J. steroid Biochem. Vol. 23, No. 5A, pp. 537–545, 1985
Printed in Great Britain.

0022-4731/85 $3.00 + 0.00

ESTROGEN AND ANTIESTROGEN BINDING TO RAT UTERINE AND PITUITARY ESTROGEN RECEPTOR: EVIDENCE FOR AT LEAST TWO PHYSICOCHEMICAL FORMS OF THE ESTROGEN RECEPTOR

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(*Received* 12 *November* 1984)

Summary—Our laboratory has previously reported that calf uterine cytosol prepared in buffer containing 10 mM molybdate and chromatographed on DEAE-Sephadex contains two forms of the unactivated estrogen receptor, Peak I and Peak II; however, cytosol receptor bound to the high-affinity antiestrogen, H1285 (4-(*N*,*N*-diethylaminoethoxy)-4′-methoxy-α-(*p*-hydroxyphenyl)-α′-ethylstilbene), eluted only as Peak I. We have extended these studies to the rat uterus and pituitary in order to determine the organ and species specificity of this phenomenon. Cytosol prepared in Tris–molybdate buffer from immature and adult rat uteri or pituitaries was labelled with 10 nM [^{3}H]estradiol or [^{3}H]H1285 and chromatographed on QAE-Sephadex. Uterine estrogen receptors bound to either [^{3}H]estradiol or [^{3}H]H1285 eluted from QAE-Sephadex as a large Peak I (~0.21 M KCl) and a smaller Peak II (~0.25 M KCl). Analyses of these partially purified estrogen receptor fractions using high-salt sucrose density gradients showed that Peak I [^{3}H]estradiol–receptor complexes sedimented predominantly as a lighter form (4.0S). In contrast, Peak I [^{3}H]H1285–receptor complexes sedimented primarily as a heavier form (5.5S) often accompanied by a smaller lighter form (4.0S). Peak II [^{3}H]estradiol– and [^{3}H]H1285–receptor complexes sedimented as the heavier form (5.3–5.5S). These data suggest a monomer–dimer relationship between estrogen receptor forms with antiestrogen binding favoring the formation of the dimeric form. Further analysis of these Peak I receptor complexes by gel filtration chromatography yielded molecular forms of approx 70 KDaltons for [^{3}H]estradiol–receptor complexes and 73 KDaltons and 165 KDaltons for [^{3}H]H1285–receptor complexes, supporting the monomer–dimer concept. Data from experiments with the pituitary also suggest that H1285 causes the formation of the dimeric receptor form whereas estradiol interaction with the receptor results only in the monomeric form. These differences in estrogen receptor forms when bound by estrogen versus antiestrogen may be related to the different biological responses induced by these ligands.

INTRODUCTION

Our laboratory has recently reported that the molybdate-stabilized calf uterine estrogen receptor, whether bound to [^{3}H]estradiol [1] or unoccupied by ligand [2], eluted from DEAE-Sephadex columns as two sharp estrogen-saturable binding peaks at approx 0.21 and 0.25 M KCl (Peak I and Peak II, respectively). Analyses of these two [^{3}H]estradiol–receptor complex forms by sucrose density gradients indicated that Peak I estradiol–receptor complexes sedimented at approx 4.8S whereas Peak II sedimented as a heavier form at approx 6.3S [2]. These two forms are in apparent equilibrium with each other, as rechromatography of either Peak I or Peak II [^{3}H]-estradiol–receptor complexes on DEAE-Sephadex columns yielded both Peak I and Peak II forms. The possibility therefore exists that Peak II may represent a dimer of Peak I [^{3}H]estradiol–receptor complexes.

In contrast, calf uterine estrogen receptors bound to the high affinity antiestrogen [^{3}H]H1285 eluted from DEAE-Sephadex columns as a single estrogen-saturable peak (Peak I) [1, 3] with an S value of 5.5S [3]. The difference between antiestrogen and estrogen binding to the estrogen receptor may reside in the initial interaction between the ligand and the receptor since postlabeling experiments with [^{3}H]H1285 showed that this antiestrogen bound to both forms of the calf uterine estrogen receptor but altered the non-activated forms of the estrogen receptor to produce a single component on DEAE-Sephadex columns.

It is not clear how the different forms of the estrogen receptor relate to biological function. However, the observation that H1285 induces a difference in the physicochemical characteristics of the estrogen receptor suggests that the differences in the biological response between estrogens and antiestrogens, e.g. upon true uterine growth [4–6], may at least partly result from these differences in the physicochemical characteristics of the ligand–receptor complexes.

Because in the calf system it would be difficult and expensive to examine the *in vivo* relationship between the effects of estrogens and antiestrogens upon the forms of the estrogen receptor and the biological responses produced by these two compounds, we have begun to examine and characterize the forms of

the estrogen receptor in a model system more amenable to future experimental manipulation, the rat. We report here that, as in the calf-uterus, estrogen–receptor complexes from the rat uterus as well as the pituitary are physicochemically different from antiestrogen–receptor complexes.

EXPERIMENTAL

Chemicals

The high-affinity antiestrogen [^{3}H]H1285 (sp. act, 20 Ci/mmol) was prepared in our laboratory [7] from unlabeled H1285 (4-(*N*,*N*-diethylaminoethoxy)-4′-methoxy-α-(*p*-hydroxyphenyl)-α′-ethylstilbene), a gift of C. W. Emmens. 17β-[6,7-^{3}H]Estradiol (53 Ci/mmol) and [^{14}C]ovalbumin and [^{14}C]γ-globulin standards were purchased from New England Nuclear Corporation (Boston, MA). QAE-Sephadex Q-25 and DEAE-Sephadex A-25 were purchased from Sigma Chemical Company (St Louis, MO). Trisacryl-M DEAE resin was purchased from LKB Instruments, Inc. (Rockville, MD). Agarose-1.5 m and DEAE Bio-Gel A were obtained from Bio-Rad Laboratories (Richmond, CA).

Animals and tissue preparation

Immature (19-day old) and mature (55–60 days of age) Sprague–Dawley female rats (Sasco, St Louis, MO) were housed in group cages in a temperature (22–23°C)—and light (lights on between 0600–1800 h)—controlled environment with food and water available at all times. Ovariectomy of mature animals was performed under Methoxyflurane anesthesia and the animals were allowed to recover for 1–2 weeks.

The rats were killed by decapitation at 24–27 days of age (immature) or at 70–100 days of age (mature). The uteri were quickly removed, stripped of adhering fat, and placed into ice-cold TEGM buffer (10 mM Tris, 1.5 mM EDTA, 10 mM monothioglycerol, 10 mM sodium molybdate, pH 7.5). Whole pituitaries were removed from the sella turcica and also placed into ice-cold buffer. The uteri and pituitaries were used fresh or immediately frozen on dry ice and stored at −80°C. There was no detectable deterioration of estrogen receptor content during 2-month storage at −80°C.

Cytosol preparation

The uteri or pituitaries from several rats were pooled and homogenized in ice-cold TEGM buffer. All subsequent procedures were conducted at 4°C. The homogenate was centrifuged at 170,000 g_{av} (50,000 rpm, 70.1 Ti rotor) for 45 min to obtain cytosol. The cytosol was incubated with 10 nM [^{3}H]estradiol or 10 nM [^{3}]H1285 for 90 min at 4°C. Labeled cytosol was treated with a pellet from an equal volume of 1% dextran-coated charcoal (1% (w/v) Norit A activated charcoal and 0.1% (w/v) dextran in buffer) for 10 min, centrifuged, and the supernatant used immediately for ion-exchange column chromatography.

Ion-exchange column chromatography

Ten-ml columns (17 mm i.d. × 35 mm) containing QAE-Sephadex, DEAE-Sephadex, DEAE Bio-Gel A, or Trisacryl-M DEAE were prepared and washed with 50 ml of TEGM buffer. Charcoal-treated cytosol samples were loaded onto the columns and the columns were washed with an additional 25 ml of TEGM buffer alone or TEGM buffer containing 0.05 M KCl. Estrogen receptor was then eluted from the columns using a 90-ml linear KCl gradient (0.05–0.30 M or 0.00–0.40 M KCl) at a flow rate of 1 drop/3–4 s (8 ml/10 min). Fractions (usually 45-drop; 1.875 ml) were collected and the radioactivity in 500-μl aliquots was determined by adding 4 ml of scintillation fluid (0.4% Omnifluor, 25% Triton X-114 in xylene) and counting at 46% efficiency. KCl concentrations were determined by conductivity measurements using a Markson Model 4503 conductivity meter.

Sucrose density gradients

Linear 5–20% sucrose gradients containing TEGM buffer and 300 mM KCl were prepared and chilled to 4°C. Receptor peak fractions from the ion-exchange columns (160 μl) were combined with 40 μl of cytosol which had been heat-denatured at 37°C for 2 hr (to prevent loss of ligand during centrifugation) and layered onto the sucrose gradients. Sedimentation values obtained without the addition of heat-denatured cytosol were the same; however, the peaks were not always as sharp. The gradients were centrifuged for 16 h in a SW 56 rotor at 44,000 rpm (190,000 g_{av}). Fractions (3 or 4 drops) were collected from the top and counted in 4 ml of scintillation fluid (0.4% Omnifluor, 25% Triton X-114, 5% water (w/v/v) in xylene). [^{14}C]Ovalbumin (3.7S) and [^{14}C]γ-globulin (6.6S) standards were centrifuged in parallel gradient tubes.

Gel filtration column chromatography

Gel filtration chromatography was performed as described by Sherman *et al.*[7]. Aliquots from the ion-exchange ligand-receptor peak fractions were loaded onto Agarose A-1.5 m columns (100–200 mesh; 1.5 cm i.d. × 90 cm) and 30-drop fractions were collected at a flow rate of approx 10 ml/h in TEGM buffer containing 300 mM KCl and 20 mM sodium molybdate. Ethanol (100 μl) was added to each fraction tube, the contents mixed, and the radioactivity was determined in 0.5 ml aliquots of the column fraction. The Stokes' radii (R_s) of the standards used to calibrate the columns were: ovalbumin, 3.05 nm; bovine serum albumin, 3.59 nm; catalase, 5.22 nm; ferritin, 6.15 nm; and thyroglobulin, 8.61 nm. Blue dextran (2 mg/ml) was used to determine the void volume of the column. The molecular weight (M_r) of each receptor form was calculated as 4224 × sedi-

mentation value(S) × R_s as described by Sherman *et al.*[7].

Relative binding affinity

Cytosol was prepared in TEGM buffer and incubated for 90 min at 0–4°C with 10 nM [^{3}H]estradiol with various concentrations of unlabeled H1285 (10^{-11}–10^{-7} M) in a TEGM buffer containing 10% (v/v) dimethylformamide. A 150-μl aliquot of a hydroxylapatite slurry (1 vol packed hydroxylapatite to 3 vol TEGM buffer) was added to each tube and mixed intermittently during a 45-min incubation on ice. The hydroxylapatite pellet was then washed 4 times with TEGM buffer, and the radioactivity was extracted for 30 min at room temperature with 1 ml of ethanol, added to scintillation fluid, and counted. Specific binding was determined by subtracting nonspecific binding ([^{3}H]estradiol + 100-fold excess H1285 or estradiol) from the total binding ([^{3}H]estradiol alone). The relative cytosol binding affinity of H1285 was determined by the ratio of the concentration of unlabeled estrogen required to inhibit 50% of specific [^{3}H]estradiol binding to the concentration of H1285 required to inhibit 50% of specific [^{3}H]estradiol binding.

RESULTS

Ion-exchange column chromatography

Our laboratory has reported that the ability to distinguish two forms of the calf uterine estrogen receptor is dependent upon the characteristics of the ion-exchange resin [2]. In the following two experiments we have investigated the effects of salt and ion-exchange resin upon the ability to distinguish different ionic forms of the estrogen receptor in the rat uterus.

The effects of salt upon the elution of mature female rat uterine [^{3}H]estradiol–receptor complexes from DEAE-Sephadex columns are shown in Fig. 1. Two peaks of [^{3}H]estradiol binding were consistently eluted from DEAE-Sephadex columns using a KCl gradient (at approx 0.20 M and 0.24 M). However, elution of the DEAE-Sephadex columns with ammonium sulfate resulted in only a single [^{3}H]estradiol binding peak (at approx 0.14 M), suggesting that the type of salt used to elute estrogen receptors from the ion-exchange columns is important for the resolution of multiple forms of the receptor. NaCl gave elution patterns similar to those obtained with KCl.

In order to optimize the separation of the binding forms of the estrogen receptor, we also investigated the effects of several different ion-exchange resins. Using immature female rat uterine cytosol labeled with [^{3}H]estradiol and eluted from DEAE-Sephadex columns with a KCl gradient, two peaks of [^{3}H]estradiol binding were detected (a large Peak I fraction eluting at approx 0.20 M KCl and a smaller Peak II fraction eluting at approx 0.24 M KCl; Fig. 2A). The separation between Peak I and Peak II was greatly improved by using QAE-Sephadex as the ion-exchange resin (Fig. 2B), while Peak II [^{3}H]estradiol–receptor complex binding was completely absent when cytosol was chromatographed on

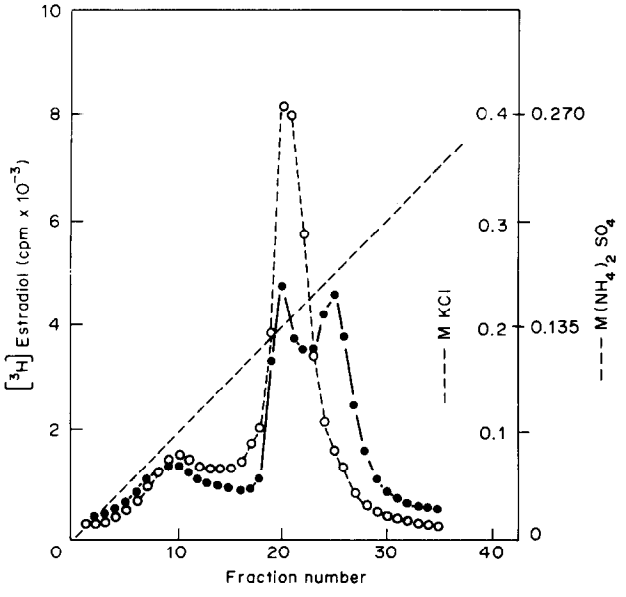

Fig. 1. Elution of [^{3}H]estradiol–receptor complexes from DEAE-Sephadex using various salt gradients. Cytosol from mature rat uteri was prepared in TEGM buffer and incubated with 10 nM [^{3}H]estradiol for 90 min at 4°C. Following treatment with dextran-coated charcoal, samples were applied to columns containing 10 ml of DEAE-Sephadex and eluted with linear 0.0–0.4 M KCl (●) or 0.00–0.27 M ammonium sulfate (○) gradients in TEGM buffer. Fractions (60 drops; 2.5 ml) were collected and the radioactivity was determined in a 500-μl aliquot. These elution profiles are representative of seven separate experiments.

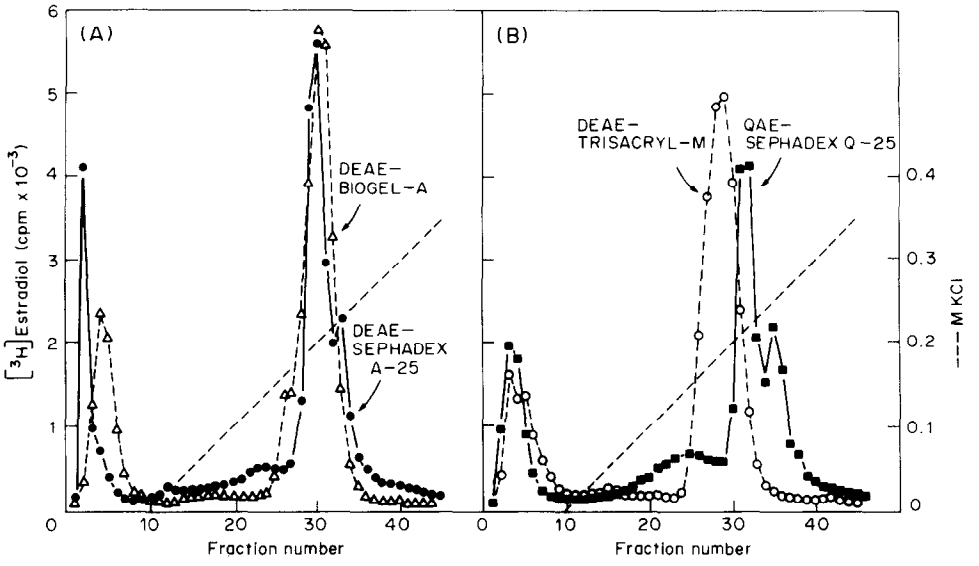

Fig. 2. Comparison of the elution of [^{3}H]estradiol–receptor complexes from various anion-exchange resins. Cytosol from immature rat uteri was prepared in TEGM buffer and incubated with 10 nM [^{3}H]estradiol for 90 min at 4°C. Following treatment with dextran-coated charcoal, samples were applied to columns containing 10 ml of DEAE-Sephadex (●), DEAE Bio-Gel A (▲), Trisacryl-M DEAE (○), or QAE-Sephadex (■). Columns were washed with 25 ml of TEGM buffer and the [^{3}H]estradiol–receptor complexes were eluted with linear 0.0–0.4 M KCl gradients in TEGM buffer. The arrow at fraction number 10 indicates the beginning of the KCl gradient. Fractions (60 drops) were collected and the radioactivity was determined in a 500-μl aliquot. The elution profiles are representative of 2–4 separate experiments.

either DEAE Bio-Gel A (Fig. 2A) or Trisacryl-M DEAE (Fig. 2B). Further experiments (not shown) indicated that the optimal separation of Peaks I and II on QAE-Sephadex could be obtained using a 0.05–0.30 M KCl gradient, and we therefore utilized this experimental procedure for most subsequent experiments.

Interaction in vitro *of* [^{3}*H*]*estradiol and* [^{3}*H*]*H1285 with the estrogen receptor*

Our laboratory has previously reported that the antiestrogen H1285 has at least a 10-fold increased affinity for binding to the rat uterine estrogen receptor as does estradiol [8, 9]. To evaluate the relative affinity of H1285 for the pituitary estrogen receptor, we incubated immature female rat pituitary cytosol with [^{3}H]estradiol and increasing concentrations of unlabeled estradiol or H1285. As shown in Fig. 3, H1285 was approx 10-fold more effective than estradiol in competing with [^{3}H]estradiol for binding to the estrogen receptor. These data are similar to those obtained in the immature rat uterus [8, 9], and these data demonstrate a similarity of H1285 binding to the estrogen receptor in a different estrogen target tissue in the rat, i.e. the pituitary.

Since we have established that both estradiol and H1285 will compete with [^{3}H]estradiol for binding to the estrogen receptor from both the rat uterus and pituitary, we wanted to determine whether estrogen receptor bound to [^{3}H]estradiol or [^{3}H]H1285 showed similar elution patterns from QAE-Sephadex columns. Uteri were labeled with 10 nM [^{3}H]estradiol or [^{3}H]H1285 and fractionated on QAE-Sephadex columns. As shown in Fig. 4, both Peak I and Peak II estrogen receptor forms are demonstrated when uterine cytosol is incubated with either [^{3}H]estradiol (Fig. 4A) or [^{3}H]H1285 (Fig. 4B). Coincubation of the cytosol fractions with excess unlabeled estradiol abolished the binding of the radioligands to both Peak I and Peak II forms of the estrogen receptor, indicating that the Peak I and Peak II estrogen-binding fractions represent two forms of an estrogen-

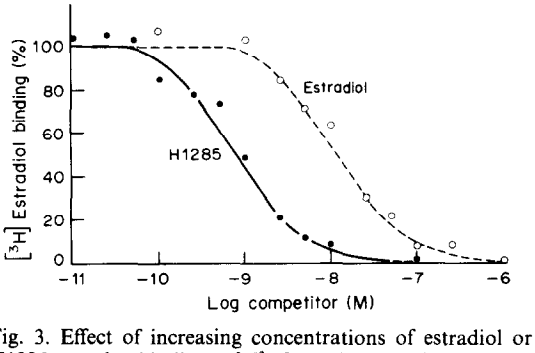

Fig. 3. Effect of increasing concentrations of estradiol or H1285 on the binding of [^{3}H]estradiol to the estrogen receptor from immature rat pituitary cytosol. Cytosol from immature female rat pituitaries was prepared in TEGM buffer and incubated with 10 nM [^{3}H]estradiol in the presence of increasing concentrations of competitor for 90 min at 4°C. Aliquots of the cytosol were assayed for specific binding by the hydroxylapatite method.

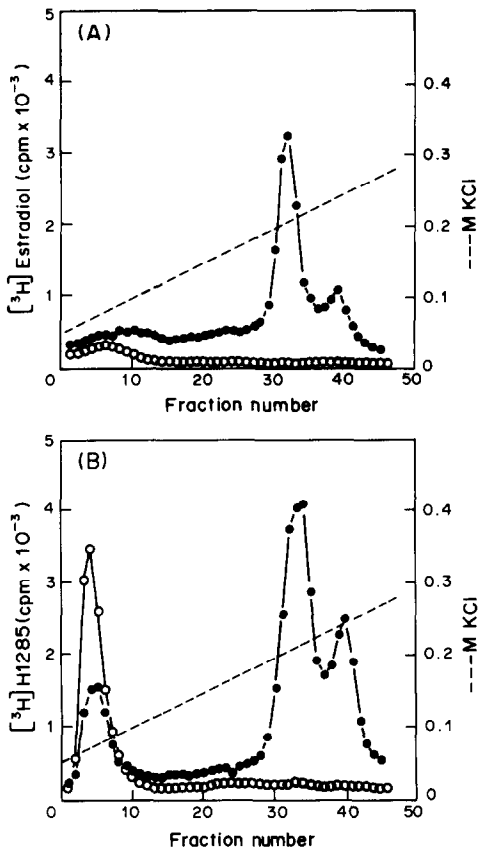

Fig. 4. Effect of competition by unlabeled estradiol on the elution of [^{3}H]estradiol– and [^{3}H]H1285–receptor complexes from QAE-Sephadex. Immature rat uterine cytosol was prepared in TEGM buffer and incubated with 10 nM [^{3}H]estradiol (Panel A) or [^{3}H]H1285 (Panel B) for 90 min at 4°C in the absence (●) or the presence (○) of excess unlabeled estradiol (1 μM and 5 μM estradiol for competition with [^{3}H]estradiol and [^{3}H]H1285, respectively). After treatment with dextran-coated charcoal, samples were applied to 10-ml QAE-Sephadex columns and eluted with linear 0.05–0.30 M KCl gradients in TEGM buffer. Fractions (45 drops; 1.875 ml) were collected and the radioactivity was determined in a 500-μl aliquot.

saturable estrogen receptor. Uterine proteins which bound [^{3}H]H1285 non-specifically eluted from QAE-Sephadex with low concentrations of KCl and this peak was not abolished by coincubation of the cytosol with 500-fold excess unlabeled estradiol (Fig. 4B). Similar binding of [^{3}H]estradiol and [^{3}H]H1285 to Peak I and Peak II forms of the estrogen receptor, and the abolishment of these radioligand-binding peaks with excess unlabeled estradiol, was observed using the rat pituitary gland as the source of the estrogen receptor (data not shown).

High-salt sucrose density gradients of Peak I and Peak II forms of the estrogen receptor

Our ability to separate two differently charged forms of the estrogen receptor permitted us to further characterize these forms. In the following study we examined the sedimentation characteristics of the Peak I and Peak II forms of the estrogen receptor when bound to either [^{3}H]estradiol or to [^{3}H]H1285. Sedimentation profiles for [^{3}H]estradiol and [^{3}H]H1285 binding to Peak I and Peak II forms of the estrogen receptors are shown in Fig. 5 and 6 for the immature rat uterus and pituitary, respectively. Peak I [^{3}H]estradiol–receptor complexes obtained from immature rat uteri sedimented primarily as a 4.0S form (Fig. 5B). In a few experiments a smaller peak sedimenting at approx 5.3S was also seen. In contrast, Peak II [^{3}H]estradiol–receptor complexes sedimented predominantly as a heavier form (approx 5.3S), although a smaller shoulder of receptor binding in the 4S region was also detected in some instances. In marked contrast, both Peak I and Peak II [^{3}H]H1285–receptor complexes sedimented as a heavier form (approx 5.5S) although a shoulder in the 4S region of Peak I was detected in several experiments (Fig. 5D). For all the above experiments, similar sedimentation values were obtained when NaCl was used instead of KCl in the sucrose gradients.

QAE-Sephadex chromatographic and sucrose density gradient centrifugation analyses of uterine [^{3}H]estradiol– and [^{3}H]H1285–receptor complexes obtained from mature intact and mature ovariectomized rats yielded data similar to that obtained with the immature rat. In the mature rat, whether intact or ovariectomized, the uterine [^{3}H]estradiol–receptor complexes eluted from QAE-Sephadex columns as two forms (at approx 0.18–0.21 M KCl and 0.23–0.25 M KCl for Peaks I and II, respectively). Peak I [^{3}H]estradiol–receptor complexes generally produced two binding peaks on sucrose density gradients (at 4.0S and 5.4S), while Peak I [^{3}H]H1285–receptor complexes sedimented only as a heavier form (5.0–5.2S). Peak II receptor complexes, whether bound to [^{3}H]estradiol or to [^{3}H]H1285, sedimented as the heavier form (5.0–5.6S).

Sedimentation properties of the estrogen receptor forms were also examined in another estrogen target tissue, the pituitary. Peak I [^{3}H]estradiol–receptor complexes from immature rat pituitaries sedimented as the lighter form (4.2S) without detectable binding activity in the 5–6S region (Fig. 6B). Similar results were obtained using mature rat pituitaries. However, the binding of the antiestrogen [^{3}H]H1285 to the estrogen receptor resulted in a sedimentation profile of Peak I receptor complexes containing two binding peaks, a larger one at approx 4.3S and another peak at approx 5.5S (Fig. 6D).

Gel filtration analysis of Peak I and Peak II estrogen receptor binding

Peak I [^{3}H]estradiol– and [^{3}H]H1285–receptor complexes were further analyzed by gel chromatography to ascertain whether the observed sedimentation differences between these two forms would also be reflected in differences in molecular weights and Stokes' radii. As shown in Table 1, immature rat

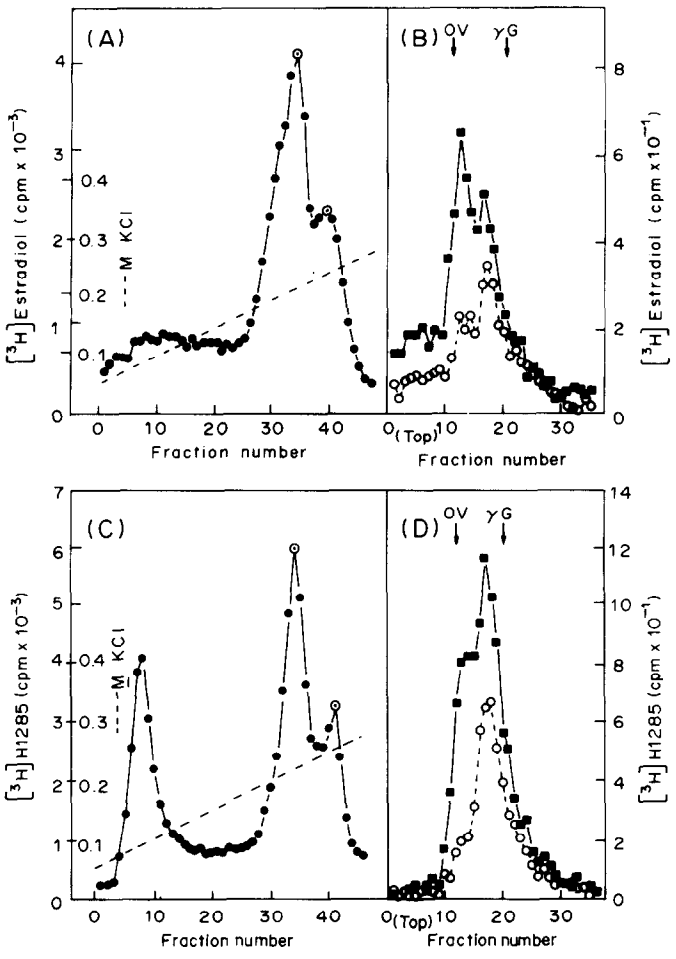

Fig. 5. QAE-Sephadex and sucrose density gradient analysis of uterine [^{3}H]estradiol– and [^{3}H]H1285–receptor complexes. Immature rat uterine cytosol was prepared in TEGM buffer and incubated for 90 min at 4°C with [^{3}H]estradiol (Panels A and B) or [^{3}H]H1285 (Panels C and D). After treatment with dextran-coated charcoal, samples were applied to 10-ml QAE-Sephadex columns and eluted as 45-drop fractions with linear 0.05–0.30 M KCl gradients in TEGM buffer (●) (Panel A for [^{3}H]estradiol; Panel C for [^{3}H]H1285). Aliquots of Peak I (■) and Peak II (○) [^{3}H]-ligand–binding fractions (160 μl), indicated by the circled fractions in Panels A and C, were mixed with 40-μl of heat-denatured cytosol and layered on 5–20% sucrose density gradients in TEGM buffer containing 300 mM KCl. The tubes were centrifuged at 190,000 g_{av} (SW56 rotor) for 16 h.[^{14}C]Ovalbumin (3.7S; 0V) and [^{14}C]γ-globulin (6.6S; γG) were centrifuged in parallel tubes as sedimentation markers. Gradient fractions (4-drop) were collected, counted, and plotted as total bound cpm.

Table 1. Gel filtration analysis of estrogen– and antiestrogen–receptor complexes*

Receptor form	R_s (nm)	M_r (KDaltons)
[^{3}H]*estradiol–receptor complexes*		
Peak I—monomer	4.1 ± 0.2	70
PeakII—dimer	5.4 ± 0.3	120
[^{3}H]*H1285–receptor complexes*		
Peak I—monomer	4.3 ± 0.1	73
Peak I—dimer	7.4 ± 0.2	165
Peak II—dimer	5.7 ± 0.2	132

*Immature rat uterine cytosol was prepared in TEGM buffer, incubated with 10 nM [^{3}H]estradiol or [^{3}H]H1285, and fractionated on QAE-Sephadex. Peak fractions were loaded onto Agarose 1.5 m columns and fractions collected in TEG buffer containing 20 mM molybdate and 0.3 M KCl. Calculations were according to the formula $K_{av} = V_e - V_0/V_t - V_0$ where V_e represents the elution vol, V_0 the void vol, and V_t the total vol of packed bed. The data for Stokes' radii (R_s) are the mean of 4–6 experiments ± SEM. The molecular weight (M_r) was calculated from the Stokes' radius and sedimentation value as described previously [7], i.e. 4224 × R_s × S.

uterine Peak I [^{3}H]estradiol–receptor complexes had one major form with an estimated molecular weight of approx 70 KDaltons (R_s = 4.1 nm). However, Peak I [^{3}H]H1285–receptor complexes were resolved into two forms with molecular weights of approx 165 KDaltons and 73 KDaltons. Peak II [^{3}H]-estradiol– or [^{3}H]H1285–receptor complexes from immature rat uteri were resolved as similar forms of approx 120–130 KDaltons. These data support the concept of a monomer–dimer relationship [2, 3]. In addition, H1285 seems to cause the formation of an altered dimer.

DISCUSSION

The existence of distinct physicochemical forms of steroid hormone receptors has recently been in-

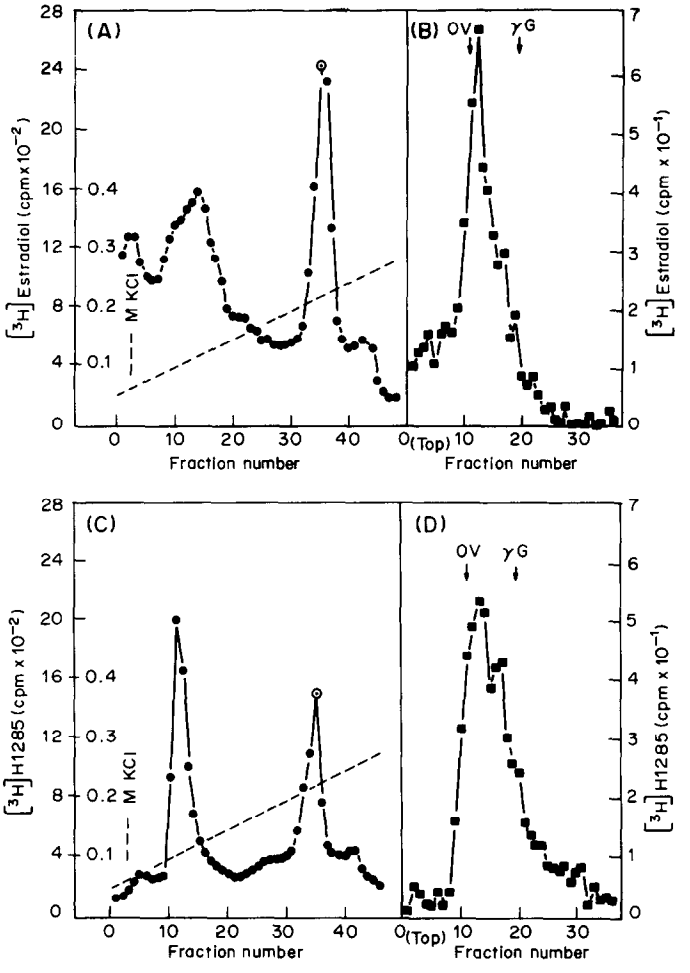

Fig. 6. QAE-Sephadex and sucrose density gradient analysis of pituitary [^{3}H]estradiol– and [^{3}H]H1285–receptor complexes. Immature rat pituitary cytosol was prepared in TEGM buffer and incubated for 90 min at 4°C with [^{3}H]estradiol (Panels A and B) or [^{3}H]H1285 (Panels C and D). After treatment with dextran-coated charcoal, samples were applied to 10-ml QAE-Sephadex columns and eluted as 45-drop fractions with linear 0.05–0.30 M KCl gradients in TEGM buffer (●) (Panel A for [^{3}H]estradiol; Panel C for [^{3}H]H1285). Aliquots of Peak I (■) [^{3}H]-ligand-receptor fractions (160 μl), indicated by the circled fractions in Panels A and C, were mixed with 40 μl of heat-denatured cytosol and layered on 5–20% sucrose density gradients in TEGM buffer containing 300 mM KCl. The tubes were centrifuged and counted as described in Fig. 5.

vestigated and described for several different steroid systems [10–12]. Our laboratory has previously reported that the molybdate-stabilized non-activated calf uterine estrogen receptor bound to [^{3}H]estradiol elutes from DEAE-Sephadex as two distinct forms (Peak I at 0.21 M KCl; Peak II at 0.25 M KCl) [1]. These two estrogen-binding peaks also sedimented in sucrose density gradients at two distinctly different S values (4.8S and 6.3S for Peak I and Peak II, respectively), suggesting a monomer (Peak I)-dimer (Peak II) relationship for the two forms of the calf uterine estrogen receptor [2].

In this present study we investigated the molybdate-stabilized non-activated forms of the estrogen receptor when bound by estrogen versus antiestrogen (1) in another species, the rat; (2) in another target tissue, the pituitary; and (3) with relation to age or endocrine state of the animal, i.e. immature, mature intact, mature ovariectomized. We report here that non-activated, molybdate-stabilized rat uterine and pituitary estrogen receptors bound to [^{3}H]estradiol are also resolved into two different forms by ion-exchange chromatography. The detection of both forms is, however, dependent upon both the type and the concentration range of salt which is used to elute the receptor peaks from the ion-exchange resin. In addition, the characteristics of the ion-exchange resin itself are also important. Stronger ion-exchange resins linked to Sephadex seem to be required to optimally separate the two ionic forms of the rat uterine estrogen receptor. QAE-Sephadex is a strongly basic anion exchanger which separates the two forms of the receptor better than DEAE-Sephadex, a more weakly basic anion

exchanger. DEAE-Biogel A and DEAE-Trisacryl M are weak ion exchange resins with backbones different from Sephadex which may account for their inability to separate the two forms. Therefore, these data suggest that the detection of estrogen receptor forms, and possibly the different physicochemical forms of other steroid hormone receptors, is highly dependent upon the characteristics of the resin and the salt which are chosen to elute and separate the receptor forms.

Our data are consistent with the hypothesis that a monomer–dimer equilibrium relationship exists for estrogen receptor forms. In addition, it appears that the binding of [^{3}H]H1285 to the estrogen receptor alters the equilibrium between the two forms to favor the stability of the heavier form of the estrogen receptor. This is most clearly evident in the difference in sedimentation values between uterine Peak I [^{3}H]estradiol– and [^{3}H]H1285–receptor complexes which sediment predominantly at 4.0S vs 5.5S, respectively. These data are supported by the results obtained with gel filtration which also suggests that H1285 causes the formation of an altered dimer. The degree to which the equilibrium is shifted by [^{3}H]H1285 is different for different tissues. In the pituitary, binding by [^{3}H]estradiol seems to result in only a monomeric form whereas [^{3}H]H1285 binding seems to result in both the monomeric and dimeric forms. These data are not identical to the results obtained from the calf, in which [^{3}H]H1285 binds to the total estrogen receptor and transforms the total estrogen receptor population to the Peak I form [1, 3] and alters the sedimentation behavior to an intermediate value (5.5S) between the Peak I (4.5S) and Peak II (6.3S) sedimentation values for [^{3}H]estradiol–receptor complexes. Nevertheless, it is clear that antiestrogen alters the physicochemical properties of the estrogen receptor in both the rat and the calf.

Multiple forms of the estrogen receptor are also evident in tissue from animals of different ages and endocrine states. There was little difference in data obtained from immature, mature intact, and mature ovariectomized rats. Thus, these two forms of the non-activated estrogen receptor were obtained regardless of the circulating estrogen levels.

The differences in the sedimentation behavior of molybdate-stabilized non-activated estrogen receptors when bound by estrogen versus antiestrogen which eluted in Peak I are similar to earlier observations of salt-activated [8, 13] and heat-activated [14] cytosol estrogen–receptor complexes. Immature rat uterine cytosol labeled with [^{3}H]estradiol sedimented primarily as a lighter form (4.4S) whereas tritiated high affinity antiestrogens altered the sedimentation of the estrogen–receptor complexes to a predominantly heavier (5.6S) form. It is possible that the alteration in the physicochemical properties of the estrogen receptor by antiestrogens is the result of an interaction of the antiestrogen–receptor complex with another protein moiety. Eckert and Katzenellenbogen[15] reported that the nuclear (activated) form of the MCF-7 cell antiestrogen–receptor complex (5S) differs from the nuclear estrogen–receptor complex (4S) by an association of the antiestrogen–receptor complex with an additional protein moiety with a molecular weight of approx 55KDaltons. However, more recently Miller *et al.*[16] reported that the heavier (5S) nuclear form of the MCF-7 cell antiestrogen–receptor complex is likely a homodimerization of the lighter (4S) form of the estrogen receptor. Finally, Tate *et al.*[17] have suggested that the shift in the sedimentation properties and ligand affinities of MCF-7 cell estrogen–receptor complexes versus antiestrogen receptor complexes, when the complexes are bound to antibodies produced against nuclear estrogen receptors, are possibly the result of a conformational change induced in the receptor when bound to antiestrogen which is different than that produced by estrogen.

These data and our data are consistent with the possibility that antiestrogen binding to the estrogen receptor could result in a conformational change in the receptor which is different than that produced by the binding of estrogens. Such a difference in estrogen receptor conformation induced by estrogens versus antiestrogens could subsequently affect the monomer–dimer equilibrium and render the antiestrogen–receptor complexes incapable of inducing the complete estrogenic responses in the target tissue. These changes could at least partly explain the observed differences in salt-resistant nuclear binding between estrogens and antiestrogens [18] and the inability of antiestrogen–receptor complexes to bind to the total complement of estradiol–receptor complex acceptor sites on calf [19] or rabbit [20] uterine chromatin. Additional studies will further characterize the forms of the estrogen receptor and determine the *in vivo* relationship between the forms of the estrogen receptor and the biological responses induced by estrogens and antiestrogens.

Acknowledgements—The authors wish to thank Mrs Terri Nally for typing the manuscript and Miss Chris Paulson for excellent technical assistance. This work was supported by NIH HD13425 and HD06362.

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