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Dissociation Kinetics of the Estrogen Receptor Immobilized by Hydroxylapatite†

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ABSTRACT: The estrogen receptor from the calf uterine cytosol was adsorbed to hydroxylapatite to provide a simpler and more controlled model for investigating in vitro the [^{3}H]estradiol dissociation kinetics of the estrogen receptor–nuclear complex. The dissociation of [^{3}H]estradiol at 29 °C from the estrogen receptor immobilized by hydroxylapatite showed biphasic kinetics with fast and slow components characteristic of the nonactivated and activated states of the receptor, respectively. The dissociation rate constant of the fast component (k_{-1} = 0.059 ± 0.004 min^{-1}), measured by exchange with estradiol, was one-third slower than that for the receptor free in the cytosol (k_{-1} = 0.162 ± 0.011 min^{-1}). The magnitude of the fast component was 17% when the receptor was bound to hydroxylapatite and 26% when in cytosol. The decrease in the rate of [^{3}H]estradiol dissociation and the reduction in the magnitude of the fast component suggest that receptor binding to hydroxylapatite facilitates activation of the estrogen receptor. The dissociation rate constant of the slower component, k_{-2}, at 29 °C was not significantly different whether the receptor was bound to hydroxylapatite (k_{-2} = 6.62 ± 0.26 × 10^{-3} min^{-1}), free in cytosol (k_{-2} = 7.25 ± 0.46 × 10^{-3} min^{-1}), or preactivated before being bound to the hydroxylapatite (k_{-2} = 6.93 ± 0.52 × 10^{-3} min^{-1}). The [^{3}H]estradiol dissociation rate from the activated estrogen receptor bound to hydroxylapatite, when measured by exchange, was not influenced by a 150- to 1500-fold excess of estradiol (0.5–5 μM estradiol), although massive quantities (10 or 200 μM) of estradiol increased the dissociation rate (k = 12.2 × 10^{-3} min^{-1} and 0.088 min^{-1}, respectively). Combinations of 1 μM estradiol plus 200 μM progesterone increased the dissociation rate (k = 0.067 min^{-1}), while 200 μM progesterone alone did not displace the bound [^{3}H]estradiol or affect receptor stability. High concentrations of steroids exerted a nonspecific detergent action on the receptor. Triton X-100 (0.01%) markedly increased [^{3}H]estradiol dissociation (k = 0.11 min^{-1}). The use of Triton X-100 to isolate nuclei containing receptors affects the [^{3}H]estradiol dissociation kinetics of the receptor and may influence the receptor's interactions with the nucleus. The hydroxylapatite-immobilized estrogen receptor is useful in investigating the kinetics and mechanism of estrogen receptor activation and serves as a tool for understanding the estrogen receptor–nuclear interactions.

The existence of a specific estrogen-binding protein or receptor and the receptor's relationship to the tissue responses initiated by the estrogens have been reviewed (Gorski et al., 1968; Jensen & De Sombre, 1973; Gorski & Gannon, 1976). The complex formed between estradiol and the receptor results in activation (transformation) and the translocation of the receptor from the cytoplasm to the nucleus (Jensen et al., 1968; Shyamala & Goski, 1969). We have described the molecular properties and mechanism of the transformation of the cytoplasmic 4S form of the receptor, a monomer with a molecular weight of (7–8) × 10^4, into the activated or nuclear 5S receptor, a dimer with a molecular weight of (13–14) × 10^4 (Notides & Nielsen, 1974, 1975; Notides et al., 1975). We have recently found that the receptor exists in two estrogen-binding affinity states and that estrogen binding shifts the equilibrium between the monomer and dimer toward the higher affinity state, the activated 5S form of the receptor (Weichman & Notides, 1977). The two affinity states of the receptor are readily measured by the unique, biphasic [^{3}H]-estradiol dissociation kinetics of the receptor. The fast [^{3}H]estradiol dissociating phase is generated by the nonactive 4S receptor, while the second, slower dissociating component is a property of the activated 5S receptor (Weichman & Notides, 1979).

In the light of these findings, it is preferable to use the [^{3}H]estradiol dissociation kinetics which are sensitive and specific as a probe of the receptor interactions with the nucleus. To establish the validity of this approach, we report here the properties and kinetics of the [^{3}H]estradiol dissociation from the receptor that has been immobilized to hydroxylapatite. These findings serve as an important prerequisite and model for understanding the interactions of the estrogen receptor with the nucleus as described in the accompanying paper (de Boer and Notides, 1981).

Experimental Procedures

Materials. The 17β-[2,4,6,7-^{3}H]estradiol (108 Ci/mmol), 17β-[2,4,6,7,16,17-^{3}H]estradiol (160 Ci/mmol), and Triton X-100 (scintillation grade) were obtained from Amersham/Searle. The radiochemical purity of the estrogens was verified by thin-layer chromatography in a chloroform–ethyl acetate (3:1) solvent system. Unlabeled 17β-estradiol and progesterone

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were obtained from Steraloids. Ultrapure grade Tris was from Schwarz/Mann, and hydroxylapatite (Bio-Gel HT) was purchased from Bio-Rad Laboratories. All other reagents used were analytical grade.

Preparation of Calf Uterine Cytosol. The cytosole fraction was prepared as previously described (Weichman & Notides, 1977). Briefly, calf uteri cooled in liquid nitrogen were reduced to a powder with a steel mortar and pestle precooled with liquid nitrogen and then homogenzied at 0 °C with a Polytron PT-10 in 5 volumes of 40 mM Tris/1 mM dithiothreitol (buffer TD). The buffer pH was preadjusted to 7.4 at the temperature of the dissociation assay. All subsequent procedures were performed at 0–4 °C unless otherwise indicated. The homogenate was centrifuged at 20000*g* for 10 min; then the supernatant was centrifuged at 220000*g* for 30 min. The resulting supernatant is referred to as the cytosol.

Assay of [^{3}H]Estradiol Dissociation from the Estrogen Receptor of the Cytosol. Uterine cytosol was equilibrated with 10–15 nM [^{3}H]estradiol for 60 min at 0 °C. Nonspecific [^{3}H]estradiol binding was measured by a parallel incubation of the cytosol with [^{3}H]estradiol containing an additional 200-fold excess of unlabeled estradiol. Aliquots of the cytosol–[^{3}H]estradiol mixture were then incubated at either 0 °C or 29 °C to obtain, respectively, the nonactivated and activated forms of the receptor. In some experiments cytosol aliquots were made 0.01% with respect to Triton X-100 before performing the dissociation assay. The dissociation of [^{3}H]estradiol from the receptor was measured by rapidly increasing the temperature to 29 °C in the presence of an excess of unlabeled estradiol. For measurement of receptor stability, the temperature was increased to 29 °C without the addition of an excess of estradiol. The details of the analysis of the dissociation were previously described (Weichman & Notides, 1977, 1979).

Binding the Estrogen Receptor to Hydroxylapatite: [^{3}H]Estradiol Dissociation from the Immobilized Receptor. The uterine cytosol–[^{3}H]estradiol mixture was incubated with charcoal (0.3% Norit A–0.03% dextran 500) at 0 °C for 5 min to adsorb the unbound estradiol. The charcoal was removed by centrifugation at 1500*g* for 5 min. Aliquots of cytosol in the ratio of 1 mL to 0.7 mL of a hydroxylapatite suspension (1:1 in buffer TD) were incubated at 0 °C for 30 min. The hydroxylapatite was centrifuged and washed twice in 1 mL of buffer TD. To initiate the dissociation assay, 1 mL of the hydroxylapatite-immobilized receptor suspension was pipetted at 29 °C into 7 or 50 mL of buffer TD (a 7- or 50-fold dilution) containing the indicated concentration of estradiol or 0.01% Triton X-100. A magnetic stirrer maintained the hydroxylapatite in uniform suspension during the dissociation assay. In experiments in which the [^{3}H]estradiol receptor dissociation was measured only by dilution, 0.25 mL of the hydroxylapatite-immobilized receptor was added to 250 mL of buffer TD at 29 °C. At the time cited, 0.2-5 mL aliquots were pipetted into 3 mL of buffer TD at 0 °C and centrifuged, and the resulting hydroxylapatite pellet was washed with 3 mL of buffer TD and again centrifuged. The hydroxylapatite obtained after centrifugation was extracted with 2 mL of ethanol, and the [^{3}H]estradiol content was measured in 10 mL of Liquiscint (National Diagnostics); the efficiency of counting was 36%. The equilibrium concentration of the [^{3}H]estradiol–receptor complex, $\bar{x}$, achieved after the dilution-promoted dissociation was calculated (Maelicke et al., 1977) from the relationship

$$\bar{x} = \frac{R + E + K_d}{2} - \left[\frac{1}{4}(R + E + K_d)^2 - RE\right]^{1/2} \quad (1)$$

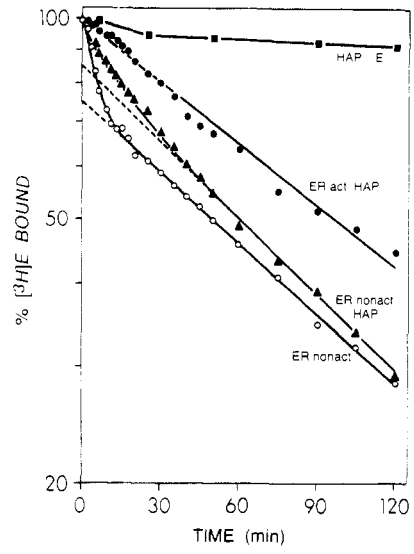

FIGURE 1: Dissociation kinetics of the [^{3}H]estradiol–receptor complex (ER) in cytosol and when bound to hydroxylapatite (HAP). Uterine cytosol in buffer TD was equilibrated with 14 nM [^{3}H]estradiol, without and with 3 μM unlabeled estradiol, at 0 °C for 1 h. Aliquots were incubated at 0 °C for 30 min (■, ▲, ○) or at 29 °C for 30 min (●). The estrogen receptor was bound by hydroxylapatite at 0 °C for 30 min (■, ●, ▲), washed with buffer TD, and then resuspended in buffer TD containing 1 μM unlabeled estradiol (▲, ●) at 29 °C; the control for receptor inactivation (■) was incubated at 29 °C without unlabeled estradiol. The specifically bound [^{3}H]estradiol was measured at the times cited by extracting the buffer TD washed hydroxylapatite pellets with ethanol. The dissociation of [^{3}H]estradiol from the receptor in the cytosol was measured at 29 °C after the addition of 1 μM unlabeled estradiol (○). The data presented are the specific [^{3}H]-estradiol-bound values (difference between incubations with and without unlabeled estradiol) corrected for receptor inactivation or dissociation from the hydroxylapatite. The receptor concentrations and dissociation rate constants were 0.09 nM, $k_{-2} = 7.11 \times 10^{-3}$ min^{-1} (●); 0.09 nM, $k_{-1} = 0.07$ min^{-1}, $k_{-2} = 8.78 \times 10^{-3}$ min^{-1} (▲); 3.1 nM, $k_{-1} = 0.286$ min^{-1}, $k_{-2} = 8.15 \times 10^{-3}$ min^{-1} (○).

where R and E denote the concentration of receptor and estradiol, respectively; K_d is the dissociation constant of the complex (the activated state) and is 3.6×10^{-11} M (Weichman & Notides, 1977).

Results

[^{3}H]Estradiol Dissociation Kinetics of the Hydroxylapatite-Bound Receptor. Immobilization of the nonactivated form of the estrogen receptor to hydroxylapatite does not inhibit its activation. The dissociation of [^{3}H]estradiol from the immobilized receptor showed biphasic kinetics having fast and slow components characteristic of the nonactivated and activated states of the receptor, respectively (Figure 1). The dissociation rate constant of the fast component, measured by isotopic ([^{3}H]estradiol–estradiol) exchange in the presence of an excess of unlabeled estradiol, was reduced by one-third (k_{-1} = 0.059 ± 0.004 min^{-1}) when the receptor was bound to hydroxylapatite as compared with the receptor free in the cytosol (k_{-1} = 0.162 ± 0.011 min^{-1}). The decrease in the [^{3}H]estradiol dissociation rate of the fast component of the hydroxylapatite-bound receptor results in a more efficient activation of the receptor (Weichman & Notides 1977, 1979), as indicated by the decreased magnitude of the fast component (in cytosol = 25.6 ± 1.4%; hydroxylapatite-bound = 16.7 ± 0.7%). The dissociation rate constant of the slower component, k_{-2}, at 29 °C was not significantly different whether the estrogen receptor was bound to hydroxylapatite (k_{-2} = 6.62 ± 0.26 × 10^{-3} min^{-1}) or free in the cytosol (k_{-2} = 7.25 ± 0.46 × 10^{-3} min^{-1}). The estrogen receptor, after being temperature activated and then bound to hydroxylapatite, showed a single,

slow [^{3}H]estradiol-dissociating component, $k_{-2} = 6.93 \pm 0.52 \times 10^{-3}$ min^{-1} (Figure 1). The nonactivated estrogen receptor bound to hydroxylapatite and incubated for 30 min at 29 °C (to induce receptor activation) showed a single, slow [^{3}H]-estradiol dissociation process which indicated that the receptor could be activated while bound to hydroxylapatite (data not shown).

The hydroxylapatite-bound [^{3}H]estradiol receptor after incubation at 29 °C without the unlabeled estradiol (the control) showed a small decrease in specific binding (Figure 1). The loss of specifically bound [^{3}H]estradiol was proportional to the volume of buffer in which the hydroxylapatite-bound receptor was suspended at 29 °C. Thus, dilution induced the dissociation of [^{3}H]estradiol from the hydroxylapatite-bound receptor (see below).

Hydroxylapatite was superior to other insoluble supports for characterizing the [^{3}H]estradiol dissociation kinetics of the immobilized estrogen receptor. It bound 90% of the receptor, whether in the nonactivated or the activated form, without significant release of the receptor at 29 °C when compared with 0 °C. Phosphocellulose and DNA–cellulose bound 35% and 24% of the estrogen receptor, respectively, preferentially in the activated form; DEAE-cellulose bound 70–90% of the receptor in both the activated and the unactivated forms. These cellulose exchangers showed significant release of the receptor at 29 °C.

Effect of High Steroid Concentration and Triton X-100 on [^{3}H]Estradiol Dissociation from the Receptor. The dissociation rates of [^{3}H]estradiol from the activated estrogen receptor free in the cytosol or immobilized by hydroxylapatite were identical; measured by isotopic exchange with 0.5–5.0 μM estradiol (a 150- to 1500-fold excess), the k_{-2} value was between 6.75×10^{-3} min and 7.93×10^{-3} min (figure 2A). However, the dissociation of [^{3}H]estradiol from the receptor in the presence of 10 and 200 μM estradiol, whether free or immobilized, showed a significant increase in [^{3}H]estradiol dissociation; the dissociation rate constants were 12.2×10^{-3} min^{-1} and 0.088 min^{-1}, respectively. This is a nonspecific effect of the steroid upon the receptor since very high (200 μM) but not lower (1 μM) concentrations of progesterone in the presence of 1 μM estradiol increased the rate of [^{3}H]estradiol dissociation from the receptor. The 200 μM progesterone alone did not displace the receptor-bound [^{3}H]estradiol or influence receptor stability at 29 °C (Figure 2A).

In the presence of 0.01% Triton X-100 the [^{3}H]estradiol dissociation from the activated estrogen receptor at 29 °C, whether free in cytosol or bound to hydroxylapatite, was a single exponential process with an even more rapid dissociation; the rate constants were 0.117 min^{-1} and 0.099 min^{-1}, respectively (Figure 2B). The hydroxylapatite-bound [^{3}H]estradiol–receptor complex, incubated at 29 °C without unlabeled estradiol, showed an initial dissociation and/or release of the [^{3}H]estradiol–receptor complex that reached a plateau within 45 min. In the presence of 0.01% Triton X-100 at 29 °C the estrogen receptor free in the cytosol showed a 15% loss of specific [^{3}H]estradiol binding in 2 h, while at 0 °C inactivation was insignificant.

Comparison of the [^{3}H]Estradiol Dissociation Kinetics of Hydroxylapatite-Bound Receptor Using Dilution and Isotopic Exchange. Dilution of the hydroxylapatite-bound [^{3}H]estradiol receptor complex from 1.2 nM to 2.2 pM gave identical [^{3}H]estradiol dissociation kinetics at 29 °C in the absence or presence of unlabeled estradiol (Figure 3). The dissocation rate constant of the faster component, k_{-1}, was 0.054 min^{-1} and that of the slower component, k_{-2}, was 5.61×10^{-3} min^{-1}.

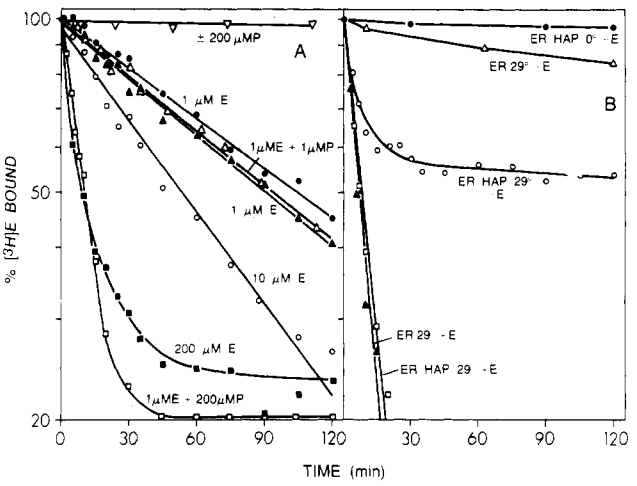

FIGURE 2: Effect of high steroid concentrations and Triton X-100 on the dissociation of the [^{3}H]estradiol–receptor complex (ER). Uterine cytosol was equilibrated with 14 nM [^{3}H]estradiol, without or with 3 μM unlabeled estradiol, at 0 °C for 1 h. The estrogen receptor was activated by incubating the cytosol at 29 °C for 30 min. Aliquots of the cytosol were then incubated with hydroxylapatite (HAP) at 0 °C for 30 min, washed, and resuspended in buffer TD. Aliquots of the hydroxylapatite-bound estrogen receptor were added to buffer TD at 29 °C containing 1 μM (△, ●, □), 10 μM (○), or 200 μM (■) unlabeled estradiol (E) and 1 μM (△) progesterone (P) or 200 μM (▽, □) progesterone. Dissociation of the [^{3}H]estradiol–receptor complex in cytosol at 29 °C was measured after the addition of 1 μM unlabeled estradiol (▲). The receptor concentrations and dissociation rate constants were 0.57 nM, $k_{-2} = 6.75 \times 10^{-3}$ min^{-1} (●); 0.71 nM, $k_{-2} = 7.39 \times 10^{-3}$ min^{-1} (▲); 0.79 nM, $k_2 = 7.3 \times 10^{-3}$ min^{-1} (△); 0.57 nM, $k = 12.2 \times 10^{-3}$ min^{-1} (○); 0.57 nM, $k = 0.088$ min^{-1} (■); 0.79 nM, $k = 0.067$ min^{-1} (□) (A). Aliquots of the hydroxylapatite-bound estrogen receptor were suspended in buffer TD containing 0.01% Triton X-100 at 0 °C (●) or at 29 °C (○), or with 0.01% Triton X-100 and 1 μM unlabeled estradiol (□). The [^{3}H]estradiol–receptor dissociation in cytosol at 29 °C was measured in 0.01% Triton X-100 and 1 μM unlabeled estradiol (▲). Receptor inactivation in the presence of Triton X-100 at 29 °C was measured without the addition of unlabeled estradiol (△). The receptor concentrations and dissociation rate constants were 0.5 nM, k 0.173 min^{-1} (○); 0.5 nM, $k = 0.099$ min^{-1} (□); and 2.4 nM, $k = 0.117$ min^{-1} (▲) (B).

When a new equilibrium was established after dilution at 29 °C, the concentration of the receptor remaining as the [^{3}H]estradiol–receptor complex was calculated by using eq 1. Only 5% (0.12 pM) of the 2.2 pM of receptor was present as the [^{3}H]estradiol–receptor complex; at this concentration of [^{3}H]estradiol–receptor complex, the probability was small that a molecule of the dissociated [^{3}H]estradiol reassociated with the receptor. Following the dilution-promoted dissociation of the [^{3}H]estradiol from the receptor, the hydroxylapatite-bound estrogen receptor was reincubated with [^{3}H]estradiol. Approximately 80 to 85% of the [^{3}H]estradiol-receptor binding was recovered associated with the hydroxylapatite, indicating that the dilution induced [^{3}H]estradiol dissociation—not receptor dissociation—from the hydroxylapatite (data not shown).

In contrast, dilution of the hydroxylapatite-bound receptor in the absence of estradiol to a final receptor concentration of only 50 pM produced signficant reassociation of [^{3}H]estradiol with the receptor. The [^{3}H]estradiol dissociation kinetics are complex (Figure 4A), going from the initial equilibrium (established at 0 °C with all the receptor binding sites occupied) to a second equilibrium after 400 to 500 min (where [^{3}H]estradiol reassociation with the receptor occurred). Equation 1 indicated that 22 pM, or 44%, of the available receptor binding sites were occupied when equilibrium was reached after dilution at 29 °C, which is in good agreement

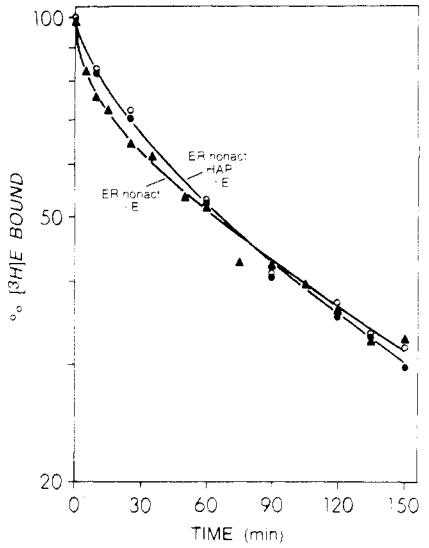

FIGURE 3: Dissociation kinetics of the [^{3}H]estradiol–receptor complex (ER) bound to hydroxylapatite (HAP), measured by dilution or isotopic exchange. Cytosol was equilibrated with 7 nM [^{3}H]estradiol, without or with 2 μM unlabeled estradiol (E), at 0 °C for 1 h. The [^{3}H]estradiol–receptor complex was incubated with hydroxylapatite at 0 °C for 30 min; then the hydroxylapatite pellet was washed and resuspended in buffer TD. Aliquots of the hydroxylapatite-bound estrogen receptor were diluted 1000-fold in the buffer TD, 29 °C (●), or in buffer TD, 29 °C, containing 500 nM unlabeled estradiol (○). The dissociation of the [^{3}H]estradiol–receptor complex in the cytosol was measured at 29 °C after the addition of 1 μM unlabeled estradiol (▲). The receptor concentrations and dissociation rate constants were 1.2 nM, k_{-1} = 0.108 min^{-1}, k_{-2} = 5.47 × 10^{-3} min^{-1} (▲); 2.2 pM, k_{-1} = 0.054 min^{-1}, k_{-2} = 5.61 × 10^{-3} min^{-1} (○, ●).

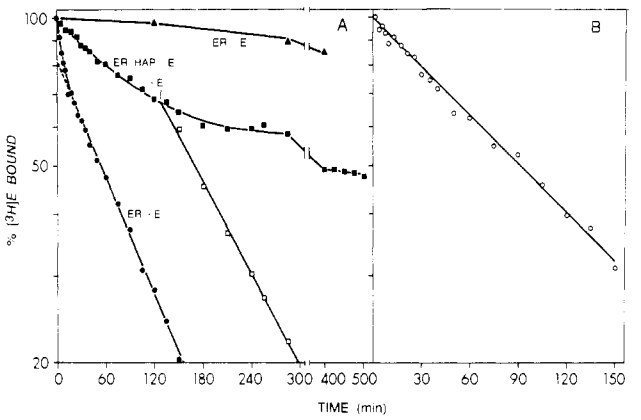

FIGURE 4: The dissociation kinetics of the [^{3}H]estradiol–receptor complex (ER) bound to hydroxylapatite (HAP) following insufficient dilution. Uterine cytosol was equilibrated with 14 nM [^{3}H]estradiol, without or with 3 μM unlabeled estradiol (E), at 0 °C for 1 h. The cytosol was incubated with hydroxylapatite at 0 °C for 30 min; the hydroxylapatite was then washed and resuspended in buffer TD. The hydroxylapatite–bound estrogen receptor was diluted 50-fold in buffer TD, 29 °C (■); at 135 min, 1 μM unlabeled estradiol was added to an aliquot of the hydroxylapatite-bound estrogen receptor (□). The [^{3}H]estradiol dissociation at 29 °C from the receptor in cytosol was measured after the addition of 1 μM estradiol (●); receptor inactivation was measured at 29 °C without the addition of unlabeled estradiol (▲). The receptor concentrations and dissociation rate constants were 3.9 nM, k_{-1} = 0.204 min^{-1}, k_{-2} = 9.01 × 10^{-3} min^{-1} (●); 50 pM, k_{-2} = 7.20 × 10^{-3} min^{-1} (□) (A). The equilibrium value of the [^{3}H]-estradiol–receptor dissociation reached between 400 and 500 min was subtracted from the initial dissociation curve, 0 to 150 min (■), to yield a dissociation curve (○) that is corrected for rebinding of [^{3}H]estradiol. The receptor concentration was 50 pM and the dissociation rate constant was 7.52 × 10^{-3} min^{-1} (B).

with the experimental value of 48% (Figure 4A). The equilibrium reached by the [^{3}H]estradiol–receptor complex that was diluted to 50 pM—48% of the initial [^{3}H]estradiol binding—was subtracted from the initial [^{3}H]estradiol dissociation curve at each determination between 0 and 150 min. The adjusted dissociation curve was monophasic, with a dissociation rate constant of 7.52 × 10^{-3} min^{-1} (Figure 4B), which is similar to the k_{-2} value of the activated estrogen receptor in cytosol (Figures 1 and 2). The addition of unlabeled estradiol at 135 min to the diluted hydroxylapatite-bound receptor resulted in isotopic exchange with the [^{3}H]estradiol–receptor complex and a monophasic dissociation process. The dissociation rate constant was 7.20 × 10^{-3} min^{-1}, which is identical with the k_{-2} value of the [^{3}H]estradiol–receptor complex in the cytosol (Figure 4A).

Discussion

The data demonstrate that hydroxylapatite is superior to DEAE-, DNA-, and phosphocellulose as an insoluble support for immobilizing the estrogen receptor. It facilitates measurements of [^{3}H]estradiol dissociation kinetics of the receptor by dilution or isotopic ([^{3}H]estradiol–estradiol) exchange and the analysis of receptor–hydroxylapatite interactions that serve as a model of estrogen receptor–nuclear interactions. The hydroxylapatite-immobilized receptor also yields data on the kinetics and mechanism of receptor activation.

The [^{3}H]estradiol dissociation kinetics of the estrogen receptor bound to hydroxylapatite, when compared with that of the receptor free in solution, showed a decrease in the magnitude of the k_{-1} component which reflected an enhancement of receptor activation. The transition of the biphasic to the monophasic [^{3}H]estradiol dissociation kinetics is a qualitative change in the receptor's estrogen binding mechanism that is associated with receptor activation (Weichman & Notides 1977, 1979). No change in the specific [^{3}H]estradiol binding concentration was observed with receptor activation; the concentration of the receptor adsorbed to the hydroxylapatite was not affected by whether the receptor was in the activated or nonactivated state (Figure 1). It remains to be determined whether receptor activation enhanced by hydroxylapatite binding is brought about by the same mechanism or conformational changes by which DNA–cellulose binding stimulates estrogen receptor activation (Yamamoto & Alberts, 1972).

The dissociation kinetics of the estrogen receptor when measured by isotopic exchange may limit the potential information contained in the dissociation process, since rebinding of the unlabeled estradiol may modify the dissociation properties of the receptor (De Meyts et al., 1973; Boeynaems, 1976). Therefore, we measured [^{3}H]estradiol dissociation promoted by dilution of the hydroxylapatite-bound [^{3}H]estradiol–receptor complex to a concentration at which the probability of [^{3}H]estradiol rebinding with the receptor was very small. Equation 1 was used to estimate the fraction of the receptor present as the [^{3}H]estradiol–receptor complex following dilution and thereby the extent of [^{3}H]estradiol dissociation that would take place.

De Meyts et al. (1973) demonstrated that a protein having negative cooperative binding may show an acceleration of ligand dissociation when dissociation is promoted by dilution in the presence of unlabeled ligand. We observed that the dissociation of [^{3}H]estradiol was identical whether dissociation was promoted by dilution alone or by dilution accompanied by an excess of unlabeled estradiol. The biphasic [^{3}H]estradiol dissociation measured by isotopic exchange accurately reflects the estrogen–receptor dissociation process and the absence of negative cooperative binding, in contrast to another report (Rosner et al., 1979). We previously reported (Weichman &

Notides, 1977) that the biphasic [^{3}H]estradiol dissociation kinetics of the estrogen receptor are consistent with a positive cooperative binding structure in which the receptor has an increased affinity for estradiol.

We have shown that the dissociation kinetics of [^{3}H]estradiol from the estrogen receptor reflects the different conformational states of the receptor (Weichman & Notides, 1977, 1980; de Boer et al., 1981). However, nonspecific perturbations of the receptor structure can induce alterations of the [^{3}H]estradiol dissociation rate. This was particularly evident when very high (200 μM) concentrations of estradiol or progesterone, but not lower (1 μM) concentrations of progesterone, increased the rate of [^{3}H]estradiol dissociation from the receptor. These data suggest that very high concentrations of a steroid, sufficient to produce micelle, exert a nonspecific detergent effect on the receptor. Suthers et al. (1976) reported that 25 μM, but not 2.5 μM, unlabeled progesterone, dihydrotestosterone, or estradiol significantly increased the rate of [^{3}H]dexamethasone from the glucocorticoid receptor; they prematurely proposed the existence of a second nonglucocorticoid binding site on the receptor of biological significance.

These data indicate that it is necessary to avoid reagents having detergent action in buffers used to isolate nuclei. Triton X-100 at 0.01% is below the critical micelle concentration and readily remains complexed with proteins, even after chromatography with styrene–divinylbenzene beads (Holloway, 1973). Triton X-100 markedly influences the ligand–receptor interaction; i.e., it induces release of [^{3}H]estradiol from the receptor and may cause receptor release from the hydroxylapatite. Triton X-100 also interferes with the interactions of the receptor with its nuclear acceptor sites (de Boer & Notides, 1981).

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