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# EFFECTS OF ANTIESTROGEN VERSUS ANTIPROGESTIN ON TRANSFORMED AND NONTRANSFORMED STEROID RECEPTORS

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**Summary**—In order to determine if different physicochemical properties exist among antihormone–receptor complexes, we have compared the interaction of the antiprogestin RU486 with progesterone receptor (PR) versus the triphenylethylene antiestrogen H1285 (4-(*N*,*N*-diethylaminoethoxy)-4′-methoxy-α-(*p*-hydroxyphenyl-α′-ethylstilbene)) with estrogen receptor (ER) from rabbit uterine tissue. Contrary to other reports, we observed no difference in the sedimentation properties of transformed PR (4S) when bound by the antagonist RU486 versus the progesterone agonist R5020 in either cytosol or DEAE partially-purified receptor preparations analyzed on sucrose gradients containing 0.3 M KCl. In addition, we found no difference in the sedimentation properties of these receptor preparations in the presence of 10 mM sodium molybdate: the nontransformed RU486–PR and nontransformed R5020–PR both sedimented as a 6S species. These same results were obtained when the receptor preparation and gradient analysis were performed in the absence of monothioglycerol. Likewise, there was no change in the sedimentation properties of the transformed PR when the receptor, partially purified in the absence of molybdate, was analyzed on sucrose gradients containing 10 mM sodium molybdate to prevent receptor alteration during centrifugation. From DNA–cellulose assays performed with partially purified PR in the absence of molybdate we determined that the 4S form of R5020–PR and RU486–PR is transformed receptor; whereas in the presence of molybdate, the 6S species is nontransformed. In contrast, we found a different pattern of sedimentation when comparing transformed antiestrogen–receptor complexes with transformed estrogen–receptor complexes. In this case, transformed H1285–ER sedimented as 6S and estradiol–ER sedimented as 4S. We conclude from these experiments that these two antihormones, RU486 and H1285, may have different mechanisms of action in their antagonism of steroid hormone action. Antiestrogen stabilizes the salt-transformed ER as a dimer while antiprogestin appears to permit dissociation of the oligomeric form of the receptor to the monomeric form.

## INTRODUCTION

The first step in steroid hormone action is the binding of steroid hormone to receptor proteins in target tissues. Upon binding the hormone, the steroid receptor undergoes a structural transformation such that nonsteroid-binding proteins dissociate from the hetero-oligomeric complex and the DNA-binding domain is exposed [1]. The transformed receptor then has an increased affinity for DNA and chromatin (reviewed in Ref. [2]). Presently there is evidence that the receptor binds tightly to DNA as a dimer [3] and that transformed estrogen and progesterone receptors bind with high affinity to specific chromatin acceptor sites (reviewed in Refs [4, 5]). It is through the specific interaction of steroid receptors with DNA that steroid hormones regulate gene transcription and elicit responses from target tissues [2]. It has been suggested that nuclear processing of steroid receptors [6, 7] and the equilibrium between receptor monomers and dimers [8] are additionally important mechanisms regulating responses to steroid hormones.

Antihormones are compounds which inhibit hormone-induced responses; therefore, they are important and useful tools in the study of hormone action. Our laboratory has in the past studied the triphenylethylene antiestrogen H1285 and its effects on the estrogen receptor. H1285 is a high affinity antiestrogen with partial agonist activity as well as strongly antagonistic activity [9, 10]. We previously reported that the mammalian estrogen receptor, when bound by

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H1285, has different physicochemical properties than estrogen receptor bound by estradiol prior to transformation [11–13]. More recently, we characterized partially purified transformed rabbit uterine estrogen receptor bound by estradiol versus H1285 and found that H1285 stabilizes the 6S dimeric form of the transformed estrogen receptor [14]. Antiestrogen, by stabilizing the receptor dimer and disturbing the monomer–dimer equilibrium, may possibly inhibit estrogenic responses by preventing rapid nuclear processing of receptor.

The progestin antagonist RU486 is of current interest as it is the first synthesized antagonist for the progesterone receptor. RU486 binds with high affinity to the progesterone receptor [15] and most reports indicate that it has no agonist activity, although in some tissues partial agonist activity has been reported (reviewed in Ref. [16]). The molecular mechanisms responsible for antagonism by this compound are not yet understood. One model for the mechanism of action of RU486 is that the antagonist prevents the dissociation of the 90 kDa heat shock protein from the 8S hetero-oligomeric progesterone receptor, thereby inhibiting transformation [17]. However, other studies have suggested either directly or indirectly that RU486 does not impede progesterone receptor transformation and DNA binding *in vitro* or *in vivo* [15,18–21]. Therefore, it has not been resolved whether RU486 prevents transformation of the progesterone receptor *in vivo* or forms abortive complexes with transformed receptor. In either case, it appears that this antihormone may have a different mechanism of antagonism than antiestrogen.

In order to compare possible different effects of steroid hormone antagonists on the physicochemical properties of steroid receptors, we compared the effect of antiprogestin on the progesterone receptor versus the effect of antiestrogen on the estrogen receptor in the rabbit uterus. We characterized the sedimentation properties of transformed receptors versus molybdate-stabilized, nontransformed receptors bound by agonist and antagonist.

## EXPERIMENTAL

### *Chemicals*

[17α-methyl-$^3$H]R5020 (87 Ci/mmol), 17β-[6,7-$^3$H]estradiol (60 Ci/mmol), [$^{14}$C]ovalbumin and [$^{14}$C]gamma-globulin were obtained from New England Nuclear (Boston, Mass). [6,7-$^3$H]RU486 (46.8 Ci/mmol) was obtained as a gift from Roussel–Uclaf (Romaineville, France). The antiestrogen [$^3$H]H1285 {H1285 = 4-(*N*,*N*-diethylaminoethoxy)-4′-methoxy-α′-ethylstilbene} (20 Ci/mmol) was prepared in our laboratory from H1285 [22]. Trizma base, monothioglycerol (MTG), phenylmethylsulfonyl fluoride (PMSF), DNA–cellulose and dextran were all purchased from Sigma Chemical Co. (St Louis, Mo.). Sodium molybdate and ultrapure sucrose were purchased from Fisher Scientific (St Louis, Mo.) and ICN Biomedicals Inc. (Costa Mesa, Calif.), respectively. DEAE–cellulose (DE-52) was purchased from Whatman (Clifton, N.J.). All other chemicals were of analytical grade and all procedures were carried out at 0–4°C unless otherwise indicated.

### *Buffers*

The following buffers were used in the various experiments: buffer A = 10 mM Tris–HCl, 1.5 mM EDTA, 5% glycerol, 12 mM monothioglycerol (MTG), and 5 mM PMSF, (pH 7.5 at 4°C); buffer B = buffer A containing 10 mM sodium molybdate; buffer C = buffer A minus MTG.

### *Cytosol preparation*

Mature rabbit uteri were purchased from Pel-Freez (Rogers, Ark.). Uterine tissue was partially thawed, minced with a scalpel, and homogenized using a glass–glass homogenizer in 3 times vol buffer A (unless otherwise stated). The homogenate was then centrifuged at 170,000 ***g*** for 30 min at 5°C to obtain cytosol. For progesterone receptor, cytosol was incubated with 10 μM cortisol followed by 10–20 nM radiolabeled [$^3$H]R5020 or [$^3$H]-RU486 for 90 min. For estrogen receptor, cytosol was incubated with 10–20 nM radiolabeled [$^3$H]estradiol or [$^3$H]H1285 for 90 min. The cytosol was then treated with 1% dextran-coated charcoal, centrifuged, and the supernatant used for DEAE–cellulose chromatography or sucrose density gradient analysis.

### *DEAE column chromatography*

DEAE-step elution was performed as previously described [22]. DE-52 columns (5 ml, 20 × 18 mm) were equilibrated with the appropriate buffer. The prepared cytosol samples were loaded onto the packed columns and the bulk of the protein was eluted with 40 ml buffer with 0.1 M KCl for estrogen receptor prep-

arations or without KCl for progesterone receptor preparations. The receptor was then eluted with 20 ml buffer containing 0.35 M KCl. Fractions (3 ml) were collected and radioactivity determined in 50 $\mu$l aliquots in 4 ml scintillation fluid (Beckman Ready-Solv) at 46% efficiency (Beckman LS-7500) to determine the peak receptor fraction.

*Sucrose density gradients*

Linear 5–20% sucrose gradients (3.6 ml) containing either 50 or 300 mM KCl in buffer were prepared and chilled at 4°C. Some gradients contained 10 mM molybdate. DEAE–cellulose peak fractions or cytosol aliquots (200 $\mu$l) were layered on the gradients and centrifuged for 16 h in a SW60 rotor at 190,000 *g*. Fractions (4 drops) were collected from the top and counted in 4 ml scintillation fluid. Sedimentation markers [$^{14}$C]ovalbumin (3.7S) and [$^{14}$C]gamma-globulin (6.6S) were added as internal standards or in parallel tubes.

*DNA–cellulose assay for receptor activation*

10 mg DNA–cellulose (5.7 mg DNA/g cellulose) was prehydrated in 0.5 ml of appropriate buffer for at least 4 h. The peak fraction from ion exchange chromatography was adjusted with the appropriate buffer to a KCl concentration of 100 mM. Samples (0.5 ml) containing equal [$^3$H]ligand–receptor complexes were combined with 10 mg DNA–cellulose (57 $\mu$g DNA/tube) in 0.5 ml buffer in 1.5 ml microfuge tubes. The mixture was vortexed and incubated 60 min at 4°C. The tubes were then centrifuged at 8730 *g* for 10 s in a Beckman microfuge B, the supernatants discarded, and the resin washed twice by resuspension and centrifugation with 1 ml of appropriate buffer. The bound [$^3$H]ligand was extracted with 0.5 ml ethanol for 10 min, and radioactivity was measured (4 ml Beckman Ready-Solv scintillation fluid) at 46% efficiency.

## RESULTS

*Transformation of rabbit uterine progesterone receptor*

Our past studies with the estrogen receptor bound by estrogen versus antiestrogen demonstrated the ability of the rabbit uterine estrogen receptor to undergo salt-induced transformation when partially purified by DEAE chromatography or on sucrose density gradients containing 300 mM KCl [23]. To determine whether or not rabbit uterine progesterone receptor bound by progestin and antiprogestin would undergo transformation under these same experimental conditions, we performed DNA–cellulose binding assays with partially purified progesterone receptor bound by [$^3$H]R5020 or [$^3$H]RU486. In the absence of 10 mM sodium molybdate the agonist– and antagonist–progesterone receptor complexes bound to DNA–cellulose, indicating receptor transformation (Table 1). However, partially purified receptors obtained in the presence of 10 mM molybdate were not transformed despite the presence of 300 mM KCl in the peak fraction.

*Sucrose density gradient analysis*

We previously determined that in the absence or presence of 10 mM molybdate the estrogen-bound receptor sedimented on high salt density gradients as 4S and 6S, respectively, while the antiestrogen-bound receptor sedimented only as the 6S form [14]. Thus, antiestrogen appears to stabilize the transformed estrogen receptor as a dimer. In this present study we examined the agonist- and antagonist-bound progesterone receptor in the absence and presence of 10 mM molybdate. In the presence of molybdate, cytosolic progesterone receptor bound by [$^3$H]R5020 sedimented as a 6S species and in the absence of molybdate it sedimented as a 4S species on sucrose density gradients containing 300 mM KCl (Fig. 1A). Cytosolic progesterone receptor bound by [$^3$H]RU486 in the presence of molybdate likewise sedimented as 6S. But unlike the antiestrogen–receptor complex, the cytosolic progesterone receptor bound by the antagonist, [$^3$H]RU486, sedimented as 4S in the absence of molybdate rather than 6S (Fig. 1B). Similar results were obtained when high salt sucrose density gradient analysis was performed on partially purified progesterone receptor bound by radiolabeled agonist and antagonist in the presence or absence of 10 mM molybdate

Table 1. Salt transformation of progesterone receptor

| [$^3$H]Ligand | 10 mM Sodium molybdate | cpm Bound |
|---|---|---|
| [$^3$H]R5020 | + | 1100 |
| | − | 6070 |
| [$^3$H]RU486 | + | 794 |
| | − | 4554 |

Cytosol was incubated with 10 $\mu$M cortisol followed by 10 nM of either [$^3$H]R5020 or [$^3$H]RU486 for 90 min at 4°C prior to DEAE chromatography. 0.5 ml Aliquots of peak fractions containing 0.4 pmol of receptor were incubated with DNA–cellulose (5.7 $\mu$g DNA) in a final concentration of 50 mM KCl.

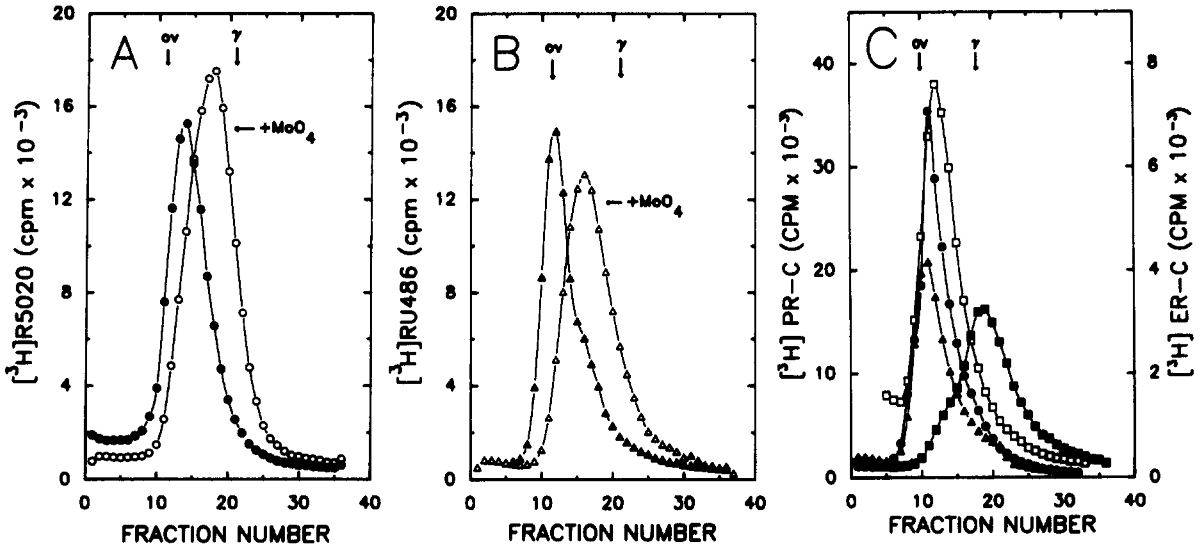


Fig. 1. Sucrose density gradient analysis of cytosolic progesterone– and estrogen–receptor complexes. Cytosol was prepared as described in Methods, in the absence or presence of 10 mM sodium molybdate. After dextran-coated charcoal-treatment 200 μl were layered on 5–20% sucrose density gradients containing 0.3 M KCl ± 10 mM sodium molybdate. [14C]Ovalbumin (3.7S) and [14C]gamma-globulin (6.6S) were used as sedimentation markers. Tubes were centrifuged at 190,000 g (SW60 rotor) in a Beckman L5-50 ultracentrifuge for 16 h. Gradient fractions were collected, counted, and plotted as total bound cpm. Panel C, ●—● = R5020–; ▲—▲ = RU486–; □—□ = estradiol; ■—■ = H1285–receptor complexes. ER-C and PR-C designate estrogen–receptor and progesterone–receptor complexes, respectively.

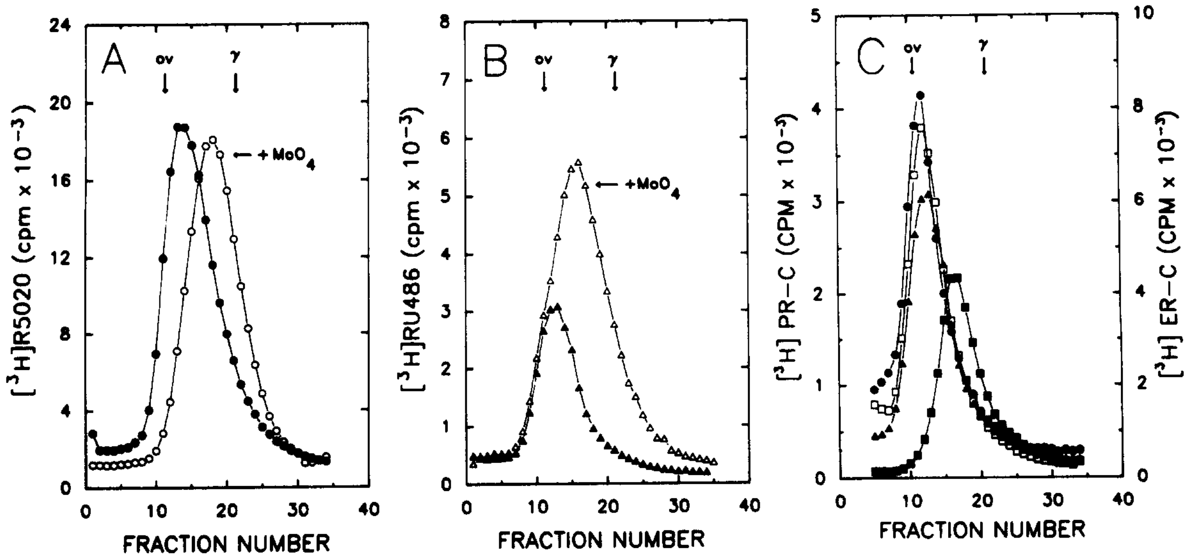


Fig. 2. Sucrose density gradient analysis of partially purified progesterone– and estrogen–receptor complexes. Cytosol was prepared as described in Fig. 1 and partially purified with DEAE–cellulose column chromatography using step-elution. Aliquots (200 μl) of the peak fraction were layered on 5–20% sucrose density gradients containing 0.3 M KCl ± 10 mM sodium molybdate. Conditions of the sucrose density gradient analysis and symbol designation were as described in Fig. 1.

(Fig. 2A and Fig. 2B). Thus, progesterone receptor in the nontransformed state in the presence of high salt and 10 mM molybdate sediments as an intermediate form of the receptor, i.e. neither the native form (9S) nor the monomeric form (4S). At the present time, the components of the 6S form are unknown. This 6S form may consist of nontransformed receptor dimer or the monomer still associated with a nonsteroid binding protein.

Figures 1C and 2C compare the sedimentation values of salt-transformed cytosolic (Fig. 1C)

and partially purified (Fig. 2C) progesterone receptor and estrogen receptor bound by their respective radiolabeled agonists and antagonists. Transformed progesterone receptor bound by [$^3$H]R5020 and [$^3$H]RU486 and transformed estrogen receptor bound by [$^3$H]estradiol all sedimented as approximately 4S, the monomeric form of steroid receptor. However, transformed estrogen receptor bound by antagonist, [$^3$H]H1285, sedimented as 6S, suggestive of the dimeric form of estrogen receptor.

In order to further verify that antiprogestin did not stabilize the 6S form of the transformed progesterone receptor as seen with antiestrogen and the estrogen receptor, we performed sucrose density analysis on the transformed partially purified progesterone receptor bound by [$^3$H]-RU486 in the absence of sulfhydryl reagents (Fig. 3A). Contrary to other reports [24], we found that even in the absence of reducing agents the antagonist-bound transformed progesterone receptor sedimented as 4S rather than 6S. We also considered the possibility that the transformed antiprogestin–receptor complex was undergoing further dissociation to the 4S form during prolonged centrifugation in the absence of molybdate. We therefore partially purified progesterone receptor bound by [$^3$H]RU486 in the absence of molybdate and then analyzed an aliquot of the peak fraction on a sucrose density gradient containing 10 mM molybdate (Fig. 3B). Again, the transformed antiprogestin–receptor complex sedimented as the 4S monomer, suggesting that antiprogestin does not act like antiestrogen in stabilizing the 6S receptor form of the transformed receptor.

## DISCUSSION

Currently there are two proposed molecular mechanisms of action for the antiprogestin effects of RU486. Renoir *et al.* [17] suggest that RU486 blocks progesterone receptor transformation *in vivo* by preventing dissociation of the 90 kDa heat-shock protein from the receptor. Others propose that transformation of antiprogestin–receptor complexes does occur *in vivo* and that the antagonistic action of RU486–receptor complexes takes place at the level of the genome [15, 18–21]. In our study we were able to readily achieve transformation of the antagonist-bound rabbit uterine progesterone receptor *in vitro* using salt. Also, we observed that in the absence of 10 mM sodium molybdate the RU486-bound progesterone–receptor complex had relatively less stability than did R5020-bound progesterone receptor.

Even though RU486 did not prevent receptor transformation in our studies, it is still possible that antiprogestin inhibits receptor dissociation

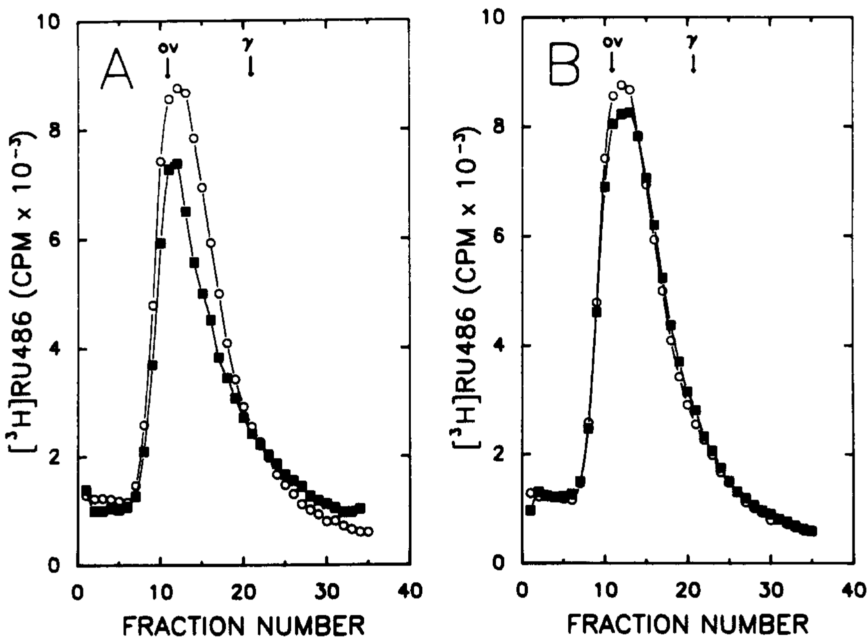


Fig. 3. Sucrose density gradient analysis of [$^3$H]RU486–receptor complexes. In both panels, ○—○, represents cytosol prepared as in Fig. 1 and analyzed in the absence of molybdate. (A) ■—■, cytosol prepared without 12 mM MTG; (B) ■—■, sucrose density gradients prepared with 10 mM molybdate. These data are representative of six experiments each.

from the nonsteroid binding proteins *in vivo*. As RU486 has little or no agonist activity [16], this suggests that progesterone receptor bound by this antihormone may not undergo transformation *in vivo*. This is in contrast to antiestrogens which have partial agonist activity [9, 25, 26]. Furthermore, antiprogestin has no known responses of its own, unlike antiestrogens which have been shown to have their own specific responses [27, 28]. We have demonstrated in other studies that salt-transformed, partially purified antiestrogen–receptor complexes bind to antiestrogen-specific chromatin acceptor sites [4, 29,30]. These antiestrogen-specific acceptor sites may be responsible for such antiestrogen-specific responses. We are presently performing similar experiments with rabbit uterine progesterone receptor bound by agonist and antagonist with the goal of better characterizing the chromatin binding activity of RU486-bound receptor.

Our present study as well as a recent report [14], suggest that the triphenylethylene antiestrogen H1285 stabilizes the 6S form of the transformed rabbit uterine estrogen receptor and that this receptor conformation appears to be a homodimer. We previously proposed that although the transformed agonist-bound receptor may bind to chromatin as a dimer, the receptor must dissociate to monomers in order to undergo nuclear processing. With antiestrogen, however, dissociation to monomers may be impaired; therefore, without receptor dissociation, nuclear processing is prevented and the target tissue becomes refractory to further estrogenic responses. This notion of an off-response is much like receptor systems in which desensitization occurs. Supportive of this hypothesis are earlier *in vivo* studies with antiestrogens which demonstrated prolonged nuclear retention and nuclear processing of antiestrogen–receptor complexes compared to estrogen-receptor complexes [6, 25, 26, 31, 32]. As our results indicate, RU486 does not stabilize the salt-transformed progesterone receptor in the 6S dimeric form as does antiestrogen with the estrogen receptor. Although this study is not a direct examination of the mechanism by which antiprogestin antagonizes progestin effects, it does suggest that RU486 may not act by inhibiting nuclear processing.

Mullick *et al.* [24] reported that transformed, salt-extracted nuclear progesterone receptor from human breast cancer cells bound by RU486 sedimented as a 4S form in the presence of reducing agents but that in the absence of these sulfhydryl reagents the antagonist-bound receptor sedimented as both 4S and 6S species, with varying amounts of receptor sedimenting as 6S depending on the cell line examined. This 6S form was not characterized and may consist of receptor associated with another nuclear component. We found that in the absence of reducing agents the cytosolic salt-transformed rabbit uterine antiprogestin–receptor complex sedimented as 4S only (Fig. 3A).

Also of interest are the results from our experiments with receptor partially purified in the presence of salt and 10 mM molybdate. We found that progesterone and estrogen receptors bound by agonist or antagonist sedimented as an intermediate nontransformed 6S species. This complex may be a receptor monomer still associated with a nonsteroid binding protein; however, preliminary experiments in our laboratory suggest that this intermediate form of receptor may be a nontransformed homodimer.

In conclusion, our results demonstrate that transformed rabbit uterine antiprogestin– and antiestrogen–receptor complexes have different physicochemical properties. That antiestrogen bound to receptor stabilizes a transformed dimer, whereas antiprogestin bound to receptor results in a transformed monomer, is suggestive of two different antihormone effects.

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