Interaction of the radiolabelled high-affinity anti-oestrogen [³H]H1285 with the cytoplasmic oestrogen receptor

Jeffery L. KEENE,* Frederick SWEET,† Mary F. RUH* and Thomas S. RUH*

*Department of Physiology, St. Louis University Medical School, St. Louis, MO 63104, U.S.A., and

†Department of Obstetrics and Gynecology, Washington University Medical School, St. Louis, MO 63110, U.S.A.

(Received 12 July 1983/Accepted 17 October 1983)

The high-affinity triarylethylene anti-oestrogen H1285 [4-(*NN*-diethylaminoethoxy)- β -ethyl- α -(*p*-hydroxyphenyl)-4'-methoxystilbene] was tritiated to high specific radioactivity (35 Ci/mmol). Competition experiments between [³H]H1285 and H1285 or oestradiol demonstrated that both compounds would compete with [³H]H1285 for oestrogen-specific binding sites in rat uterine cytosol. [³H]H1285 had at least 10 times the affinity for the receptor compared with oestradiol at the 50% competition level. [³H]H1285 appeared to have at least twice the association rate for the oestrogen receptor compared with [³H]oestradiol. In addition, the dissociation half-life ($t_{1/2}$) of specific binding of [³H]H1285 to oestrogen receptors at 0°C was about 220 h compared with a value of 60 h for [³H]oestradiol. Because of the extremely slow dissociation of [³H]H1285 from the oestrogen receptor, we were able to compare the sedimentation profiles of [³H]H1285–receptor complexes with those of [³H]oestradiol–receptor complexes in the presence of 0.4 M-KCl on 5–20% sucrose density gradients. [³H]Oestradiol–receptor complexes had a major peak at 4.4S with a smaller peak at 5.6S, whereas with [³H]H1285–receptor complexes the 5.6S peak was always higher than the 4.4S peak. There was significant variation between the dissociation behaviour at 20°C of [³H]H1285–receptor complexes and [³H]oestradiol–receptor complexes pre-activated at 25°C for 30 min in the presence and in the absence of 10 mM-sodium molybdate. The dissociation $t_{1/2}$ of [³H]oestradiol–receptor complexes at 20°C decreased from 1.5 h to 0.5 h when molybdate was present during heat treatment whereas the dissociation $t_{1/2}$ for [³H]H1285–receptor complexes was 5 h for both conditions. These observations indicate that there are fundamental differences in the initial interaction of H1285 and oestradiol with the oestrogen receptor.

Certain triarylethylene derivatives are anti-oestrogens, compounds that inhibit oestrogen action at oestrogen-responsive target tissues. Interest in these compounds stems from their clinical usefulness in the treatment of hormone-dependent breast tumours (Katzenellenbogen *et al.*, 1979). The molecular details of anti-oestrogen action in both the cytoplasm and the nucleus remain unclear. Work in this area was hampered until recently because of the lack of available radiolabelled anti-oestrogens with sufficient affinity for the oestrogen receptor to allow direct binding studies to be performed. Tamoxifen, for example,

has an affinity 10 times less than that of oestradiol-17 β for the oestrogen receptor (Jordan *et al.*, 1977), creating problems of dissociation and subsequent re-association with other cellular components during experiments. Recently, radiolabelled monohydroxylated forms of two commonly used anti-oestrogens, C1628 (Katzenellenbogen *et al.*, 1981a) and tamoxifen (Borgna & Rochefort, 1980), were shown to possess affinities for the oestrogen receptor nearly equivalent to that of oestradiol. By using these high-affinity compounds it has been possible to perform kinetic analyses of anti-oestrogen–receptor interactions (Katzenellenbogen *et al.*, 1981b) and to examine directly the mechanisms involved in anti-oestrogen-induced receptor transformation (Rochefort & Borgna, 1981).

Abbreviations used: DCC, dextran-coated charcoal; h.p.l.c., high-pressure liquid chromatography.

Our interest has focused on the manner in which the anti-oestrogen-receptor complex interacts with nuclei (Ruh & Baudendistel, 1977, 1978) and chromatin (Ruh *et al.*, 1981). Therefore we searched for an anti-oestrogen with sufficiently high affinity for use in chromatin-binding studies. The advantage of a high-affinity compound would be to decrease ligand dissociation and therefore to inhibit anti-oestrogen-receptor complex decomposition during lengthy chromatin-binding assays. We have previously characterized the anti-uterotrophic properties of the substituted triarylethylene H1285 [4-(*NN'*-diethylaminoethoxy)- β -ethyl- α -(*p*-hydroxyphenyl)-4'-methoxystilbene] and found H1285 to inhibit oestrogen-induced increases in uterine wet weight, total DNA content and rate of DNA and protein synthesis (Ruh *et al.*, 1979). Since H1285 has been shown to possess an affinity 10 times greater than that of oestradiol for the rat uterine cytoplasmic oestrogen receptor (Ruh *et al.*, 1979), we radiolabelled this compound. In the present study we describe the direct interaction of [^3H]H1285 with the cytoplasmic oestrogen receptor from the immature rat uterus. The high affinity and extremely low dissociation rate make [^3H]H1285 a useful tool for further studies on the mechanism of action of anti-oestrogens, particularly at the level of the nuclear interaction.

Experimental

Chemicals

H1285, synthesized by the method of Collins *et al.* (1971), was a gift from Professor C. W. Emmens. [^3H]Toluene, [6,7- ^3H]oestradiol (52 Ci/mmol), [^{14}C]methyl- γ -globulin, [^{14}C]methyl-ovalbumin and Omnifluor were purchased from New England Nuclear Corporation (Boston, MA, U.S.A.). Oestradiol-17 β , dextran and Triton X-114 were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Ultrapure sucrose for density-gradient analyses was purchased from Schwarz-Mann Co. (Spring Valley, NY, U.S.A.). Norit-A and dextran ($M_{r(\text{av.})}$ 20000) were obtained from Fisher Scientific (St. Louis, MO, U.S.A.). All other chemicals were reagent-grade.

Preparation of [^3H]H1285

[^3H]H1285 was prepared from an iodinated precursor prepared in the St. Louis University Medical School (by T. S. R.). H1285 was iodinated by the introduction of radioinert iodine into the positions *ortho* to the phenolic function (Fig. 1). To do this, 65 μmol (30 mg) of H1285 dissolved in 37.5 ml of benzene was added to a 500 ml reaction flask fitted with a rubber septum and previously

Fig. 1. Structure of the triarylethylene anti-oestrogen H1285

The asterisks represent the location of the ^3H labels.

flushed with dry N_2 . To this, 65 μmol (1.87 ml) of freshly made sodium ethoxide (0.8 mg of sodium metal/ml of ethanol) was added with a syringe. This was followed by the injection of 65 μmol of elemental iodine dissolved in 3.75 ml of benzene. The additions of sodium ethoxide and iodine were repeated. The reaction was maintained at 22°C for 5 min with magnetic stirring. The dark red solution was evaporated to dryness *in vacuo* at 30°C, and the residue redissolved in 10 ml of benzene, filtered and again evaporated to dryness. This residue was redissolved in 0.5 ml of benzene/diethylamine (7:1, v/v) for further purification.

The redissolved reaction products were layered on 20 ml of Whatman DC-1 silica gel (Whatman, Clifton, NJ, U.S.A.) contained in a glass column (1 cm $\times$ 30 cm) previously equilibrated in benzene/diethylamine (7:1, v/v). The products were eluted with the equilibration solvent into 4 ml fractions. Iodinated H1285 (R_F 0.29) was clearly separated from unreacted H1285 (R_F 0.53) and iodine as shown by t.l.c. The t.l.c. system consisted of Eastman Chromagram no. 13181 silica-gel plastic sheets with fluorescent indicator (Eastman Kodak Co., Rochester, NY, U.S.A.) with benzene/diethylamine (7:1, v/v) as the mobile phase. The purified iodinated product could be converted back into H1285 by exposure to an H_2 atmosphere in the presence of palladium-on-alumina catalyst (Alfa Products, Danvers, MA, U.S.A.). Fractions containing the iodinated precursor were combined and dried under N_2 . The resulting 40 mg of amber oil was packed in solid CO_2 and immediately sent to New England Nuclear for radiolabelling. The compound was tritiated by the method of Blackburn (1972) with 5% palladium on alumina as the catalyst and dimethylformamide as the solvent.

The radiochemical purity of the crude product received from New England Nuclear was determined to be 78% by t.l.c. with the above system.

Sections (3mm) of the t.l.c. sheet were added to 4ml of 0.4% Omnifluor in xylene for scintillation counting of radioactivity. Further purification was achieved by using silica-gel column chromatography. $[^3\text{H}]\text{H1285}$ [475mCi in 2ml of benzene/ethanol (1:1, v/v)] was dried under a stream of N_2 and redissolved in 0.5ml of benzene/diethylamine (7:1, v/v). This was chromatographed as described above for the iodinated precursor except that 0.5ml fractions were collected. Fractions with a radiochemical purity in excess of 95% were recombined, dried under N_2 and stored at -20°C at a concentration of 1.5mCi/ml in anhydrous tetrahydrofuran with 0.025% butylated hydroxytoluene (Aldrich Chemical Co., Milwaukee, WI, U.S.A.). After purification, 287mCi of $[^3\text{H}]\text{H1285}$ was recovered.

H.p.l.c. was performed with the use of a Waters model M-45 solvent-delivery system (Waters Associates, Milford, MA, U.S.A.), a Waters model 440 absorbance-detector (280nm) and a Waters $\mu\text{Bondapak C}_{18}$ reverse-phase column (3.9mm $\times$ 300mm). Tetrahydrofuran/50mM-sodium phosphate buffer (2:3, v/v), pH5.0, comprised the mobile phase. Typically, 40nCi of $[^3\text{H}]\text{H1285}$ was injected with 20 μg of unlabelled H1285 as carrier and the chromatography was performed at a flow rate of 0.5ml/min at 6.9MPa (1000lbf/in²). Twenty-five-drop fractions were collected and mixed with 4ml of 0.4% (w/v) Omnifluor and 25% (v/v) Triton X-114 in xylene for scintillation counting of radioactivity.

Cytosol preparation

Female Sprague-Dawley rats (21–26 days old) purchased from Sasco (Omaha, NB, U.S.A.) were used for all studies. The animals were killed by decapitation and the uteri were rapidly removed. After adhering fat and connective tissues were stripped away the uteri were placed in ice-cold TE buffer (10mM-Tris/1.5mM- Na_2EDTA , pH7.4). All subsequent procedures were performed at 4°C unless noted. The uteri were homogenized (five to ten uteri/ml of TE buffer) in Duall all-glass tissue grinders (Kontes Glass Co., Vineland, NJ, U.S.A.). Cytosol was obtained after centrifugation of the homogenate at 105000g (r_{av} 5.9cm) for 1h at 4°C in a Beckman model L5-50 ultracentrifuge with a Beckman type 40 rotor (Beckman Instruments, Palo Alto, CA, U.S.A.). The supernatant (cytosol) was diluted to one to two uteri/ml with TE buffer (0.7–2.2mg of protein/ml) for binding assays.

Binding assays

Stock solutions of $[^3\text{H}]\text{oestradiol}$, $[^3\text{H}]\text{H1285}$ and oestradiol were prepared as 100-fold-concentrated solutions in ethanol and diluted with uterine

cytosol to a final ethanol concentration of 1% (v/v) to yield the desired ligand concentrations. After the appropriate incubation, ligand not bound to receptor was removed by adsorption. DCC [0.1vol., 5% (w/v) Norit A-activated charcoal and 0.5% (w/v) dextran in TE buffer] was added to cytosol at 4°C . After rapid mixing, the samples were incubated on ice for 15min, at which time the charcoal was removed by centrifugation in a Beckman TJ-6 refrigerated centrifuge at 1500g for 10min at 4°C . Portions of the supernatants were each mixed with 4ml of scintillation fluid containing 25% (v/v) Triton X-114 and 0.4% Omnifluor (w/v) in xylene. Liquid-scintillation counting of radioactivity was performed on a Beckman model LS7500 scintillation spectrometer at a counting efficiency of 48%.

U.v. spectra

Absorbance measurements were made on a Beckman Instruments model 25 recording spectrophotometer. Quartz cuvettes with a 1cm light-path were used. $[^3\text{H}]\text{H1285}$ and H1285 were each dissolved in ethanol (10 $\mu\text{g}/\text{ml}$) and scanned from 400nm to 200nm.

Determination of specific radioactivity

The specific radioactivity of $[^3\text{H}]\text{H1285}$ was determined by comparing the absorbance at 290nm of the purified labelled compound in ethanol with a standard curve of known concentrations of H1285. The radioactivities of portions of the $[^3\text{H}]\text{H1285}$ solution were counted in 4ml of a 0.4% (w/v) Omnifluor in xylene solution. The counting efficiency was 58%. The specific radioactivity was determined to be 35Ci/mmol in this manner.

Relative binding affinity

To assess the relative binding affinity of H1285 and oestradiol, portions of the 105000g cytosol were incubated with 20mM- $[^3\text{H}]\text{H1285}$ plus increasing concentrations of H1285 or oestradiol (0.1–10mM). The ligands were added as dimethylformamide solutions and the final dimethylformamide concentration was 10% (v/v). All tubes were then incubated for 18h at 4°C .

Unbound ligand was removed by DCC adsorption, as described above.

Association rate constant (k_{+1}) at 0°C

The association reaction at 0°C was initiated by adding 0.5nM- $[^3\text{H}]\text{oestradiol}$ or 0.5nM- $[^3\text{H}]\text{H1285}$ to uterine cytosol obtained as described above. Non-specific binding was measured in a parallel incubation containing 100 μM -oestradiol in addition to the labelled ligands. At various times indicated portions were removed and added to the

DCC suspension to stop the reaction, and treated further as described above. Alternatively, the reaction was quenched by the addition of 1 μ M-oestradiol, and subsequently treated with DCC to removed unbound [3 H]H1285 and [3 H]oestradiol. Even though the ligand-receptor interaction is reversible, over brief time periods ligand dissociation is negligible. Therefore the association rate constant (k_{+1}) can be obtained from the following equation:

$$[1/(R_0 - RL)] - 1/R_0 = k_{+1}t$$

where R_0 is the total receptor site concentration, RL is the concentration of ligand-bound receptor and t the time in seconds. After re-arrangement of the equation, the data are plotted as t versus $1/(R_0 - RL)$, and k_{+1} equals the slope. The total amount of bound receptor was measured after 16 h with saturating amounts of the ligands.

Dissociation rate constant (k_{-1}) at 0°C

Cytosol was incubated with either 20 nM-[3 H]-H1285 or 20 nM-[3 H]oestradiol in the presence or in the absence of 2 μ M-oestradiol at 0°C. After 2 h unbound ligand was removed by DCC adsorption, and 2 μ M-oestradiol was added to all tubes to mask any re-association of label. This was designated as time zero. Portions were removed at the indicated times for DCC adsorption of free ligand and scintillation counting of the radioactivities of bound [3 H]H1285 and [3 H]oestradiol. Specific binding is represented as the difference between samples with and without 2 μ M-oestradiol in the original 2 h incubation. The dissociation half-life ($t_{1/2}$) is the length of time necessary for 50% of the time zero specifically bound ligand to dissociate from the receptors. The dissociation rate constant is calculated as: $k_{-1} = (\ln 2)/t_{1/2}$.

Receptor transformation by [3 H]H1285 and [3 H]oestradiol

Cytosol prepared both in the presence and in the absence of 10 mM-sodium molybdate was incubated with either 5 nM-[3 H]oestradiol or 5 nM-[3 H]-H1285 for 2 h at 0°C. To assess non-specific binding, parallel incubation tubes contained 2 μ M-oestradiol in addition to the labelled ligands. All samples were then warmed at 25°C for 0.5 h. After cooling to 0°C, unbound ligand was removed by DCC adsorption. After transferring all samples to 20°C, dissociation was initiated by adding 2 μ M-oestradiol to mask re-association of the label to the receptor. Binding was measured at various times by DCC adsorption. Specific binding was taken as the difference between determinations with and without 2 μ M-oestradiol in the original 2 h incubation. Receptor stability was assessed at 3 h by maintaining parallel incubations with the original

concentrations of 3 H-labelled ligand with and without 2 μ M-oestradiol. Under all conditions receptor stability at 3 h was found to be 95% or greater.

Sucrose density gradients

Cytosol was incubated at 0°C for 2 h with either [3 H]oestradiol or [3 H]H1285 both in the presence and in the absence of a 100-fold molar excess of oestradiol. Samples (150 μ l) were treated with DCC just before being layered on 3.6 ml of 5–20% (w/v) linear sucrose density gradients containing 0.4 M-KCl prepared in TE buffer. The tubes were centrifuged in a Beckman Instruments model L5-50 ultracentrifuge with a Beckman SW 56 rotor at 225 000 g (r_{av} 8.8 cm) for 19 h. Fractions (four drops each) were collected by ascending flow by using 30% (w/v) sucrose infused through the bottom of each tube. [14 C]Methylated ovalbumin (3.7 S) and [14 C]methylated γ -globulin (6.6 S) were used as external sedimentation-coefficient markers.

Results

H1285 was radiolabelled to high specific radioactivity (35 Ci/mmol). The u.v.-absorbance spectra for [3 H]H1285 and H1285 are shown in Fig. 2(a). Both compounds display similar spectra with absorbance peaks at 245 and 290 nm. The purified radioactive compound co-chromatographed with freshly prepared H1285 as a single peak in both t.l.c. (Fig. 2b) and h.p.l.c. (Fig. 2c) systems.

Interaction in vitro of [3 H]H1285 with oestrogen receptor

To evaluate the affinity of the receptor for [3 H]H1285 we incubated cytosol with the radioligand and concurrently with increasing concentrations of unlabelled oestradiol and H1285 (Fig. 3b). It can be seen that approximately a 10-fold molar excess of oestradiol is needed to decrease specific binding to the 50% level. As expected, equimolar unlabelled H1285 diminished specific [3 H]H1285 binding to 50%. These data indicate that the affinity of the oestrogen receptor for [3 H]-H1285 appears to be 10 times its affinity for [3 H]-oestradiol. These results confirm previous studies from this laboratory with [3 H]oestradiol as the associated ligand and H1285 as the competitor, i.e. H1285 was approx. 10-fold more effective as a competitor of [3 H]oestradiol binding than was oestradiol (Fig. 3a).

Having established that the receptor has a higher affinity for [3 H]H1285 than for [3 H]oestradiol, we wanted to determine if this enhanced affinity was due to an increased association rate, decreased dissociation rate, or both. Fig. 4 shows the results of an experiment to determine the as-

Fig. 2. Assessment of radiochemical purity of [³H]H1285 by (a) u.v. spectra, (b) t.l.c. or (c) h.p.l.c.

In (a), a u.v.-absorbance spectrum was obtained for [³H]H1285 dissolved in ethanol. This was compared with a series of spectra of known amounts of unlabelled H1285 to determine the concentration of the labelled compound. Ethanol was used as the blank. [³H]H1285 was directly analysed by t.l.c. in (b) as described in the Experimental section. The mobility of the unlabelled compound is indicated by the bar above the peak. The retention of [³H]-H1285 on h.p.l.c. was compared with that of H1285 in (c) when the compounds were chromatographed simultaneously. The upper trace represents radioactivity, and the lower trace represents the absorbance at 280 nm.

sociation rate constant (k_{+1}) at 0°C. For [³H]-H1285 we measured a k_{+1} of $9.5 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$ and for [³H]oestradiol $4.8 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$.

The rate of dissociation of [³H]H1285 from the oestrogen receptor is responsible for much of the high affinity seen by the compound. As shown in Fig. 5, at 0°C [³H]H1285 dissociated only very slowly from the receptor when compared with oestradiol. Both compounds exhibited monophasic dissociation kinetics typical of receptor in the non-transformed state. The $t_{1/2}$ for [³H]H1285 is 220 h, which corresponds to a k_{-1} of $8.8 \times 10^{-7} \text{ s}^{-1}$. [³H]-Oestradiol displayed a $t_{1/2}$ of 50 h and a k_{-1} of $3.2 \times 10^{-6} \text{ s}^{-1}$. The $t_{1/2}$ for [³H]H1285 is about 4-fold greater than the $t_{1/2}$ reported for the high-affinity anti-oestrogen [³H]CI628M and 300-fold greater than the $t_{1/2}$ for [³H]CI628 (Katzenellenbogen *et al.*, 1981b). Both oestrogen and anti-oestrogen-occupied receptor complexes in our studies were found to be stable for the duration of the experiments (results not shown).

It has previously been reported that receptor

transformation triggered by the triarylethylene monohydroxytamoxifen was qualitatively different from that induced by oestradiol as determined from dissociation kinetics (Rochefort & Borgna, 1981). We performed similar experiments to determine if this was specific for monohydroxytamoxifen or if it might be a more generalized phenomenon for triarylethylene anti-oestrogens. We compared the dissociation behaviour at 20°C of [³H]H1285 and [³H]oestradiol (Fig. 6) from both the heat-transformed oestrogen receptor and from the sodium molybdate-stabilized receptor. The oestradiol-occupied molybdate-stabilized receptor displayed a lower affinity for the ligand than did the oestradiol-occupied heat-transformed receptor, as shown by dissociation kinetics. At 20°C, the oestradiol dissociated more rapidly from the molybdate-stabilized than from the transformed oestrogen receptor. In marked contrast, there was no apparent difference in the dissociation behaviour of [³H]H1285 from either the native (stabilized) or heat-treated oestrogen receptor. Both at

Fig. 3. Competition between H1285 and oestradiol for the oestrogen receptor

Cytosol from rat uteri was incubated with either 20nM- $[^3\text{H}]$ oestradiol (a) or $[^3\text{H}]$ H1285 (b) in the presence of increasing concentrations of oestradiol or H1285. After 18 h at 4°C, bound radiolabelled ligand was determined by DCC adsorption. The horizontal broken line represents the concentration of unlabelled compound needed to inhibit binding by 50%

Fig. 4. Determination of association rate constant for $[^3\text{H}]$ -H1285

Rat uterine cytosol was incubated with either 0.5nM- $[^3\text{H}]$ H1285 or 0.5nM- $[^3\text{H}]$ oestradiol both with and without a 100-fold molar excess of oestradiol. Specific binding was determined by DCC adsorption at the indicated times. The data are plotted as described in the Experimental section. Total oestrogen-receptor concentration was determined in a parallel incubation containing 10nM- $[^3\text{H}]$ oestradiol incubated for 18 h at 0°C.

Fig. 5. Dissociation of $[^3\text{H}]$ H1285 and $[^3\text{H}]$ oestradiol from the oestrogen receptor at 0°C

Cytosol was incubated at 0°C with 20nM- $[^3\text{H}]$ H1285 or $[^3\text{H}]$ oestradiol in the presence and in the absence of 2 μM -oestradiol. After a 2 h incubation unbound ligand was removed by DCC adsorption (time zero) and all incubation tubes were adjusted to 2 μM -oestradiol. Bound radioactivity was determined at the indicated times by DCC adsorption. 100% = oestrogen-receptor-specific binding (difference between 2 h incubation with and without excess oestradiol). The different symbols for each labelled ligand represent two different experiments.

0°C (Fig. 5) and at 20°C (Fig. 6) $[^3\text{H}]$ H1285 dissociated much more slowly from the receptor than did oestradiol. Therefore $[^3\text{H}]$ H1285-receptor complexes, like $[^3\text{H}]$ monohydroxytamoxifen-receptor complexes, did not display molybdate-sensitivity with regard to dissociation kinetics. However, $[^3\text{H}]$ monohydroxytamoxifen dissociates from the receptor 4-fold faster than does $[^3\text{H}]$ -H1285 in the rat uterus (Rochefort & Borgna, 1981).

High-salt sucrose density gradients

The high affinity of $[^3\text{H}]$ H1285 for the oestrogen receptor enabled us to perform sedimentation studies without dissociation and re-association of the anti-oestrogen during centrifugation. Sedimentation profiles for cytosol incubated with either 4nM- $[^3\text{H}]$ oestradiol or $[^3\text{H}]$ H1285 with and without excess unlabelled oestradiol are shown in Fig. 7. A major peak of $[^3\text{H}]$ oestradiol-receptor complexes consistently appeared at 4.4S, with a

Fig. 6 Dissociation of $[^3\text{H}]\text{H1285}$ and $[^3\text{H}]\text{oestradiol}$ from heat-transformed or molybdate-stabilized oestrogen receptor

Uterine cytosol was incubated for 2 h at 0°C with either 4 nM $[^3\text{H}]\text{oestradiol}$ or 4 nM $[^3\text{H}]\text{H1285}$ both in the presence and in the absence of 10 mM sodium molybdate. Parallel incubation tubes containing a 100-fold molar excess of unlabelled oestradiol were used to determine non-specific binding. The samples were then heated at 25°C for $\frac{1}{2}$ h and rapidly cooled to 0°C . All incubation tubes were then treated with DCC to remove unbound radioligand and all tubes were brought to $0.4\text{ }\mu\text{M}$ oestradiol to mask re-association of the label with the receptor. After transference of the tubes to a 20°C water bath specific binding was determined by DCC adsorption at the indicated times.

smaller peak at 5.6 S (Fig. 7b). This was in contrast with the $[^3\text{H}]\text{H1285}$ -receptor complexes, which displayed the same two sedimentation values, but the 5.6 S peak was always higher than the 4.4 S peak (Fig. 7a). The presence of a 200-fold molar excess of oestradiol abolished binding of both $[^3\text{H}]\text{oestradiol}$ and $[^3\text{H}]\text{H1285}$ to the cytosol oestrogen receptor.

Discussion

In the past direct analysis of the binding of anti-oestrogens to the oestrogen receptor was impaired by the fact that all known anti-oestrogens had a low affinity ($<10\%$) for the oestrogen receptor. It is only recently that high-affinity radiolabelled anti-oestrogens have become available for study (Borgna & Rochefort, 1980; Katzenellenbogen *et al.*, 1981b; Ruh *et al.*, 1982). In searching for a potential high-affinity anti-oestrogen we were interested in the reports from Emmen's group (Collins *et al.*, 1971; Emmen, 1973) that the triarylethylene H1285, synthesized in their labora-

tory, had very potent anti-fertility properties in the mouse, although it was not tested for anti-uterotrophic properties. Our subsequent discovery that H1285 had an extremely high affinity for the oestrogen receptor (Ruh *et al.*, 1979) suggested that H1285 would be a useful tool to study anti-oestrogen action. We therefore ^3H -labelled H1285 to use as a high-affinity probe of anti-oestrogen action.

In the present study we have used $[^3\text{H}]\text{H1285}$ to assess the interaction of anti-oestrogens with the cytoplasmic oestrogen receptor. Previous studies from this laboratory indicated that H1285 had a relative affinity 11-fold greater than oestradiol for the rat oestrogen receptor. In the present paper we have confirmed this with the use of $[^3\text{H}]\text{H1285}$ and have further been able to characterize the binding parameters of the system. The high affinity of H1285 for the oestrogen receptor is reflected in the dissociation and association reactions. H1285 has a dissociation rate approx. 4-fold times slower than oestradiol from the oestrogen receptor and an association rate approximately twice that of oestradiol.

A further difference between H1285-receptor interaction and oestradiol-receptor interaction is the dissociation rate kinetics in the presence and in the absence of 10 mM sodium molybdate. Oestrogen receptor can be transformed by heating at 25°C or by exposure to 0.4 M KCl or -NaCl . Oestrogen receptor thus transformed displays an increased affinity for nuclei, ATP-Sepharose and DNA-cellulose and sediments at 5 S on sucrose density gradients (Grody *et al.*, 1982). The presence of 10 mM sodium molybdate during warming at 25°C prevents receptor transformation (Shyamala & Leonard, 1980). This is evidenced by the more rapid dissociation rate of oestradiol from the receptor in the presence of molybdate (Rochefort & Borgna, 1981). However, the dissociation of H1285 from the receptor appears to be unaffected by the addition of molybdate. This difference between transformation of anti-oestrogen complexes and oestradiol-receptor complexes was previously seen by Rochefort & Borgna (1981) with monohydroxytamoxifen, an anti-oestrogen with a dissociation rate similar to that of oestradiol.

The very slow dissociation rate of $[^3\text{H}]\text{H1285}$ from the oestrogen receptor ($t_{1/2} = 220\text{ h}$ at 0°C) makes this compound especially attractive for studying the interaction of anti-oestrogen-receptor complexes with nuclear components. Preliminary studies from this laboratory indicate that H1285-receptor complexes interact with different chromatin fraction than do oestradiol-receptor complexes (Ruh *et al.*, 1982). Future work in this area should lead to a better understanding of the molecular mechanisms of oestrogen and anti-oestrogen action.

Fig. 7. High-salt sucrose-density-gradient analysis of cytoplasmic [³H]oestradiol-receptor and [³H]H1285-receptor complexes

Cytosol from immature rat uteri was incubated with 5 nM-[³H]H1285 (a) or 5 nM-[³H]oestradiol (b) both in the presence and in the absence of a 100-fold molar excess of unlabelled oestradiol. After 2 h at 0°C unbound ligand was removed by DCC adsorption, and the cytosol was adjusted to 0.4 M-KCl. A portion (150 µl) was layered on a 3.6 ml 5–20% linear sucrose gradient containing 0.4 M-KCl in TE buffer. Centrifugation was for 19 h at 225 000g. The open symbols represent cytosol incubated in the presence of excess oestradiol (E₂). Abbreviations used: OV, [¹⁴C]methylated ovalbumin; γG, [¹⁴C]methyl-γ-globulin.

We thank Professor C. W. Emmens for the gift of H1285, and Mrs. T. Nally for typing the manuscript. This work was supported by National Institutes of Health Grant HD 13425.

References

- Blackburn, G. E. (1972) *J. Labeled Compounds* **8**, 279–292
- Borgna, J.-L. & Rochefort, H. (1980) *Mol. Cell. Endocrinol.* **20**, 71–85
- Collins, D. J., Hobbs, J. J. & Emmens, C. W. (1971) *J. Med. Chem.* **14**, 952–957
- Emmens, C. W. (1973) *J. Reprod. Fertil.* **34**, 23–28
- Grody, W. W., Schrader, W. T. & O'Malley, B. W. (1982) *Endocrine Rev.* **3**, 141–163
- Jordan, V. C., Dix, C. J., Rowsby, L. & Prestwich, G. (1977) *Mol. Cell. Endocrinol.* **7**, 177–192
- Katzenellenbogen, B. S., Bhakoo, H. S., Ferguson, E. R., Lan, N. C., Tatee, T., Tsai, T.-L. S. & Katzenellenbogen, J. A. (1979) *Recent Prog. Horm. Res.* **35**, 259–297
- Katzenellenbogen, B. S., Rorke, E. A. & Eckert, R. L. (1981a) *J. Steroid Biochem.* **15**, 219–229
- Katzenellenbogen, B. S., Pavlik, E. J., Robertson, D. W. & Katzenellenbogen, J. A. (1981b) *J. Biol. Chem.* **256**, 2908–2915
- Rocheftort, H. & Borgna, J. L. (1981) *Nature (London)* **292**, 257–259
- Ruh, T. S. & Baudendistel, L. J. (1977) *Endocrinology* **100**, 420–426
- Ruh, T. S. & Baudendistel, L. J. (1978) *Endocrinology* **102**, 1828–1846
- Ruh, T. S., Baudendistel, L. J., Nicholson, W. F. & Ruh, M. F. (1979) *J. Steroid. Biochem.* **89**, 599–611
- Ruh, T. S., Ross, P., Jr., Wood, D. M. & Keene, J. L. (1981) *Biochem. J.* **200**, 133–143
- Ruh, T. S., Keene, J. L. & Ross, P., Jr. (1982) in *Hormone Antagonists* (Agarwal, M. K., ed.), pp. 163–178, Walter de Gruyter, Berlin
- Shyamala, G. & Leonard, L. (1980) *J. Biol. Chem.* **255**, 6028–6031