated as described in A (progesterone-treated) that responded during hourly tests for lordosis (n = 7)
decreased in the progesterone-treated group for the duration of the experiment, and the level of nuclear progestin receptors declined at a steady rate. By 20 h after capsule insertion, a time when similarly-treated animals were no longer receptive (Figure 2c), the level of nuclear progestin receptors had declined to a level near control values ($t_{10} = 1.568, p > 0.10$).

To confirm that the decline in nuclear progestin receptor levels was not secondary to a decline in blood progesterone levels, serum progesterone levels were determined in the animals used for receptor assays. Serum progesterone levels were increased to 19.4 ng/ml by 4 h after insertion and remained elevated for the duration of the experiment (Figure 3). These results demonstrate that hypothalamic nuclear progestin receptor levels decline despite the continued presence of circulating estradiol and progesterone.

**Experiment 3**

In the previous experiment, we demonstrated that hypothalamic nuclear progestin receptor levels decline even in the presence of elevated blood progesterone levels. In order to further investigate the relationship between circulating progesterone levels and nuclear progestin receptor retention, we attempted to decrease blood progesterone concentrations by removing progesterone capsules 2 h following their insertion. OVX guinea pigs were injected with 10 μg estradiol benzoate (EB). Forty h later, animals received a 1.0 cm progesterone capsule or an empty capsule. Two h after capsule insertion, capsules were removed from one-half of the animals and
Figure 3. Levels of progesterone measured in serum obtained from animals treated as described in figure 2

Progesterone levels measured in animals treated with empty capsules did not vary with time, therefore, these values were pooled. n = 3-6.
sham-removed from the others (animals were anesthesized and the wound clip was changed). Animals were killed 3, 6, 10, or 18 h after capsule insertion (1, 4, 8, or 16 h after capsule removal), and MBH-POA progestin receptor levels and serum progesterone levels were measured. Removal of the progesterone source should result in rapid loss of circulating progesterone. If nuclear retention of progestin receptors is dependent upon circulating progesterone levels, then the loss of progestin receptors from cell nuclei should parallel the decline in blood progesterone levels. Furthermore, the loss of nuclear progestin receptors should occur at a faster rate in animals in which the progesterone capsule is removed.

In the group continuously-exposed to progesterone capsules, serum progesterone concentrations were elevated by 3 h after capsule insertion ($t_{(6)} = 11.644, p<0.001$; Figure 4), and remained elevated for the duration of the time period studied. In the group exposed to capsules for 2 h, serum progesterone levels gradually declined toward control levels following capsule removal. At 1, 4, and 8 h after capsule removal (3, 6, and 10 h after capsule insertion), serum progesterone levels were slightly elevated above control levels ($t_{(6)} = 5.463, p<0.001$; $t_{(6)} = 6.090, p<0.001$; and $t_{(6)} = 4.059, p<0.005$). By 16 h after capsule removal, levels had declined to control levels ($t_{(6)} = 0.615, p>0.50$).

By 3 h after capsule insertion, cytosol progestin receptor levels were decreased in both progesterone-treated groups as compared to control levels ($t_{(7)} = 2.867$ and $2.715, p<0.05$; Figure 5b). The concentration of cytosol progestin receptors remained at this level in
Figure 4. Levels of progesterone measured in serum obtained from OVX guinea pigs exposed to progesterone capsules for 2 h or for the duration of the experiment (in situ).

Animals were injected with 10 μg estradiol benzoate, and 40 h later, they received a 1.0 cm capsule containing progesterone or an empty capsule. Two h after capsule insertion, progesterone capsules were removed from one-half of the animals (n = 5).
TIME AFTER CAPSULE INSERTION (hours)

SERUM PROGESTERONE (ng/ml)

- IN SITU
- 2 HOURS
- EMPTY
Figure 5. Progestin receptor levels following progesterone capsule removal

A: Nuclear progestin receptor levels (mean +/- S.E.M.) measured in MBH-POA of guinea pigs described in figure 4

In animals treated with empty capsules, nuclear receptors were assayed only at 3 and 18 h and cytosol receptors only at 18 h (n = 5-12).

B: Cytosol progestin receptors measured in same animals as in A

C: Percent of similarly-treated guinea pigs that responded during hourly tests for lordosis
SPECIFICALLY BOUND $[^{3}\text{H}]R\ 5020$

(fmoles/mg protein)  

BOUND $[^{3}\text{H}]R\ 5020$

(fmoles/mg DNA)

TIME AFTER CAPSULE INSERTION (hours)

PERCENT RESPONDING
the group exposed to progesterone capsules for only 2 h, but continued to decrease in the group continuously exposed to progesterone (2 hour vs. in situ: \( t_{13} = 6.651, p < 0.001 \) at 10 h; \( t_{10} = 7.999, p < 0.001 \) at 18 h).

As was seen in the previous experiment, the concentration of nuclear progestin receptors declined even in animals exposed continuously to progesterone. In the group exposed to progesterone capsules for only 2 h, nuclear progestin receptors declined more rapidly at a rate parallel to the decline in circulating progesterone levels. At 6 and 10 h after capsule insertion, nuclear progestin receptor levels in animals exposed to progesterone capsules for 2 h were elevated above control levels, but were lower than levels measured in animals continuously-exposed to progesterone (\( t_{13} = 3.273, p < 0.01 \) and \( t_{21} = 4.312, p < 0.001 \)). By 18 h, nuclear receptor levels had declined to control levels in both groups.

In order to test the effect of progesterone capsule removal on female sexual behavior, OVX guinea pigs were treated as described above and tested for lordosis. Bullock (10) has reported that exposure to progesterone capsules for as little as 30 minutes activates sexual receptivity in 100% of all guinea pigs tested. If the presence of sexual behavior is determined by the concentration of nuclear progestin receptors, then based on the results of our receptor assays, we would predict that fewer animals exposed to progesterone capsules for 2 h would have responded with sexual receptivity as compared to animals continuously-exposed. In addition, because the concentration of nuclear progestin receptors had returned to baseline by 18 h in both groups, we
would predict that heat termination would have occurred in all animals by that time.

Heat termination occurred at about the same time in both groups; however, fewer animals became sexually receptive in the group exposed to progesterone capsules for only 2 h (Table 2). As seen previously, a relationship appears to exist between nuclear progestin receptor concentration and the number of animals displaying lordosis (Figure 5a & c). At 10 h after capsule insertion, fewer animals were sexually receptive in the group exposed to progesterone capsules for only 2 h. In similarly-treated animals, nuclear progestin receptor levels were lower than those measured in animals continuously-exposed to progesterone capsules. By 18 h after capsule insertion, a time when hypothalamic nuclear progestin receptor levels had returned to control levels in similarly-treated animals, heat termination had occurred in all animals tested.
Table 2. Effect of progesterone capsule removal on lordosis

<table>
<thead>
<tr>
<th>Group</th>
<th>Percent Responding*</th>
<th>Latency to Lordosis b (h)</th>
<th>Heat Duration b (h)</th>
<th>Heat Duration b (h)</th>
<th>Last Lordosis b (h)</th>
<th>Maximum Lordosis Duration b (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>In Situ</td>
<td>24</td>
<td>63%</td>
<td>6.00</td>
<td>8.40</td>
<td>5.25</td>
<td>13.40</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+/-0.44</td>
<td>+/-0.88</td>
<td>+/-0.98</td>
<td>+/-0.47</td>
</tr>
<tr>
<td>2 Hour</td>
<td>26</td>
<td>42%</td>
<td>6.18</td>
<td>8.18</td>
<td>3.46</td>
<td>13.36</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+/-0.52</td>
<td>+/-0.85</td>
<td>+/-0.88</td>
<td>+/-0.54</td>
</tr>
</tbody>
</table>

DVX guinea pigs were injected with 10 μg estradiol benzoate. Forty hours later, a 1.0 cm progesterone capsule was inserted for either 2 h or for the duration of the experiment (in situ). Hourly tests for lordosis began prior to capsule insertion and continued until heat had terminated. Values represent means +/- S.E.M.

*Based on responding animals only.

*X² = 3.93, p < 0.05.
DISCUSSION

The purpose of these experiments was to test the hypothesis that heat termination results from the loss of progestin receptors from hypothalamic cell nuclei. From this hypothesis, it follows that hormonal manipulations that maintain elevated levels of nuclear progestin receptors should maintain the period of sexual receptivity. In Experiment 1, guinea pigs were treated with estradiol and various doses of progesterone, administered in a continuous fashion, in an attempt to delay heat termination. However, despite the continuous administration of both hormones, and regardless of the progesterone dose tested, heat termination occurred at about the same time in all groups. The dose of progesterone used did affect latency to lordosis, but the mechanisms by which this effect occurred is not known.

The fact that heat termination occurred despite the continued administration of estradiol and progesterone introduced the possibility of a dissociation between elevated nuclear progestin receptor levels and sexual receptivity. Perhaps elevated blood levels of these hormones succeeded in maintaining elevated nuclear progestin receptor levels even though they did not prolong the duration of the period of sexual receptivity. Such a dissociation would negate our hypothesis and would indicate that some process other than the loss of nuclear progestin receptors is responsible for the termination of heat. The results from Experiment 2 demonstrate that nuclear progestin receptor levels do, in fact, decline in the presence of continued administration of estradiol.
and progesterone. Nuclear progestin receptor levels declined gradually and approached control levels at about the same time that heat had terminated in similarly-treated animals. Thus, these data support the hypothesis that heat termination may result from the loss of hypothalamic nuclear progestin receptors.

One possible explanation for our failure to find an optimal dose of progesterone that maintains sexual receptivity in guinea pigs for a prolonged period may be that a species difference in progestin receptor dynamics exists between hamsters and guinea pigs. As discussed earlier, Lisk (15) has demonstrated in hamsters that progesterone, when infused at an optimal rate, can activate a period of sexual receptivity of prolonged duration. It is possible that in hamsters progestin receptor processing occurs at a different rate than in guinea pigs. It is also possible that an optimal dose of progesterone exists in guinea pigs but that this dose was not included in the present study. The wide range of doses tested, however, makes this possibility unlikely.

The results obtained in this study raise the question of what factors are responsible for the loss of nuclear-bound progestin receptors. Experiment 3 was designed, in part, to test what effect decreasing blood progesterone levels has on nuclear retention of progestin receptors. It was found that upon removal of the progesterone source, blood progesterone levels and nuclear progestin receptor levels decreased at parallel rates. A similar finding has been reported in guinea pigs following the subcutaneous injection of progesterone (6), and in rats following an intravenous injection of progesterone (18). These studies demonstrate that under certain conditions, circulating
levels of progesterone influence the retention of nuclear progestin receptors. As blood levels of progesterone decrease, less progesterone is available to interact with progestin receptors, resulting in a decreased level of nuclear progestin receptors.

Although blood progesterone levels may be one source of regulation of nuclear progestin receptor retention, the results from Experiments 2 and 3 indicate that other factors also may be involved. One such factor may be the level of progestin receptors available for binding to progesterone (cytosol receptors). As the level of available progestin receptors decreases, fewer receptors are available to interact with progesterone. The result is a decreased level of nuclear-bound progestin receptors. It is possible that this is the mechanism by which nuclear progestin receptors are lost in the presence of high levels of circulating progesterone. Following progesterone capsule insertion, cytosol progestin receptor levels decreased (Experiment 2) and remained lowered even after nuclear progestin receptors had declined to baseline. This is particularly interesting since these animals should have elevated blood estradiol levels and would be expected to have on-going progestin receptor induction. This raises the possibility that progesterone may act to inhibit progestin receptor induction by estradiol or that prolonged progesterone exposure increases the rate of receptor degradation.

The loss of nuclear progestin receptors could also be due to mechanisms involved in the processing of progestin receptors. It is possible that there is a substance, perhaps regulated by progesterone action, that inactivates nuclear progestin receptors directly. It is
also possible that the loss of nuclear progestin receptors is a consequence of inactivation following the uncoupling of progesterone from its receptor. Because of the apparent link between nuclear progestin receptor retention and heat duration, studying the mechanisms underlying the loss of nuclear progestin receptors may yield important new information about the regulation of heat duration.

In Experiment 3, progesterone capsule removal resulted in lower concentrations of nuclear progestin receptors as compared to concentrations measured in animals continuously-exposed to capsules. As predicted from these receptor levels, fewer animals exposed to progesterone capsules for only 2 h became sexually receptive. At 10 h after capsule insertion, only 42% of the 2 h-exposed animals were sexually receptive compared to 63% of the continuously-exposed animals. At 18 h after capsule insertion, a time when nuclear progestin receptor levels had returned to control levels in both groups, none of the animals tested displayed sexual behavior.

These results are in partial contrast to those reported by Bullock (10). In that study, Bullock reported that exposure of estrogen-treated guinea pigs to Silastic capsules containing progesterone for 0.5 h, 2 h, or for the duration of the study, resulted in sexual behavior in 100% of the animals tested. In addition, he reported that the mean heat duration was the same in all treatment groups. These data were interpreted to indicate that heat duration was determined by the initial exposure to progesterone (within 30 min of capsule insertion). Bullock's conclusions, however, were based on the assumption that removal of the progesterone capsule was equivalent to
the removal of progesterone from the circulation. Our data indicate that this assumption may be false. Upon removal of the progesterone source, circulating progesterone levels did not plummet; instead, they and consequently nuclear progestin receptor levels, declined gradually toward baseline levels over a period of 8-16 hours.

The results from this study support the hypothesis that heat termination results from the loss of hypothalamic nuclear progestin receptors. A tight correlation was found to exist between the concentration of nuclear progestin receptors and the percentage of animals that are sexually receptive. As the concentration of nuclear progestin receptors decreased, so did the number of guinea pigs displaying lordosis. Based on the nature of this correlation, one would predict that the cellular events relevant to lordosis that are induced by progesterone action in the cell nucleus are short-lived. In contrast, the correlation between elevated nuclear progestin receptors and sexual receptivity in rats appears to be rather loose. In OVX female rats treated with estradiol and progesterone, hypothalamic nuclear progestin receptor levels decrease to control levels 8-12 h before heat terminates in similarly-treated animals (Ahdieh, Brown, Wade, and Blaustein, unpublished observations). Therefore, one would predict that in rats the cellular effects of progesterone action relevant to lordosis are longer-lived. Research is currently underway in this laboratory to investigate these predictions.
REFERENCES


nucleus of the hypothalamus inhibits the activation of sexual behavior by estradiol and progesterone. Brain Res. 233:417-423.


SECTION V. ABBREVIATION OF THE PERIOD OF SEXUAL BEHAVIOR IN FEMALE
GUINEA PIGS BY THE PROGESTERONE ANTAGONIST, RU 486
Abbreviation of the period of sexual behavior in female guinea pigs by the progesterone antagonist, RU 486

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ABSTRACT

In previous studies we have tested the hypothesis that the termination of the period of female sexual behavior results from the loss of progestin receptors from hypothalamic cell nuclei. We have shown that hormonal manipulations that delay heat termination also delay loss of hypothalamic nuclear progestin receptors. In order to determine if accelerated nuclear receptor loss results in attenuation of the period of sexual behavior, we tested the effect of RU 486, a progesterone antagonist, on heat termination. Ovariectomized guinea pigs were treated with 10 µg estradiol benzoate. Forty h later, animals received 100 µg progesterone followed 4 h later by an injection of RU 486 or vehicle. RU 486 injected 4 h after progesterone caused heat termination to occur earlier than in vehicle-injected animals. We have found that RU 486 administration to estradiol-treated guinea pigs causes accumulation of progestin receptors in cell nuclear extract. Because this accumulation can be detected only when assay conditions are used that promote exchange of RU 486 progestin receptor complexes (15 °C incubation rather than 0 °C), our routine assay conditions (at 0 °C) can be used to measure primarily receptors that are occupied by progesterone. In order to confirm that RU 486 decreased progesterone-occupied nuclear progestin receptor levels when injected 4 h after progesterone, animals treated as in the behavioral experiment were killed 6 or 10 h after progesterone injection (2 or 6 h after RU 486), and nuclear progestin receptor levels were measured. RU 486
treatment resulted in lowered nuclear concentrations of hypothalamic progestin receptors at both times. These results support our hypothesis that the termination of the period of sexual receptivity in female guinea pigs is the result of loss of progestin receptors from hypothalamic cell nuclei.
The sequential secretion of estradiol and progesterone during the estrous cycle of many rodent species, including guinea pigs, has been shown to activate a period of female sexual behavior of limited duration (13, 25, 50). Substantial experimental evidence exists suggesting that intracellular progestin receptors in mediobasal hypothalamus (MBH) neurons are involved in this activation. Hormonal implant studies of the brain have identified the MBH as the primary site for both estradiol and progesterone's actions on sexual receptivity (14, 31, 36, 43, 44). Estradiol administered to OVX rats or guinea pigs increases the concentration of progestin receptors in MBH cytosol (5, 30, 35, 37, 48), while subsequent progesterone treatment results in a transient increase in the concentration of these receptors in the cell nuclear fraction (6, 33). Concurrent with this increase in nuclear progestin receptors, the concentration of receptors in the cytosol decreases; however, cytosol receptor levels are not replenished as nuclear receptor levels decrease (6, 10). As a result of the transient increase in nuclear binding, the progesterone-receptor complex is thought to influence protein synthesis, resulting in activation of sexual behavior (15, 39, 40).

We have previously hypothesized that the termination of the period of sexual receptivity (heat) results from the loss of progestin receptors from hypothalamic cell nuclei (9, 10). It seems possible that as the concentration of progesterone-progestin receptor complexes in cell nuclei decreases, the cellular effects of progesterone diminish,
thereby resulting in the loss of sexual responsiveness. In support of this hypothesis, it has been shown that the increase and subsequent decrease in nuclear progestin receptor levels parallels the onset and decline of sexual receptivity (6, 10). Furthermore, hormonal manipulations that result in delayed heat termination also cause delayed loss of nuclear progestin receptors. A supplemental injection of progesterone, administered to hormonally-treated OVX guinea pigs, or a supplemental injection of estradiol have both been shown to result in increased heat duration and prolonged retention of elevated levels of hypothalamic nuclear progestin receptors (3, 9).

In the present study, we further tested this hypothesis by determining if accelerated nuclear progestin receptor loss results in abbreviation of the period of sexual receptivity. With the development of the antiprogestin, RU 486, such an attempted manipulation has been made possible. We have previously shown that RU 486 prevents the initiation of sexual behavior when administered one hour prior to progesterone, presumably by decreasing the concentration of available progestin receptors in hypothalamic cytosol (8). If RU 486 treatment leads to decreased levels of progesterone-bound MBH nuclear progestin receptors, then based on our hypothesis, RU 486 should abbreviate heat duration when administered following progesterone injection.
METHODS

Animals

Adult Hartley strain female guinea pigs weighing 300 - 350 g were obtained from Charles River Breeding Laboratories (Wilmington, MA) and were group-housed in 51 X 56 X 23 cm metal cages. A 14 / 10 h light-dark cycle was maintained with lights on from 0600 h to 2000 h. Food (Purina guinea pig chow) and water were freely available. Two to four weeks after arrival, animals were bilaterally ovariectomized under conditions reported previously (8). A 2 - 3 week recovery period was allowed before animals were used in this study.

Hormones

17β-Estradiol benzoate (EB) was dissolved in sesame oil; progesterone, hydrocortisone (cortisol), and RU 486 (17β-hydroxy-11β-(4-dimethylaminophenyl)-17α-(1-propyl)-estra-4,9,-dione-3-one, Roussel-Uclaf, Romainville, France) were dissolved in sesame oil containing 15% benzyl benzoate and 5% benzyl alcohol. Injection volumes were 0.1 ml for EB, progesterone, and cortisol, and 0.4 ml for RU 486. All injections were administered subcutaneously in the axillary region. In all experiments, EB injection occurred at 1700 h.
Behavioral Testing

Animals were tested hourly for lordosis by manually stimulating the flanks and perineal area and measuring the length of time the lordosis posture was held (51). All tests were conducted with the experimenter blind to the assigned treatment group. An animal was considered sexually responsive if the lordosis posture was held for at least 1 sec during two consecutive tests. Testing continued until all animals were unresponsive, or for a minimum of 14 h. The period of sexual receptivity was considered terminated when lordosis could not be elicited during two consecutive tests. Latency to lordosis, maximum lordosis duration, heat duration, and time of last lordosis were calculated as described previously (9, 16).

Combined Cytosol and Nuclear Progestin Receptor Assay

Guinea pigs were decapitated and their brains rapidly removed. The mediobasal hypothalamus (MBH) or the mediobasal hypothalamus-preoptic area (MBH-POA) was dissected on a chilled block as described previously (3).

Cytosol and nuclear progestin receptor levels were measured as described previously with minor modifications (3, 4, 6). All procedures were conducted at 0-4 °C unless otherwise noted. Tissues were placed in glass tissue grinding vessels containing 1.0 ml buffer A (0.32 M sucrose, 1 mM KH$_2$PO$_4$, 1 mM MgCl$_2$, 10% glycerol, 12 mM monothioglycerol, pH 7.2), and were homogenized with a Teflon pestle with appropriate clearance for cell nuclear isolation (32). Homogenates were transferred
to 12 X 75 mm polycarbonate tubes and the grinding vessels were rinsed with 0.5 ml A. The homogenates were centrifuged at 1000 X g for 5 min. Cytosol extract was obtained by centrifuging the resulting supernatant at 48,000 X g for 30 min. The pellet from the first centrifugation was washed twice by adding 2 ml buffer A and centrifuging at 1000 X g for 5 min. The crude nuclear pellet was then purified by dispersing in 0.3 ml buffer A and 1.0 ml buffer B (2.15 M sucrose, 1 mM KH2PO4, 1 mM MgCl2, 10% glycerol, pH 7.2), and centrifuging at 25,000 X g for 20 min. The pellicle and supernatant were removed and the walls of the tube were dried.

For the cytosol assay, 300 μl samples of cytosol extract were applied to 4.5 X 68 mm columns of Sephadex LH-20 (Pharmacia Fine Chemicals, Piscataway, NJ) to remove residual, potentially competing steroids (4). Immediately after running into the columns, samples were washed in with 100 μl buffer A and the macromolecular fraction was eluted with 700 μl A. Aliquots of 250 μl filtered cytosol were incubated with 50 μl buffer A containing 1% ethanol (final concentration) and (17α-methyl-3H)R 5020 (New England Nuclear, Boston, MA; spec. act. = 87 Ci/mmol) +/- 250-fold concentration of unlabeled progesterone. A final concentration (in 300 μl) of 0.4 nM (3H)R 5020 was used because this concentration results in binding almost exclusively to the progestin receptor with almost no specific binding to blood contaminants or low affinity binders (5).

Following an overnight incubation at 0-4 °C, bound (3H)R 5020 was separated from free by gel filtration on Sephadex LH-20 columns. Samples of incubate (250 μl) were applied to the column and washed in
with 150 µl of buffer A. Thirty min after applying the sample, the macromolecular fraction was eluted into scintillation vials with 700 µl of A. Five ml of scintillation counting fluid (0.17 g Bis MSB + 5.5 g PPO / liter scintillation grade toluene (Fisher Scientific) containing 33% Fisher’s surfactant) were added and radioactivity was counted on a Packard Tri-Carb Model 300C spectrophotometer at an efficiency of 40%. Specific binding was calculated by subtracting nonspecific binding (with unlabeled progesterone) from total binding (without unlabeled progesterone). Protein concentration was determined by the dye-binding method of Bradford (7), using BSA as the standard. Results are expressed as femtomoles (³H)R 5020 specifically bound per mg cytosol protein.

For the nuclear receptor assay, the purified nuclear pellet was dispersed with 285 µl buffer TEGT (10 mM Tris-HCl, 1.5 mM Na₂EDTA, 10% glycerol, 12 mM monothioglycerol, pH 7.4). After 15 min, an equal volume of buffer TEGT-K₁,o (TEGT + 1 M KCl, pH 7.4) was added. The tube contents were vortex-mixed and incubated for 15 min. The nuclei were then centrifuged at 48,000 X g for 5 min. Samples (250 µl) of the supernatant (nuclear extract) were incubated with 50 µl buffer TEGT-K₀.s (TEGT + 0.5 M KCl, pH 7.4) containing 1% ethanol (final concentration) and (³H)R 5020 +/- 250-fold concentration of unlabeled progesterone. A final concentration of 3.0 nM (³H)R 5020 was used (6). Following an overnight incubation at 0-4 °C, bound (³H)R 5020 was separated from free as described above, except that buffer TEGT-K₀.s was used. The DNA content of the nuclear pellet was determined by the method of Burton (11) with calf thymus DNA used as the standard.
Results are expressed as femtomoles (³H)R 5020 specifically bound per mg DNA.

Cytosol Exchange Assay

To assay progestin receptors in MBH-POA cytosol, tissues were homogenized in 3 ml TEGT (1 MBH-POA / ml) with a Polytron tissue grinder (Brinkmann Instruments, Westbury, NY). Following centrifugation at 48,000 X g, the supernatant (cytosol extract) was filtered through Sephadex LH-20 columns as described above to remove any residual steroid hormone. The filtered cytosol was pooled for each treatment group and 750 µl aliquots were incubated with 150 µl TEGT containing 0.4 nM (³H)R 5020 +/- 0.1 µM unlabeled progesterone (in a total volume of 900 µl) at 0 or 15 °C. After a 2, 4, or 8 h incubation, a 250 µl sample of incubate was applied to Sephadex LH-20 columns to separate bound (³H)R 5020 from free as described above.

Statistical Analysis

Differences in the percentage of animals responding to various hormone treatments were analyzed by Fisher’s Exact Probability Test. Other differences, both in behavioral parameters and in receptor concentrations, were analyzed by Student’s t-test. Levels of significance given are based on two-tailed comparisons unless otherwise stated.
RESULTS

Experiment 1

In addition to its antiprogestin properties, RU 486 has been shown to be a potent glucocorticoid antagonist (2, 12, 26, 34, 38). We demonstrated in earlier work that the inhibitory effect of RU 486 on sexual receptivity could be overcome with a large dose of progesterone, indicating that the inhibition was not the result of debilitation caused by impaired glucocorticoid activity (8). However, because progesterone in high doses can act as a partial glucocorticoid agonist (41,45), it remained possible that the high dose of progesterone used to overcome the inhibition was acting to restore impaired glucocorticoid activity, thereby allowing the animals to become sexually receptive. Therefore, in this experiment the ability of cortisol to overcome RU 486-induced inhibition of sexual behavior was tested. If RU 486 inhibited sexual receptivity in cortisol-treated animals, then it would serve as further evidence that RU 486 was not influencing sexual behavior through its actions on the glucocorticoid system.

Forty h after receiving 10 µg EB, OVX guinea pigs were divided into four groups and administered the following treatments: group 1 = 5 mg RU 486 and 100 µg progesterone; group 2 = oil vehicle and 100 µg progesterone; group 3 = 5 mg RU 486 and 5 mg progesterone; group 4 = 5 mg RU 486, 100 µg progesterone, and 5 mg cortisol. Beginning just prior to progesterone injection, all animals were tested hourly for the presence of lordosis.
Confirming our earlier work, RU 486 inhibited the display of female sexual behavior (20% vs. 100%, *p*=0.048; Table 1). This inhibition was overcome by the administration of a larger dose of progesterone (100% vs. 20%, *p*=0.048) but not of cortisol (0% vs. 20%). These results, therefore, support our earlier conclusions that RU 486-induced inhibition of sexual receptivity is the result of actions upon the progestin system rather than of actions on the glucocorticoid system.

Experiment 2

Previously, we reported that the concentration of hypothalamic cytosol progestin receptors decreased following administration of RU 486 to estrogen-pretreated OVX guinea pigs (8). Interestingly, no concurrent increase in nuclear progestin receptor levels was noted. One possibility for this is that RU 486-bound receptors may be present in the cytosol extract, but under our current assay conditions (at 0 °C) RU 486 does not exchange with the radiolabeled ligand, (*3H)R 5020, and therefore, the receptors are not detected. It is also possible that RU 486-bound progestin receptors may be present in the KCl-nuclear extract but are not detected for the same reason. Alternatively, it is possible that by binding RU 486, progestin receptors may be rapidly degraded or lose their ability to bind ligand.

In order to determine if RU 486-bound receptors are present in the cytosol fraction, MBH-POA progestin receptors were assayed at a slightly elevated incubation temperature of 15 °C. Incubation at elevated temperatures has been shown to facilitate nuclear estrogen
Table 1. Failure of cortisol to overcome RU 486-induced inhibition of lordosis

<table>
<thead>
<tr>
<th>Treatment                  </th>
<th>n</th>
<th>Percent Responding</th>
<th>Latency to Lordosis&lt;sup&gt;b&lt;/sup&gt; (h)</th>
<th>Heat Duration&lt;sup&gt;b&lt;/sup&gt; (h)</th>
<th>Heat Duration (h)</th>
<th>Last Lordosis&lt;sup&gt;b&lt;/sup&gt; (h)</th>
<th>Maximum Lordosis Duration&lt;sup&gt;b&lt;/sup&gt; (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Veh + Po.&lt;sub&gt;1&lt;/sub&gt;</td>
<td>5</td>
<td>100%</td>
<td>4.60 +/-1.03</td>
<td>7.40 +/-1.63</td>
<td>11.00 +/-1.18</td>
<td>9.60 +/-2.60</td>
<td></td>
</tr>
<tr>
<td>RU&lt;sub&gt;6&lt;/sub&gt; + Po.&lt;sub&gt;1&lt;/sub&gt;</td>
<td>5</td>
<td>20%</td>
<td>4.00</td>
<td>10.00</td>
<td>2.00</td>
<td>13.00</td>
<td></td>
</tr>
<tr>
<td>RU&lt;sub&gt;6&lt;/sub&gt; + Po&lt;sub&gt;5&lt;/sub&gt;</td>
<td>5</td>
<td>100%</td>
<td>5.40 +/-0.75</td>
<td>8.80 +/-0.58</td>
<td>13.20 +/-0.37</td>
<td>11.20</td>
<td></td>
</tr>
<tr>
<td>RU&lt;sub&gt;6&lt;/sub&gt; + Po.&lt;sub&gt;1&lt;/sub&gt; + Corts</td>
<td>5</td>
<td>0%</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>OVX guinea pigs were treated with 10 μg EB. Forty h later, animals were divided into 4 groups, and were administered one of the 4 treatments listed. Values given represent group means +/- S.E.M. (Veh = vehicle, P = progesterone, RU = RU 486, Cort = cortisol; subscripts are doses in mg).

<sup>b</sup>Based on responding animals only.
receptor ligand exchange (42), and we have found that incubation of hypothalamic cytosol at up to 15 °C for 8 h does not result in loss of progestin binding (see Appendix A). Six OVX guinea pigs were treated with 10 µg EB forty h before receiving an injection of 5 mg RU 486 or oil vehicle. Three hours later, animals were killed and cytosol progestin receptors were measured. As described in Methods, cytosol was incubated with (3H)R 5020 at both 0 °C and 15 °C for 2, 4, or 8 h. If RU 486-bound progestin receptors are present in the cytosol, and RU 486 exchanges with (3H)R 5020 at elevated temperatures, then the level of receptors measured in MBH-POA from RU 486-treated animals at 15 °C should approach the level measured in vehicle-injected animals.

In a separate experiment, the possibility that RU 486-bound progestin receptors are present in 0.5 M KCl-nuclear extract was investigated. Twelve OVX guinea pigs were treated with 5 mg RU 486 or the oil vehicle 40 h after receiving 10 µg EB. Three h after RU 486 injection, animals were killed and MBH-POA, KCl-nuclear extracts were obtained. Aliquots of extract were incubated with (3H)R 5020 at 0 or 15 °C for 8 hours. We have found that incubation of nuclear extract at up to 15 °C for 8 h does not result in loss of progestin binding (see Appendix B). If RU 486-bound progestin receptors are present in the nuclear extract, and RU 486 exchanges with (3H)R 5020 at 15 °C, then the level of nuclear progestin receptors measured at 15 °C should be greater in RU 486-injected animals than in vehicle-injected animals.

Confirming our previous study, RU 486 injection decreased cytosol progestin receptor levels by about 50%. While incubation of cytosol at 15 °C resulted in slightly greater measured progestin receptor levels in
both groups, the percent decrease in receptor concentration caused by RU 486 was not altered by the elevated incubation temperature (Figure 1). In contrast, incubation of MBH-POA nuclear extract from RU 486-injected animals at 15 °C revealed a 100% increase in progestin receptor concentration over levels measured in vehicle-injected animals ($t_{10}=4.68, p<0.001$; Figure 2). These data suggest that RU 486-bound progestin receptors are located primarily in the nuclear extract and not in the cytosol.

**Experiment 3**

In order to determine if RU 486 treatment decreases progesterone-occupied nuclear progestin receptor concentrations when injected 4 h after progesterone, MBH progestin receptor levels were measured in OVX guinea pigs treated with 100 μg progesterone 40 h after receiving 10 μg EB. Four h after progesterone injection, animals received 5 mg RU 486 or the oil vehicle. Animals were killed 2 or 6 h later (6 or 10 h after progesterone injection), and progestin receptor concentrations were measured in MBH cytosol and KCl-nuclear extract. A third group of estradiol treated animals which received only vehicle injections was included as a control group. Because we found in Experiment 2 that only a very slight amount of RU 486-bound nuclear progestin receptors was detected with our routine assay procedure (at 0 °C), the level of nuclear progestin receptors measured in RU 486 treated animals in this experiment represents primarily receptors that were occupied by progesterone.

Progesterone treatment resulted in decreased cytosol progestin receptor concentrations and increased nuclear receptor concentrations at
Figure 1. Failure to detect RU 486-bound progestin receptors in MBH-POA cytosol

OVX guinea pigs were treated with 5 mg RU 486 or oil vehicle 40 h after receiving 10 μg EB. Two h later, animals were killed and MBH-POA cytosol was obtained and pooled from similarly-treated animals. Aliquots of cytosol were incubated with (³H)R 5020 +/- unlabeled progesterone at 0 °C or 15 °C for 2, 4, or 8 h. Results are expressed as a percent ratio of (³H)R 5020 specifically bound / (³H)R 5020 specifically bound in cytosol incubated at 15 °C (for 8 h) from vehicle-injected animals.
$[^3 \text{H}] \text{R 5020 SPECIFICALLY BOUND}$

(percent of vehicle at 15°C)

INCUBATION PERIOD (hours)

- RU 486: 15°C
- VEHICLE: 0°C

177
Figure 2. RU 486 causes the accumulation of progesterin receptors in MBH-POA cell nuclear fraction

OVX guinea pigs were treated with 5 mg RU 486 or oil vehicle 40 h after receiving 10 μg EB. Nuclear extract was obtained and aliquots were incubated with (3H)R 5020 +/- unlabeled progesterone at 0 °C or 15 °C for 8 h. Results are expressed as a percent ratio of (3H)R 5020 specifically bound / (3H)R 5020 specifically bound in nuclear extract incubated at 15 °C (for 8 h) from vehicle-injected animals (n = 6 per group).
both 6 and 10 h after progesterone injection (Figure 3; cytosol: \( t_{20} = 2.67, p < 0.02 \) at 6 h, and \( t_{17} = 4.10, p < 0.001 \) at 10 h; nuclear: \( t_{20} = 3.77, p < 0.01 \) at 6 h and \( t_{24} = 5.57, p < 0.001 \) at 10 h). Cytosol progestin receptor concentration was further decreased by 2 h after RU 486 injection (6 h after progesterone; \( t_{21} = 4.63, p < 0.001 \)), and was still decreased 4 h later (\( t_{17} = 3.16, p < 0.01 \)). RU 486 decreased the level of progesterone-occupied nuclear progestin receptors as measured at both 6 (\( t_{20} = 2.10, p < 0.05 \)) and 10 h (\( t_{24} = 1.75, p < 0.05 \), 1-tailed comparison) after progesterone injection.

**Experiment 4**

In order to determine if the observed decrease in nuclear progestin receptors was due to a decrease in receptor concentration or to a decrease in binding affinity of receptors for \( ^{3}H \) R 5020, MBH nuclear extract obtained from animals treated as in experiment 3 were assayed with a range of \( ^{3}H \) R 5020 concentrations, and the results were analyzed by the method of Scatchard (46). Twenty-two OVX guinea pigs were treated with 100 \( \mu \)g progesterone 40 h after receiving 10 \( \mu \)g EB. Four h later, animals received either 5 mg RU 486 or the oil vehicle. Two h later (6 h after progesterone injection), animals were killed and MBH nuclear extract was obtained as described. Extracts were pooled for each group and 250 \( \mu \)l aliquots were incubated with a range of \( ^{3}H \) R 5020 concentrations (0.04 - 11 nM) in the presence or absence of a 250-fold excess of unlabeled progesterone. Following an overnight incubation, bound \( ^{3}H \) R 5020 was separated from free as described.

The binding isotherms obtained were analyzed with the Lundon-1
Figure 3. Effect of RU 486, administered 4 hours after progesterone, on progestin receptor levels

A: Progestin receptor levels measured in MBH nuclear fraction

OVX guinea pigs were treated with 100 μg progesterone or oil vehicle 40 h after receiving 10 μg EB. Four h later, animals received 5 mg RU 486 or oil vehicle. Animals were killed 6 or 10 h after progesterone injection, and progestin receptor levels were determined in the cytosol and nuclear fractions of the MBH. Values given represent mean +/- S.E.M. with levels measured in vehicle-injected animals (63.0 fmoles / mg DNA at 6 h, and 35.0 fmoles / mg DNA at 10 h) subtracted. n = 11 to 14 per group.

B: Progestin receptor levels measured in MBH cytosol

Values given represent fmoles (3H)R 5020 specifically bound / mg cytosol protein (mean +/- S.E.M.). n = 9 to 11 per group.
program for ligand binding analysis (Lundon Software, Cleveland, OH). When analyzed using the Lundon one site model (29), which was the model that best fit the data, RU 486 treatment caused a 23% decrease in the concentration of nuclear KCl-extractable progestin binding sites and had little effect on binding affinity (Figure 4).

Experiment 5

To further test our hypothesis that heat termination results from loss of hypothalamic nuclear progestin receptors, the effect of RU 486 on heat termination was tested. Our hypothesis predicts that RU 486 administered after progesterone should, by decreasing nuclear progestin receptor levels, cause heat abbreviation. OVX guinea pigs were treated with 10 μg EB. Forty h later, animals received 100 μg progesterone followed 4 h later by an injection of 5 mg RU 486 or oil vehicle. A third group which was treated with RU 486 at the time of progesterone treatment, was included for comparison.

As expected, RU 486 treatment at the time of progesterone inhibited the activation of sexual receptivity (31% vs. 92%, as compared to vehicle-injected controls, p=0.005; Table 2). RU 486 treatment at 4 h did not affect the latency to lordosis, but, as predicted, caused heat termination to occur at an earlier time than in vehicle-injected animals (t_{18}= 2.27, p<0.05). By 13 h after progesterone injection, heat had terminated in animals treated with RU 486 at 4 h, whereas in vehicle-treated animals heat termination occurred by 16 h (Figure 5). No effect of RU 486 on maximum lordosis duration of the responding animals was noted.
Figure 4. Scatchard representation of (³H)R 5020 binding to MBH nuclear extract

OVX guinea pigs were treated with 100 µg progesterone 40 h after receiving 10 µg EB. Four h later, animals received 5 mg RU 486 or oil vehicle, and were killed 2 h later (6 h after progesterone injection). Aliquots of MBH nuclear extract obtained were incubated with a range of (³H)R 5020 (0.04 - 11 nM) +/- unlabeled progesterone. Following an overnight incubation, bound (³H)R 5020 was separated from free

Inset: Saturation analysis of specifically bound (³H)R 5020
[\text{\textsuperscript{3}H}R5020] SPECIFICALLY BOUND (fmoles/mg DNA)

\[ [\text{\textsuperscript{3}H}R5020] \text{CONCENTRATION (nM)} \]

RU 486
Kd = 0.082 nM

VEHICLE
Kd = 0.059 nM
Table 2. RU 486 abbreviates the period of sexual receptivity

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Percent Responding</th>
<th>Latency to Lordosis^b</th>
<th>Heat Duration^b</th>
<th>Heat Duration (h)</th>
<th>Last Lordosis^b</th>
<th>Maximum Lordosis Duration^b (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Veh</td>
<td>12</td>
<td>92%</td>
<td>4.91 +/-0.41</td>
<td>7.55 +/-0.96</td>
<td>6.92 +/-0.88</td>
<td>11.36 +/-0.72</td>
<td>11.64 +/-1.69</td>
</tr>
<tr>
<td>RU - 0h</td>
<td>13</td>
<td>31%</td>
<td>5.25 +/-1.38</td>
<td>5.00 +/-0.71</td>
<td>1.54 +/-0.69</td>
<td>9.25 +/-1.11</td>
<td>9.25 +/-2.29</td>
</tr>
<tr>
<td>RU - 4h</td>
<td>13</td>
<td>69%</td>
<td>4.67 +/-0.41</td>
<td>5.22 +/-1.06</td>
<td>3.62 +/-0.82</td>
<td>8.89 +/-0.82</td>
<td>12.33 +/-2.07</td>
</tr>
</tbody>
</table>

^OVX guinea pigs were treated with 100 μg progesterone 40 h after receiving 10 μg EB. Animals were divided into 3 groups and administered one of the three treatments listed at the time of progesterone injection or 4 h later (Veh = vehicle, RU = RU 486).

^Based on responding animals only.
Figure 5. Percent of animals exhibiting lordosis during hourly tests for lordosis

OVX guinea pigs were treated with 100 µg progesterone 40 h after receiving 10 µg EB, and were divided into three groups. Two groups received 5 mg RU 486 administered at the time of progesterone, or 4 h later. The third group received the oil vehicle 4 h after progesterone injection. All animals were tested hourly for lordosis beginning just prior to progesterone injection.
TIME AFTER PROGESTERONE (hours)

PERCENT RESPONDING

- VEHICLE
- RU 486 at 0 h
- RU 486 at 4 h
DISCUSSION

RU 486 has been shown to act as a potent antiprogestin and antiglucocorticoid both in vivo and in vitro (1, 2, 8, 12, 17, 19-22, 26, 34, 38, 47). These actions are thought to be the result of a direct interaction of the synthetic steroid with the intracellular progestin or glucocorticoid receptor (1, 8, 17, 22, 26, 34, 38). Previously, we reported that RU 486 prevented the onset of progesterone-facilitated female sexual behavior in OVX hormonally-treated guinea pigs. This inhibition could be overcome by the administration of a larger dose of progesterone, suggesting that the inhibition was the result of the antiprogestin action of RU 486 rather than of a debilitating effect caused by impaired glucocorticoid action (8). However, because progesterone, under certain conditions, has been shown to act as a suboptimal glucocorticoid agonist (41, 45), the possibility still remained that the large dose of progesterone used was acting to restore impaired glucocorticoid activity, thereby allowing animals to exhibit lordosis. In the first experiment of this study, we found that an injection of cortisol at the time of RU 486 injection could not prevent RU 486-induced inhibition of progesterone-facilitated sexual receptivity. Therefore, it is highly unlikely that the large dose of progesterone overcame the inhibition by acting to restore glucocorticoid activity. Thus, this experiment confirms the interpretation that RU 486 inhibits sexual behavior through its actions on the progestin system.

In previous studies, we have provided evidence to support the
hypothesis that heat termination results from the loss of progestin receptors from hypothalamic cell nuclei. We have shown that hormone treatments that result in delayed heat termination also result in a delayed loss of hypothalamic nuclear progestin receptors (3, 9). In addition, we recently reported that, in the presence of continued administration of both estradiol and progesterone, sexual receptivity decreased parallel to a decrease in hypothalamic nuclear progestin receptor levels (10). In the present study, we provided further support for this hypothesis by demonstrating that a treatment that accelerated the loss of MBH nuclear progestin receptors also accelerated heat termination.

Administration of RU 486 four hours after progesterone injection decreased progestin binding in MBH-nuclear extract within 2 hours. This decrease in binding was a result of a decrease in the concentration of progesterone-bound receptors rather than a decrease in receptor affinity as demonstrated by Scatchard analysis. It is not known why the concentration of progestin receptor levels measured by Scatchard analysis in this assay were higher than those we normally measure. However, the relative decrease in nuclear progestin receptor concentration caused by RU 486 was the same as what was found with the one-point assay (23 - 28%). This decrease represents approximately one-half of the progesterone-dependent nuclear progestin receptor concentration.

In similarly-treated animals, RU 486 administered four hours after progesterone injection abbreviated the period of sexual receptivity. Two hours after RU 486 injection, the percentage of
sexually receptive animals began to decline. By 9 hours after RU 486 injection (13 hours after progesterone injection), heat had terminated in all animals treated with RU 486, whereas heat termination in vehicle-injected animals did not occur until 2 to 3 hours later. This suggests that accelerated loss of hypothalamic nuclear progestin receptors results in accelerated heat termination.

In addition to decreasing the concentration of progesterone-bound nuclear progestin receptor levels, RU 486 injection also further decreased the level of cytosol progestin receptors. It is not known if the decrease in nuclear receptor concentration occurred secondarily to this decrease in cytosol concentration, or if RU 486 acted directly to displace progesterone from the nuclear-bound receptor. Surprisingly, nuclear progestin receptor levels did not appear to decrease further between 2 and 6 hours after RU 486 injection. It is possible that if receptor levels were measured at a later time point, a further decrease would have been observed. Nevertheless, it seems that nuclear progestin receptor levels were decreased to a level below that required to continue induction of progesterone's lordosis-relevant cellular events.

The finding that RU 486 administered to OVX estradiol-pretreated animals results in accumulation of nuclear progestin receptors suggests that RU 486 binds to progestin receptors in vivo, and is capable of allowing them to be transformed, a process thought to be necessary for nuclear binding (18). A similar finding has been reported after RU 486 treatment in human endometrial cells (17). Similarly, Jung and Baulieu (26) have reported that exposure of cultured mouse fibroblast cells to RU 486 resulted in nuclear accumulation of glucocorticoid receptors;
however, Moguilewsky and Philibert (34) have reported that glucocorticoid receptors bound to RU 486 are not transformed in vitro.

The characteristics of the RU 486-accumulated nuclear progestin receptor complex that determine why RU 486 acts as an antagonist on progesterone-facilitated sexual behavior are not yet known. We have found no difference between the salt extractability of RU 486-accumulated nuclear progestin receptors and progesterone-accumulated progestin receptors (see Appendix C), suggesting that both types of receptor-ligand complexes interact with nuclear binding components with similar affinities (27). However, it is possible that RU 486-progestin receptor complexes interact with different nuclear binding components or at different sites, or that they do not undergo receptor processing, proposed as a necessary step for nuclear estrogen receptor action (23, 24).

In a recent study by Gravanis et al. (17) it was found that RU 486 acted as a partial progestin agonist with regard to progesterone’s effects on estradiol-dehydrogenase activity, DNA polymerase activity, and secretory cell transformation in the postmenopausal human uterus. In the presence of progesterone, however, RU 486 acted to inhibit progesterone’s effect. In addition, Horwitz (22) has reported that in T47D cells, RU 486 acts as a progestin agonist to inhibit cell proliferation. These findings raise the possibility that RU 486 may act as a progestin antagonist by acting as a suboptimal progestin receptor agonist. However, because RU 486 administered in combination with progesterone resulted in no progestin activity attributable to either steroid in the human uterus (17), it is unlikely that all of RU 486’s
inhibitory effects can be ascribed to its acting as a suboptimal agonist. In addition, RU 486 has been shown to act as a pure progestin antagonist in the rat and rabbit uterus (38). Further study of any possible agonistic actions of RU 486, and of the characteristics of RU 486-bound progestin receptors is required to determine the mechanism of RU 486 inhibition of progesterone action.

It should be noted that recent studies suggest that steroid hormone receptors, both occupied and unoccupied, reside primarily in the cell nucleus (23, 49). According to the proposed model of steroid hormone action resulting from these studies, steroid hormone receptors are in loose association with nuclear components in the absence of bound ligand. Upon binding steroid hormone, the receptor complex becomes tightly associated with specific nuclear components. Receptors measured in the cytosol fraction by routine techniques such as those used in the present study, therefore, may represent loosely associated, unoccupied receptors released from cell nuclei during tissue homogenization, whereas receptors in the nuclear fraction may represent tightly associated, occupied receptors. In any event, our results indicate that RU 486 administration caused a reduction in the concentration of hypothalamic nuclear-bound, progesterone-occupied progestin receptors.

In summary, as predicted based on our hypothesis that heat termination results from the loss of progestin receptors from hypothalamic cell nuclei, injection of RU 486 four h after progesterone administration resulted in abbreviation of heat duration. These data support our hypothesis that heat termination results from the loss of progestin receptors from hypothalamic cell nuclei. Maintenance of
progesterone action on sexual receptivity may require elevation of nuclear progestin receptor levels. However, as receptor levels decline, progesterone action diminishes, resulting in the decline of sexual behavior and termination of the receptive period.
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DISCUSSION AND SUMMARY

Three major hypotheses were tested and are supported by the experiments presented in this dissertation. These are:

1) Intracellular hypothalamic progestin receptors are involved in the facilitation of sexual receptivity by progesterone;

2) Estradiol induction of intracellular progestin receptors may be necessary for the activation of sexual behavior by progesterone; and

3) The mechanism responsible for heat termination may include the loss of progestin receptors from hypothalamic cell nuclei.

Although all of the studies included in this dissertation provide support for the involvement of intracellular progestin receptors in the facilitation of sexual receptivity, the best evidence is provided by experiments in the first and fifth studies, in which the antiprogestin, RU 486, was used. RU 486 administered one hour before, or at the same time as progesterone, inhibited the facilitation of sexual receptivity by progesterone. This inhibition could be overcome by administration of a higher dose of progesterone, but not cortisol, indicating that the inhibition was the result of RU 486 acting on the progestin-sensitive system and not on the glucocorticoid-sensitive system. RU 486 was shown to compete for progestin binding sites in vitro, and to decrease the availability of hypothalamic cytosol progestin receptors in vivo. In addition, RU 486 decreased the concentration of nuclear progestin receptors when injected four hours after progesterone. These findings strongly suggest that RU 486 inhibits progesterone facilitation of
sexual behavior by blocking the interaction of progesterone with its intracellular receptor.

These data, however, do not exclude the possibility that progesterone's effects on sexual behavior are mediated by membrane-bound receptors. It is possible that RU 486 interacts with membrane-bound progestin receptors to prevent progesterone action. Investigation of the binding characteristics of the membrane-bound progestin receptors, described by Towle and Sze (169), should help in determining if RU 486 could be acting in this manner. Nevertheless, the data presented in this dissertation provide strong evidence to support further the hypothesis that intracellular progestin receptors are involved in the activation of progesterone-facilitated sexual behavior.

The hypothesis that estradiol induction of intracellular progestin receptors may be necessary for the activation of sexual behavior by progesterone is supported by experiments presented in the second study. Etgen (56) had previously reported that the polychlorinated pesticide, o,g'-DDT, could substitute for estradiol in priming OVX female rats to respond to subsequently administered progesterone. However, o,g'-DDT did not appear to increase the concentration of hypothalamic intracellular progestin receptors. These results were interpreted as evidence that intracellular progestin receptor induction during the estrogen priming period is not essential for the facilitation of sexual receptivity by progesterone. Therefore, it was concluded that a sufficient concentration of estrogen-independent progestin receptors is available to mediate progesterone's effects on sexual behavior.
In contrast to the above study (56), it was found in the second study of this dissertation that, under experimental conditions similar to those of Etgen (56), o,p'-DDT treatment increased the concentration of hypothalamic intracellular progestin receptors. Although the dose used in this study was higher than that used by Etgen, it was found to be a threshold dose for behavioral responsiveness in the animals used in this study. The most likely reason for the discrepancy between the results obtained in these two studies is that in the present study, hypothalamic cytosol was filtered prior to assaying progestin receptors in order to remove any residual o,p'-DDT. o,p'-DDT was shown to compete for cytosol progestin binding sites in vitro; therefore, failure to remove residual amounts of o,p'-DDT in the cytosol prior to assaying progestin receptors may have resulted in Etgen's failure to detect an increase in receptor concentration. The results presented in this dissertation suggest that the estrogen priming action of o,p'-DDT includes the induction of hypothalamic intracellular progestin receptors. Therefore, these results are consistent with, and provide further support for, the hypothesis that induction of intracellular progestin receptors by estrogen action is a prerequisite for progesterone facilitation of sexual receptivity to occur.

In addition to mediating the onset of the period of sexual receptivity, progesterone interaction with intracellular progestin receptors may also lead to the termination of the receptive period. We have hypothesized that the mechanism responsible for heat termination may involve the loss of progestin receptors from hypothalamic cell nuclei (58). This hypothesis was originally based on the observation...
that a temporal correlation exists between the transient elevation of hypothalamic nuclear progestin receptor levels and the expression of sexual receptivity. Nuclear progestin receptor levels have been shown to increase prior to the onset of sexual receptivity and to decrease to baseline levels as the period of receptivity terminates (20). It seems possible that as nuclear progestin receptor levels decrease, the cellular effects of progesterone action diminish, resulting in the loss of receptivity. This hypothesis has been tested by investigating how various hormonal treatments affect both heat duration and the retention of progestin receptors in hypothalamic cell nuclei. Based on this hypothesis, hormonal manipulations that delay heat termination should also delay the decline in nuclear progestin receptor levels. Blaustein (13) has shown that administration of supplemental estradiol, at the time of progesterone administration to estradiol-pretreated OVX guinea pigs, delayed the loss of progestin receptors from hypothalamic cell nuclei. This treatment also resulted in delayed heat termination (13, 38, 82), indicating that estradiol may increase heat duration by increasing the concentration of intracellular progestin receptors, thereby delaying the loss of nuclear progestin receptors.

The studies on heat termination included in this dissertation were conducted to determine the effect of progesterone on heat termination and on the retention of nuclear progestin receptor levels. Several studies have indicated that progesterone can influence heat duration. Morin and Feder (115), have reported that injections of progesterone administered every three hours to estradiol-pretreated OVX guinea pigs increased heat duration by more than 2 hours. Similarly,
Hansen and Sodersten (74) found that heat duration in the rat is dependent upon the dose of progesterone as well as the dose of estradiol. In addition, Lisk (93) has shown that the dose of progesterone regulates heat duration in female hamsters. In the third study of this dissertation, evidence was provided demonstrating that a supplemental injection of progesterone, administered 8 hours after an initial progesterone injection, delayed heat termination by more than two hours. This treatment was also shown to result in a delayed loss of hypothalamic nuclear progestin receptors in similarly-treated animals.

In a further test of the hypothesis that heat termination results from the loss of progestin receptors from hypothalamic cell nuclei, an attempt was made to find an optimum dose of progesterone that would result in greatly prolonged heat durations. In experiments described in the fourth study, estradiol and various doses of progesterone were administered in a continuous fashion to OVX guinea pigs. It was reasoned that at an optimal level of progesterone, and with the continued administration of estradiol, nuclear progestin receptor levels might remain elevated for a greatly extended period of time. This could occur by a balance being attained between cytosol progestin receptor induction by estradiol action and nuclear accumulation of receptors by progesterone binding. Based on our hypothesis, such an extended retention of nuclear progestin receptors should result in delayed heat termination. However, heat termination occurred at about the same time in all animals regardless of the dose of progesterone, and despite the continued administration of estradiol and progesterone.

This finding raised the possibility of a dissociation between
nuclear progestin receptor levels and the termination of sexual behavior. It was possible that continued administration of estradiol and progesterone maintained high levels of nuclear progestin receptors, but failed to maintain sexual responsiveness. Such a dissociation would negate our hypothesis and indicate that some process other than the loss of nuclear progestin receptors is responsible for heat termination. However, it was found that nuclear progestin receptors levels did, in fact, decrease despite the continued administration of both estradiol and progesterone.

These findings raised the question of what factors regulate the retention of progestin receptors in cell nuclei. One such factor is circulating levels of progesterone. Blaustein and Feder (20) and McGinnis et al. (106) have reported that the transient increase in nuclear progestin receptor levels following the injection of progesterone parallels the rise and fall of blood progesterone levels. To investigate this relationship further, progesterone capsules were removed from OVX estradiol-pretreated guinea pigs two hours after their insertion, and the effect on hypothalamic nuclear progestin receptor levels was monitored. Following capsule removal, nuclear progestin receptor levels gradually decreased in parallel with the decline in blood progesterone concentration. By 18 hours after capsule insertion, nuclear progestin receptor levels as well as serum progesterone concentrations had returned to baseline levels. In animals that were continuously exposed to progesterone, serum progesterone concentrations remained elevated throughout the course of the experiment; however, nuclear progestin receptor levels also decreased gradually to near
baseline levels by 18 hours after capsule insertion. These results demonstrate that under certain conditions, circulating progesterone levels influence the nuclear retention of progestin receptors; however, because nuclear receptor levels decrease despite elevated circulating progesterone levels, other factors must be involved.

One factor that may be involved in regulating retention of nuclear progestin receptors is the level of available cytosol receptors. As the level of progestin receptors in the cytosol decreases due to progesterone binding or to degradation, fewer receptors are available to accumulate in the cell nucleus; thus nuclear progestin receptor levels decline. This is most likely the mechanism by which nuclear progestin receptor levels decline in the presence of high levels of circulating progesterone. Following progesterone capsule insertion, cytosol progestin receptor levels decreased, and remained lowered even as nuclear progestin receptor levels declined to baseline.

This interpretation suggests that heat termination may be the result of the same cellular events that lead to sequential inhibition. As discussed in the General Introduction, evidence exists which suggests that sequential inhibition may be a period of hyposensitivity to progesterone (13, 14, 22, 77). This hyposensitivity is thought to result from a diminished level of cytosol progestin receptors due to prior progesterone exposure (19, 112). Because fewer cytosol progestin receptors are available to interact with progesterone, an insufficient level of nuclear progestin receptor accumulation for activation of sexual behavior occurs (13, 14, 101). This same mechanism appears to be responsible for heat termination; in fact, the hyposensitivity to
progesterone can be thought of as an extension of heat termination. This idea is further supported by the finding that supplemental progesterone injection delays heat termination. In the absence of the initial injection of progesterone, the second dose of progesterone would be expected to activate a receptive period that would not terminate until 12 to 16 hours after injection. Instead, in the presence of the initial progesterone, heat termination occurred 4 to 8 hours after the second injection of progesterone.

It is probable that in intact estrous-cycling guinea pigs, both a decrease in circulating progesterone levels and a decrease in hypothalamic progestin receptor concentrations are responsible for the decline in nuclear progestin receptors, and therefore, heat termination. During the estrous cycle, the appearance of sexual behavior parallels the transient preovulatory increase in circulating progesterone levels (64). As progesterone levels decrease, the period of sexual behavior terminates, indicating that availability of progesterone influences heat duration, and possibly nuclear retention of hypothalamic progestin receptors. Goy et al. (71) have demonstrated that in intact female guinea pigs, estradiol and progesterone administered near the end of the receptive period or during the luteal phase of the estrous cycle, was unable to stimulate sexual receptivity. Based on the findings discussed above, it is likely that this refractoriness is due to decreased cytosol progestin receptor availability. Therefore, it seems likely that in the intact animal, availability of cytosol progestin receptors may play a role in regulating nuclear retention of progestin receptors, and therefore, heat duration.
In the fourth study of this dissertation, exposure of guinea pigs to progesterone capsules for 2 hours resulted in fewer animals becoming sexually receptive as compared to the number of animals continuously exposed to progesterone. Of those animals that did become sexually receptive, heat termination occurred at about the same time regardless of treatment group. However, a tight correlation was found to exist between the percentage of animals sexually receptive at a given time following progesterone exposure, and the level of hypothalamic nuclear progestin receptors. As the concentration of nuclear progestin receptors decreased, so did the number of guinea pigs displaying lordosis. In contrast, the correlation between elevated nuclear progestin receptors and sexual receptivity in rats indicates a time lag may exist between nuclear progestin receptor loss and heat termination (1). In OVX female rats treated with estradiol and progesterone, hypothalamic nuclear progestin receptor levels decreased to baseline levels 8 to 12 hours before heat terminated in similarly-treated animals.

Rainbow and coworkers (144, 145) have shown that progesterone-facilitated sexual receptivity is protein synthesis dependent. Based on the results of the studies presented here, one would predict that in guinea pigs, the proteins that may be induced by progesterone action have a short half-life, whereas in the rat, the induced protein, or perhaps induced RNA, has a longer half-life. This prediction could be tested by inhibiting RNA or protein synthesis at various times after progesterone injection. If the putative induced proteins are short-lived, then inhibition of protein synthesis toward
the end of the receptive period should result in premature heat termination. Alternatively, if the induced proteins are longer-lived, then administration of protein synthesis inhibitors toward the end of the receptive period would be without effect. By comparing the effect of protein synthesis inhibition with the effect of RNA inhibition, it may be possible to determine if the maintenance of sexual receptivity in rats in the absence of nuclear-bound progesterone is the result of induction of proteins with a long half-life, or of induction of mRNAs with a long half-life.

In the fifth study of this dissertation, the effect of accelerating the loss of hypothalamic nuclear progestin receptors on heat termination was examined. RU 486 administered four hours after progesterone injection was found to accelerate the loss of progesterone-occupied progestin receptors from hypothalamic cell nuclei. This treatment also accelerated the termination of sexual receptivity, providing strong support for the involvement of hypothalamic nuclear progestin receptor loss in heat termination.

In addition to the mechanism for heat termination examined in these studies, other mechanisms for heat termination have been proposed. Hansen et al. (76, 77) and Sodersten et al. (167) have proposed that a circadian factor is responsible for heat termination. This hypothesis originated in the finding that injection of progesterone early on the day of behavioral estrus advanced the onset of sexual receptivity in female rats, but did not affect when the receptive period ended (165). Hansen et al. (76, 77) have reported a circadian rhythm in the display of lordosis in estrogen-treated OVX rats, with high levels of
receptivity being exhibited during the dark phase of the light / dark cycle. This circadian cyclicity was abolished by lesions of the suprachiasmatic nucleus (SCN) (76). In a recent study, Sodersten et al. (167) reported that the circadian signal responsible for inhibition of lordosis during the photoperiod, and perhaps for heat termination, is arginine vasopressin. Vasopressin is produced by the SCN and has been shown to be present in cat cerebral spinal fluid in a diurnal pattern (150). Sodersten et al. (167) have found that arginine vasopressin administered intraventricularly 3 hours after progesterone injection inhibited sexual behavior in a dose-dependent manner. This effect of vasopressin was independent of any systemic effect on blood pressure, and could be blocked by intraventricular administration of antisera to arginine vasopressin. While it is possible that a circadian signal plays a role in heat termination, some researchers have reported an absence of a circadian rhythm in the display of sexual behavior in estrogen-treated OVX rats (32, 53). In addition, sequential estradiol and progesterone treatment is capable of activating lordosis during the light phase of the light / dark cycle (unpublished observations), when cerebral spinal levels of vasopressin would be expected to be elevated. Further study is required to determine if a circadian factor is involved in heat termination. It is conceivable that several independent mechanisms are involved in heat termination in the estrous-cycling female. Such redundancy could serve to ensure that the animal would not remain receptive beyond the onset of light, when the danger of predation could be increased.

Erskine (51) has suggested that heat termination in
estrous-cycling female rats is the result of 5α-reduction of testosterone that is secreted in significant amounts just prior to the onset of sexual behavior (59). To test this, Erskine (51) has administered 5α-reductase inhibitors or the androgen receptor antagonist, flutamide, to estrous-cycling female rats. These treatments did not affect the onset of sexual receptivity but did delay heat termination. However, flutamide was injected daily for 8 days prior to testing, and the 5α-reductase inhibitors were administered 4 days prior to testing. It is not apparent why such prolonged drug treatments were used. Although no effect of these treatments were noted on serum estradiol concentrations or on the duration of the estrous cycle, it seems possible that these extreme treatments may have altered heat termination in a manner not directly related to their effects on the androgen system.

Rubin and Barfield (157) have proposed that progesterone activates two systems within the brain. One system is activated in the mediobasal hypothalamus and is responsible for the facilitation of sexual behavior, the other system is activated in the midbrain and is responsible for the inhibition of sexual behavior. The facilitory system, they further suggest, may react to progesterone with a short latency, while the inhibitory system is activated with a longer latency. In this way, progesterone action first facilitates and then terminates the period of sexual receptivity. This dual site / dual function explanation for progesterone’s actions has been suggested by other researchers as well (52, 114). However, as discussed in the General Introduction, both of progesterone’s effects on sexual behavior can be
ascribed to the same neural site. Interestingly, Rubin and Barfield's data demonstrate this fact most clearly. Progesterone implants placed in the VMN both facilitated sexual receptivity and prevented a subsequent progesterone injection from activating a second period of receptivity (157). A possible test of this hypothesis would be to implant RU 486 in the midbrain and determine if this treatment prolongs the period of receptivity induced by a systemic injection of progesterone. Based on the finding that RU 486 administered 4 hours after progesterone attenuated heat duration, it seems unlikely that such a manipulation would extend the receptive period.

The mechanism by which loss of nuclear progestin receptors from hypothalamic cell nuclei results in heat termination is not known. This is due primarily to the uncertainty of the mechanism by which progesterone action activates sexual receptivity. Several studies have implicated the serotonergic system in the regulation of sexual receptivity. Pharmacological agents that increase serotonin levels or activate serotonin receptors, inhibit the display of lordosis in estradiol-progesterone treated rats and guinea pigs (54, 108, 110, 127, 161). In addition, agents that decrease serotonin levels or inactivate serotonin receptors, facilitate lordosis in estradiol-pretreated animals (10, 58, 67, 151). The inhibitory effect of serotonin on lordosis appears to be specific for the progestin system. Serotonergic agents fail to inhibit lordosis that is activated by estrogen treatment alone (110), indicating that serotonin does not interfere directly with estrogen action. From these findings, it has been suggested that progesterone may facilitate sexual receptivity by inhibiting
serotonergic activity (54, 108). It seems possible, therefore, that
heat termination could result from an increase in serotonergic activity
as progesterone's cellular effects diminish. Erskine and Baum (52) have
tested this possibility and have found no changes in hypothalamic
serotonin or 5-hydroxyindoleacetic acid levels near the time of heat
termination. However, in their study, heat termination was induced by
coital stimulation which may cause heat termination through a mechanism
different from the one involved in the nonmated animal (149). The
possibility remains, therefore, that heat termination in non-mated
animals may involve an increase in serotonergic activity, or possibly an
increase in serotonergic receptors.

In addition to serotonin and vasopressin, several other hormones,
neurotransmitters, and neuropeptides have been shown to affect rodent
sexual behavior. These include LHRH (123, 135, 152, 158), prolactin
(46, 166), endogenous opioids (2, 179), oxytocin (31), catecholamines
(40, 64, 148), melatonin (44), and corticotropic releasing factor (162).
How, and if, these systems are integrated to regulate sexual behavior is
completely unknown and will be a major area for research in future
years. It is probable that until this question is answered, we will not
fully understand the process by which heat termination occurs.
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APPENDIX A. EFFECT OF INCUBATION TIME AND TEMPERATURE ON CYTOSOL PROGESTIN RECEPTOR MEASUREMENT
Figure A-1. The effect of incubation time and temperature on hypothalamic progestin receptors measurement

Hypothalamic cytosol extract was obtained from estradiol-treated OVX guinea pigs, and incubated with (3H)R 5020 +/- unlabeled progesterone, at 0, 15, or 20°C for 2, 4, 6, 8, or 22 hours. Results are expressed as a percentage of specifically bound (3H)R 5020 measured at 15°C for 6 hours.
APPENDIX B. EFFECT OF INCUBATION TIME AND TEMPERATURE ON NUCLEAR PROGESTIN RECEPTOR MEASUREMENT
Figure B-1. The effect of incubation time and temperature on nuclear progestin receptor measurement

Hypothalamic cell nuclear extract was obtained from OVX guinea pigs treated with estradiol benzoate and progesterone. Progestin receptors were measured by incubating the extract with \(^{3}H\)R 5020 +/- unlabeled progesterone at 0, 10, 15, 20, or 25°C for 2, 4, 8, or 22 h. Results are expressed as a percentage of specifically bound \(^{3}H\)R 5020 measured at 15°C for 4 h.
PERCENT $[^3H]R5020$ SPECIFICALLY BOUND

INCUBATION PERIOD (hours)

2 4 8 22
APPENDIX C. COMPARISON OF SALT EXTRACTABILITY OF RU 486- AND PROGESTERONE-ACCUMULATED NUCLEAR PROGESTIN RECEPTORS

In order to determine if RU 486-accumulated nuclear progestin receptors are as tightly-bound to cell nuclear components as progesterone-accumulated receptors, nuclear-bound receptors were extracted with a range of salt concentrations. Fourteen OVX guinea pigs were injected with 5 mg progesterone or RU 486, 40 h after receiving 10 µg EB. Three h later, animals were killed and MBH-POA were dissected. Following cell nuclear isolation, nuclear pellets were suspended in buffer TEST. Suspensions from similarly-treated animals were pooled, and aliquoted into polycarbonate tubes. Receptors were extracted by adding an equal volume of TEST containing 0, 0.2, 0.3, 0.4, or 1.0 M KCl. Receptors were then measured as described with an incubation period of 8 h at 15°C. The results are shown on the facing page, and are expressed as a percent of binding measured in the 0.5 M KCl extract.

Because a slight difference in extractability of the receptors was noted with 0.2 M KCl (Figure C-1), this finding was reexamined. Following RU 486 or progesterone injection, nuclear progestin receptors were extracted with 0.5 M or 0.2 M KCl. No difference was found between the extractability of the RU 486- and the progesterone-accumulated receptor (80.9% +/- 4.4% and 79.3% +/- 5.9% respectively, n = 6 per group).
Figure C-1. Salt extractability of RU 486- and progesterone-accumulated progestin receptors
[3H]R5020 SPECIFICALLY BOUND (% of 0.5 M extract)

KCl CONCENTRATION (M)

RU 486
PROGESTERONE