Analysis of Estrogen Receptor Activation by Its [^{3}H]Estradiol Dissociation Kinetics†

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ABSTRACT: The dissociation of [^{3}H]estradiol from the estrogen receptor of the calf uterus follows a two-component exponential process. The fast component is ascribed to [^{3}H]estradiol dissociating from the nonactivated receptor, and its rate constant is k_{-1}; the magnitude of the fast component is proportional to the fraction of the receptor in the nonactivated state. The slow exponential component, k_{-2}, represents the [^{3}H]estradiol dissociating from the activated receptor. In buffer containing 0.4 M KCl the magnitude of the fast component was inversely proportional to the initial receptor concentration; at a [^{3}H]estradiol receptor concentration of 3.6, 1.7, and 0.8 nM, the amplitude of the fast component at 28 °C was 0.10, 0.13, and 0.23, respectively. The dissociation rate constants were similar in the absence (k_{-1} = 0.18 min^{-1}; k_{-2} = 7.0 × 10^{-3} min^{-1}) or presence of 0.4 M KCl (k_{-1} = 0.14 min^{-1}; k_{-2} = 8.3 × 10^{-3} min^{-1}). The transformation of the receptor from the low to the higher affinity (activated) state is directly dependent upon the concentration of the nonactivated receptor present; the greater the concentration, the smaller the magnitude of the fast component. The dissociation of [^{3}H]estradiol from the receptor following ammonium sulfate precipitation is monophasic, with a rate constant (7.7 × 10^{-3} min^{-1}) identical with k_{-2} of the biphasic curve; sucrose gradient analysis showed the presence of the activated 5S receptor. In the presence of 0.1–0.4 M sodium thiocyanate, [^{3}H]estradiol dissociation occurred as a single exponential process, with an increase in the rate constant as the NaSCN concentration increased. Increasing the pH from 6.8 to 8.0 resulted in a decrease in the magnitude of the fast component from 0.44 to 0.23, and an increase in k_{-2} from 7.5 × 10^{-3} to 13.7 × 10^{-3} min^{-1}. Thus, the environment of the [^{3}H]estradiol–receptor complex is an important influence on receptor activation and on the dissociation kinetics. The rate of receptor activation and its reaction order were determined at a given receptor concentration in the absence or presence of 0.15 M KCl by measuring the decrease in the magnitude of the fast component with time of preincubation at 28 °C (receptor activation), prior to assaying the [^{3}H]estradiol dissociation kinetics at 28 °C. The transition of the receptor from a low to a higher affinity state follows second-order kinetics with the rate constant of receptor activation (k_{act}) increasing with receptor dilution. The nonactivated receptor is in an inhibitory complex in low ionic strength buffers from which it must dissociate before transforming to the activated state. The rate of decrease of the magnitude of the fast component is temperature dependent; the Arrhenius energy of activation for receptor transformation was 30.3 kcal mol^{-1}. The Arrhenius energy of activation for the [^{3}H]estradiol dissociation rate constant k_{-1} was 15.0 kcal mol^{-1} and 29.9 kcal mol^{-1} for k_{-2}. Two competing pathways are available to the nonactivated receptor: estradiol dissociation (k_{-1}) and receptor activation (k_{act}, having the higher energy barrier). The two-component dissociation of [^{3}H]-estradiol from the receptor provides a new and sensitive criterion for investigating receptor activation and its relationship to ligand binding. These data support our previous findings that estrogen receptor activation is a dimerization of the 4S lower affinity monomer with a second monomer (a similar or dissimilar unit) to form the higher affinity 5S dimer.

The activation of the cytoplasmic, nonactivated form of the estrogen receptor by an estrogen- and temperature-dependent reaction results in translocation of the receptor into the nucleus (Jensen et al., 1968; Shyamala & Gorski, 1969). This important regulatory step of receptor activity has been studied in vitro by sucrose gradient centrifugation and by binding to isolated nuclei, chromatin, and polyanions (Jensen & De Sombre, 1973). These studies have identified two forms of the receptor with different physical properties. The nonactivated receptor is a monomer with a molecular weight of 7 to 8 × 10^4, sediments as a 4S species on sucrose gradients containing 0.4 M KCl, and binds poorly to isolated nuclei. The in vitro incubation of the nonactivated estrogen receptor at 20–37 °C in the presence of estradiol results in receptor activation. The activated receptor is a dimer with a molecular weight of 13 to 14 × 10^4, sediments as a 5S protein into sucrose gradients containing 0.4 M KCl, and binds well to isolated nuclei (Notides & Nielson, 1974, 1975; Notides et al., 1976). When induced under in vitro conditions, the activated receptor is indistinguishable from the receptor extracted from nuclei following in vivo exposure to estradiol (Jensen & De Sombre, 1973; Notides & Nielsen, 1974).

We recently demonstrated that the dissociation of [^{3}H]-estradiol from the estrogen receptor follows a two-component exponential process which is a property of receptor activation (Weichman & Notides, 1977). The first, or fast, component (k_{-1}) results from the dissociation of [^{3}H]estradiol from the nonactivated receptor. The amplitude or magnitude of the fast component is determined by two competing processes: (1) the dissociation of [^{3}H]estradiol from the nonactivated state of the receptor and (2) the rate at which dimerization (k_{act}) occurs to produce the activated receptor. Activation of the receptor generates the second, or slower, [^{3}H]estradiol-dissociating component. These data indicate that estradiol binding modulates the receptor activation process by driving the nonactivated receptor toward the higher affinity state, the activated receptor.

The kinetics of [^{3}H]estradiol dissociation provides a sensitive method for distinguishing the two states of the receptor. In this paper we demonstrate the application of the estradiol dissociation kinetics of the receptor to measure the factors influencing the activation process and the rate and reaction order of receptor activation.

Experimental Procedures

Materials. The 17β-[6,7-^{3}H]estradiol (41 or 60 Ci/mmol) was obtained from Amersham/Searle. Its radiochemical purity was verified by thin-layer chromatography in a chloroform:ethyl acetate (3:1) solvent system. Unlabeled 17β-

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Table I: The Effect of Assay Temperature on the Amplitude of the First Exponential Component and on the Dissociation Rate Constants of the [^{3}H]Estradiol Receptor[a]

	buffer TD			buffer TD/0.15 M KCl		
temp (°C)	k_{-1} (min^{-1})	amp	k_{-2} (× 10^3 min^{-1})	k_{-1} (min^{-1})	amp	k_{-2} (× 10^3 min^{-1})
17	0.09 ± 0.01	0.12 ± 0.01	1.6 ± 0.3 (3)			
23	0.10 ± 0.01	0.19 ± 0.02	3.2 ± 0.2 (6)	0.19 ± 0.01	0.13 ± 0.04	2.9 ± 0.2 (2)
25	0.12 ± 0.01	0.21 ± 0.01	4.0 ± 0.3 (7)	0.24 ± 0.03	0.14 ± 0.02	3.6 ± 0.4 (5)
28	0.18 ± 0.01	0.31 ± 0.01	7.0 ± 0.2 (15)	0.28 ± 0.02	0.19 ± 0.02	7.1 ± 0.7 (4)
31	0.23 ± 0.01	0.36 ± 0.01	17.3 ± 0.1 (4)			

[a] Cytosol, prepared in buffer TD or buffer TD/0.15 M KCl, pH 7.4, at the temperature cited, was equilibrated with 5 nM [^{3}H]estradiol at 0 °C for 1 h. The dissociation of [^{3}H]estradiol at the indicated temperature was assayed after the addition of 5 μM unlabeled estradiol. The data are the mean ± SEM of the number of determinations (n). amp is the amplitude or magnitude of the fast exponential component of the [^{3}H]estradiol dissociation process and equals the difference between the extrapolated slow component at time zero and 100%, divided by 100%.

estradiol was obtained from Steraloids. Tris, sucrose, and ammonium sulfate were UltraPure grades from Schwarz/Mann. Dithiothreitol and Hepes[1] (*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid) were purchased from Sigma. All other reagents used were analytical grade.

Preparation of Calf Uterine Cytosol. The cytosol fraction of the calf uterus was prepared as previously described (Weichman & Noties, 1977). Briefly, calf uteri were homogenized at 0–4 °C with a Polytron PT-10 in 3–5 volumes of 40 mM Tris/1 mM dithiothreitol (buffer TD) or 40 mM Hepes/1 mM dithiothreitol. The buffer pH was preadjusted so that it was 7.4 at the temperature of the dissociation assay; exceptions are noted in Table II. The supernatant of a 220 000g centrifugation of the homogenate is referred to as the "cytosol" and had a protein concentration of 6–10 mg/mL. Cytosol prepared in buffer TD was adjusted to a specific concentration (where noted) of KCl or sodium thiocyanate (NaSCN) by the addition of a 4 M buffered solution of KCl or NaSCN.

Assay of [^{3}H]Estradiol Dissociation from the Estrogen Receptor. Uterine cytosol was equilibrated with 5–10 nM [^{3}H]estradiol for 60 min at 0 °C. Aliquots were then incubated at either 0 °C (nonactivated receptor) or the specific temperature (12–35 °C) for the time noted (activated receptor). The dissociation of [^{3}H]estradiol from the receptor, measured by the exchange of [^{3}H]estradiol with an excess of unlabeled estradiol, and the analysis of the dissociation curves were previously described (Weichman & Notides, 1977).

Sucrose Gradient Centrifugation. Separation of the 4S and 5S estrogen receptors by sucrose gradient centrifugation with an SW 56 Beckman rotor, using 5–20% linear sucrose gradients in buffer TD containing 0.4 M KCl, was described in a previous publication (Notides & Nielsen, 1974).

Results

Effect of KCl on [^{3}H]Estradiol Dissociation from the Receptor. The magnitude of the fast component of the biphasic curve of [^{3}H]estradiol dissociation from the nonactivated receptor in buffer TD/0.4 M KCl was inversely proportional to the initial receptor concentration. At [^{3}H]estradiol receptor concentrations of 3.6, 1.7, and 0.8 nM, the amplitudes of the fast component from each of the [^{3}H]estradiol dissociation curves at 28 °C were 0.10, 0.13, and 0.23, respectively (Figure 1A); dilution of the receptor with the supernatant from cytosol that had been heat denatured (100 °C for 10 min) in buffer TD/0.4 KCl showed similar values for the magnitude of the fast component. The high salt buffer was not simply diluting the action of a heat-stable inhibitor of receptor activation in the cytosol. The fast component rate constant, k_{-1}, was 0.14 ± 0.02 min^{-1} (Figure 1A, inset); the slow component, k_{-2}, was 8.3 ± 0.5 × 10^{-3} min^{-1}.

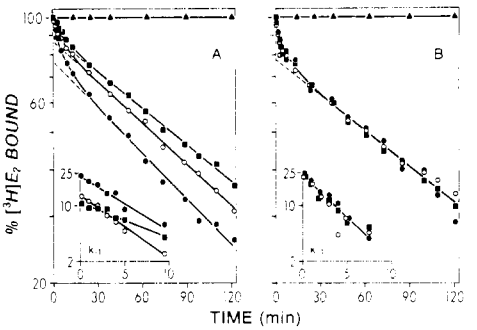

FIGURE 1: The effect of receptor concentration on the [^{3}H]estradiol dissociation kinetics in 0.15 and 0.4 M KCl. Cytosol in buffer TD was made 0.4 (A) and 0.15 M (B) with respect to KCl and then equilibrated with 8 nM [^{3}H]estradiol at 0 °C for 1 h. Aliquots were either undiluted or diluted two- and fourfold with the appropriate buffer and equilibrated at 0 °C for 20 min; 8 μM unlabeled estradiol was added, and [^{3}H]estradiol dissociation was assayed at 28 °C. Simultaneously, after the addition of buffer without unlabeled estradiol (▲), the inactivation of the receptor was assayed at 28 °C. The [^{3}H]estradiol receptor concentrations with the corresponding dissociation rate constants were: (■) 3.62 nM; k_{-1}, 0.10 min^{-1}; k_{-2}, 7.3 × 10^{-3} min^{-1}; (○) 1.69 nM; k_{-1}, 0.18 min^{-1}; k_{-2}, 8.4 × 10^{-3} min^{-1}; (●) 0.78 nM; k_{-1}, 0.15 min^{-1}; k_{-2}, 9.2 × 10^{-3} min^{-1} (A). The receptor concentrations were (■) 4.03 nM, (○) 1.84 nM, (●) 0.87 nM, and the dissociation rate constants expressed as the mean of the three curves ± SEM were k_{-1}, 0.24 ± 0.02 min^{-1}; k_{-2}, 7.3 ± 0.1 × 10^{-3} min^{-1} (B). The magnitude or amplitude (amp) of the fast component is defined as the difference between the extrapolated slow component at time zero and the total receptor, 100%. The amplitude also appears as the zero time intercept of the derived fast component k_{-1} in the inset.

Disappearance of the fast component resulted after activation of the [^{3}H]estradiol receptor (Weichman & Notides, 1977; 30-min preincubation at 28 °C) prior to dilution of the receptor in buffer TD/0.4 M KCl; whether at high or low receptor concentrations (0.8–5.3 nM) in buffer TD/0.4 M KCl, only the second component with a rate constant (k_{-2}) of 9.5 ± 0.5 × 10^{-3} min^{-1} was present.

The [^{3}H]estradiol dissociating from the nonactivated receptor at 28 °C in buffer TD/0.15 M KCl showed a 40% decrease in the magnitude of the fast component, as compared with buffer TD. A small but consistent increase in the rate constant of the fast component (k_{-1} = 0.28 ± 0.02 min^{-1}) was observed in buffer TD/0.15 M KCl compared with buffer TD (k_{-1} = 0.18 ± 0.01 min^{-1}) at 28 °C. The rate constant of the slow component was not influenced by the absence (k_{-2} = 7.0 ± 0.2 × 10^{-3} min^{-1}) or presence (k_{-2} = 7.1 ± 0.7 × 10^{-3} min^{-1}) of 0.15 M KCl at 28 °C (Table I). The decrease in the magnitude of the fast component suggests that 0.15 M KCl stimulated receptor activation; our sucrose gradient centri-

[1] Abbreviations used: Hepes, *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid; buffer TD, 40 mM Tris/1 mM dithiothreitol.

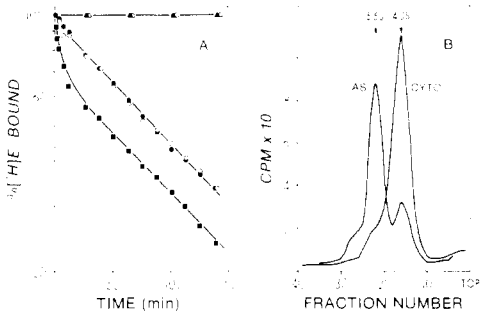

FIGURE 2: The effect of ammonium sulfate precipitation on the [^{3}H]estradiol dissociation kinetics. Uterine cytosol equilibrated with 10 nM [^{3}H]estradiol for 1 h at 0 °C was made 30% saturated with respect to ammonium sulfate. The 168 mg of ammonium sulfate per mL of cytosol was added over a 15-min period, with stirring, at 0 °C, and incubated for an additional 30 min at 0 °C before centrifugation at 10000g for 10 min. The ammonium sulfate precipitate was redissolved in one-third the original volume of cytosol. One aliquot was incubated at 0 °C for 30 min (○), and the other aliquot was incubated at 28 °C for 30 min (●). The dissociation of [^{3}H]estradiol from the receptor in the ammonium sulfate fractions and in the uterine cytosol (■) was assayed at 28 °C after the addition of 8 μM unlabeled estradiol. Simultaneously, inactivation of the receptor in the ammonium sulfate fraction without (▲) and with (△) preincubation at 28 °C was assayed at 28 °C. The dissociation rate constants were: (●) 7.8×10^{-3} min^{-1}; (○) 7.6×10^{-3} min^{-1}; (■) k_{-1}, 0.18 min^{-1}; k_{-2}, 7.5×10^{-3} min^{-1} (A). Sucrose gradient centrifugation analysis of the estrogen receptor in the cytosol (CYTO) and in a 0–30% ammonium sulfate fraction (AS). Centrifugation was for 16 h at 50000 rpm at 1 °C (B).

fugation analysis indicated that 0.1 M KCl stimulated the rate of the 4S to 5S receptor transformation (Notides et al., 1975). However, neither the rate constant nor the magnitude of the fast or slow exponential component in buffer TD or buffer TD/0.15 M KCl was influenced as a function of the initial receptor concentration (Figure 1B), as was observed in buffer TD/0.4 M KCl (Figure 1A). The decrease in amplitude of the fast component in 0.15 M KCl was not due to nonspecific [^{3}H]estradiol binding or receptor lability. The nonspecific binding of [^{3}H]estradiol (5–10% of the total bound [^{3}H]estradiol) was identical in the absence or presence of salt and did not change during the dissociation assay at 28 °C. The [^{3}H]estradiol-binding activity of the receptor was stable during incubation at 28 °C in the absence or presence of salt (Figure 1).

Effect of Ammonium Sulfate Precipitation and pH on [^{3}H]Estradiol Dissociation from the Receptor. The fast component of the [^{3}H]estradiol dissociation process was absent following ammonium sulfate precipitation of the estrogen receptor. The [^{3}H]estradiol dissociation, assayed at 28 °C, showed a single component with a dissociation rate constant of 7.7×10^{-3} min^{-1}, which was identical with the slow exponential component (k_{-2}) of the [^{3}H]estradiol dissociation process before ammonium sulfate precipitation (Figure 2A). Sucrose gradient centrifugation analysis of the estrogen receptor after ammonium sulfate precipitation showed predominantly a 5S estrogen-binding protein that was indistinguishable from the 5S receptor produced by heating (Figure 2B).

The magnitude of the fast component of the [^{3}H]estradiol dissociation curve at 28 °C decreased as the pH was increased from 6.8 to 8.0. The fraction of the receptor dissociating with the k_{-1} rate constant decreased from 0.44 at pH 6.8 to 0.23 at pH 8.0. The dissociation rate constant k_{-1} was not significantly influenced by pH, whereas the rate constant of the slow dissociating component (k_{-2}) increased as the pH was raised toward 8.0 (Table II).

Table II: The Effect of pH on the [^{3}H]Estradiol Dissociation Kinetics[a]

pH	k_{-1} (min^{-1})	amp	k_{-2} ($\times 10^3$ min^{-1})
6.8	0.21	0.44	7.5
7.1	0.17	0.40	7.2
7.4	0.18	0.33	7.2
7.7	0.22	0.31	11.3
8.0	0.29	0.23	13.7

[a] Cytosol was prepared in buffer TD that was pH 7.1, 7.4, or 7.7 at 28 °C or in 40 mM Hepes/1 mM dithiothreitol that was pH 6.8 or 8.0 at 28 °C. The cytosol samples were equilibrated with 5 nM [^{3}H]estradiol at 0 °C for 1 h. The dissociation of [^{3}H]-estradiol was assayed at 28 °C after the addition of 5 μM unlabeled estradiol. Each curve was corrected for nonspecific binding; receptor inactivation was insignificant. amp is the amplitude or magnitude of the fast exponentail component of the [^{3}H]-estradiol dissociation process.

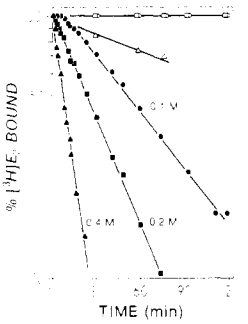

FIGURE 3: The effect of NaSCN on the [^{3}H]estradiol dissociation kinetics. Cytosol in buffer TD was equilibrated with 5 nM [^{3}H]-estradiol at 0 °C for 1 h. Aliquots of the cytosol were made 0.1 (●), 0.2 (■), and 0.4 M (▲) with respect to NaSCN and equilibrated at 0 °C for 15 min. The dissociation of [^{3}H]estradiol at 25 °C was assayed after the addition of 5 μM unlabeled estradiol. Simultaneously, receptor inactivation (○, □, △) at each corresponding NaSCN concentration was assayed at 25 °C after the addition of buffer without unlabeled estradiol. Each point has been corrected for nonspecific binding and receptor inactivation. The dissociation rate constants were (●) 0.016, (■) 0.031, and (▲) 0.090 min^{-1}.

Effect of Sodium Thiocyanate on [^{3}H]Estradiol Dissociation from the Receptor. In the presence of NaSCN, the [^{3}H]estradiol dissociating from the receptor showed a single exponential process with the rate constant increasing as the NaSCN concentration increased. In the presence of buffer TD containing 0.1 or 0.2 M NaSCN, the [^{3}H]estradiol dissociation rate constants at 25 °C were 0.016 and 0.031 min^{-1}, respectively; these values were intermediate between k_{-1} (0.12 min^{-1}) and k_{-2} (4.0×10^{-3} min^{-1}) at 25 °C in buffer TD (Figure 3; Table I). In buffer TD containing 0.4 M NaSCN, the [^{3}H]estradiol dissociation rate constant was 0.09 min^{-1}, which is similar to k_{-1} of the two-component exponential process in buffer TD without NaSCN (Weichman & Notides, 1977). The dissociation rate constants measured in the presence of NaSCN were identical whether the estrogen receptor was activated (by preincubation at 25 °C for 30 min before the addition of the NaSCN) or nonactivated. Sucrose gradient analysis showed that the receptor incubated with 0.4, 0.2, or 0.1 M NaSCN at 25 °C for 30 min showed a receptor profile of respectively 4 S only, equal amounts of 4 S and 5 S, and more 5 S than 4 S. The estrogen receptor incubated in buffer TD/0.4 M KCl at 25 °C for 30 min was completely transformed to the 5S state.

Effect of Temperature on [^{3}H]Estradiol Dissociation from the Receptor. The magnitude of the fast component, representing [^{3}H]estradiol dissociation from the nonactivated state of the receptor, correspondingly increased as the temperature at which the [^{3}H]estradiol dissociation assay was performed

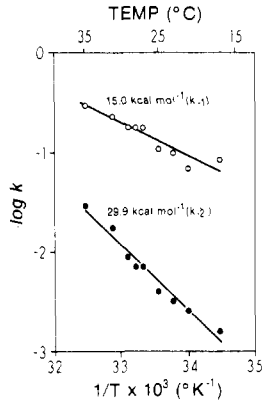

FIGURE 4: Arrhenius plot of the [^{3}H]estradiol dissociation rate constants k_{-1} (O) and k_{-2} (●), measured in buffer TD. The Arrhenius relationship is $\ln k = \ln A - E_a/RT$, where k is the dissociation rate constant (min^{-1}), A the probability factor (min^{-1}), E_a the energy of activation for the dissociation of [^{3}H]estradiol (kcal mol^{-1}), R the gas constant (1.978 cal K^{-1} mol^{-1}), and T the temperature (K). The slope times $-R$ is equal to E_a.

increased (Table I). The dissociation rate constant k_{-2} increased more than k_{-1} with each increase in temperature, indicating the greater temperature dependence of the slow component (Table I). The energy of activation measured by the Arrhenius relationship was 15.0 kcal mol^{-1} for the fast component and 29.9 kcal mol^{-1} for the slow component (Figure 4). The effect of temperature on [^{3}H]estradiol dissociation without or with 0.15 M KCl was similar; as the temperature increased, the magnitude of the fast component increased (Table I).

Analysis of Receptor Activation. Since the amplitude or magnitude of the fast exponential component of the [^{3}H]-estradiol dissociation kinetics is proportional to the amount of the nonactivated form of the receptor, the [^{3}H]estradiol dissociation kinetics can be used to measure the rate of receptor activation. The [^{3}H]estradiol receptor in buffer TD incubated at 35 °C for 0, 1, 2, 3, 4, 6, 8, and 10 min produced partial receptor activation; 8 μM of unlabeled estradiol was added, and [^{3}H]estradiol dissociation was measured at 28 °C. The time of estrogen receptor activation at 35 °C did not influence the dissociation rate constant $k_{-1} = 0.15 \pm 0.01$ min^{-1} or $k_{-2} = 4.9 \pm 0.1 \times 10^{-3}$ min^{-1}, but the magnitude of the fast component decreased as the duration of incubation at 35 °C increased. The rate of decrease of the magnitude of the fast component did not follow first-order kinetics (Figure 5A). The transition of the receptor from the nonactive to the active state of the receptor is better described as a second-order process (Figure 5B). This is consistent with previous results based upon sucrose gradient analysis that receptor activation is a macromolecular dimerization reaction (Notides et al., 1975). The apparent second-order rate constant of receptor activation, k_{act}, was 1.0×10^8 M^{-1} min^{-1} at an initial concentration of 3.9 nM in buffer TD at 35 °C.

The apparent second-order rate constant of receptor activation at 28 °C increased as the cytosol was diluted in buffer TD from 2.4×10^7 M^{-1} min^{-1} at a receptor concentration of 5.3 nM to 1.1×10^8 M^{-1} min^{-1} at 1.6 nM, calculated from the [^{3}H]estradiol dissociation kinetics at 28 °C (Table III). Unlike an ideal second-order reaction, in which the half-time ($t_{1/2}$) increases as the concentration of the reactants decreases, the $t_{1/2}$ of receptor activation changed minimally with receptor concentration. As the concentration of the receptor was reduced, the resulting apparent second-order rate constant increased; consequently, the estimated $t_{1/2}$ changed only slightly (Table III).

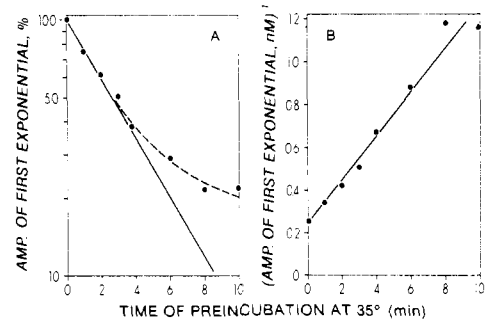

FIGURE 5: The relationship between the amplitude (amp) of the fast exponential component of the [^{3}H]estradiol dissociation kinetics and time of receptor activation. Aliquots of the uterine cytosol preequilibrated with 8 nM [^{3}H]estradiol were incubated at 35 °C for the time cited. The aliquots were recooled to 0 °C, 8 μM of unlabeled estradiol was added, and the [^{3}H]estradiol dissociation was assayed at 28 °C. The amplitude of the fast exponential component of the [^{3}H]estradiol dissociation curve was converted to nM of receptor using the assumption that the aliquots of receptor remaining at 0 °C (not preincubated at 35 °C) contained 100% nonactivated receptor at the start of the [^{3}H]estradiol dissociation assay at 28 °C. The decrease of the amplitude of the fast exponential component was plotted as a first-order function (A) and a second-order function (B) against the time of receptor activation at 35 °C.

Table III: The Effect of Estrogen Receptor Concentration on the Rate Constant, k_{act}, of Receptor Activation[a]

[^{3}H]estradiol receptor concn (nM)	k_{act} ($\times 10^{-7}$ M^{-1} min^{-1})	$t_{1/2}$ (min)
5.3	2.4	7.9
4.4	3.2	7.1
3.7	4.2	6.4
2.7	5.4	6.9
1.6	11.0	5.7

[a] Uterine cytosol prepared in buffer TD was equilibrated with 8 nM [^{3}H]estradiol for 1 h at 0 °C. The receptor concentration was estimated after adsorbing the free [^{3}H]estradiol with charcoal/dextran and correcting for nonspecific binding. At each receptor concentration 4–8 aliquots were incubated at 28 °C for various times to induce partial receptor activation; receptor activation was then stopped by recooling to 0 °C. The dissociation of [^{3}H]estradiol at 28 °C was assayed after the addition of 8 μM unlabeled estradiol. Each curve was corrected for nonspecific binding. The second-order rate constants, k_{act}, were calculated as described in Figure 5. The half-time ($t_{1/2}$) is $1/k_{act}[ER]_0$, where k_{act} is the apparent second-order rate constant of receptor activation and $[ER]_0$ is the initial nonactivated receptor concentration.

The rate of receptor activation, measured by plotting the amplitude of the fast component as a second-order function vs. the time of receptor activation, increased in buffer TD/0.15 M KCl as compared with buffer TD. At an initial receptor concentration of 2.7 nM, the apparent second-order rate constant of receptor activation at 28 °C was 5.4×10^7 M^{-1} min^{-1} in buffer TD and 1.4×10^8 M^{-1} min^{-1} in buffer TD/0.15 M KCl, a 2.6-fold stimulation. The increase in the rate of receptor activation by 0.15 M KCl was observed at all receptor concentrations.

Effect of Temperature on Rate of Receptor Activation. The rate of receptor activation was 37-fold faster at 35 °C than at 12 °C. The [^{3}H]estradiol receptor, at an initial concentration of 2.8 to 3.2 nM, was preincubated at the temperature cited for various times to produce receptor activation. Increasing the preincubation temperature increased the rate of receptor activation (Figure 6A). The activation energy estimated by the Arrhenius relationship for receptor activation in buffer TD was 30.3 kcal mol^{-1} and linear over the temperature range measured (Figure 6B). The Arrhenius acti-

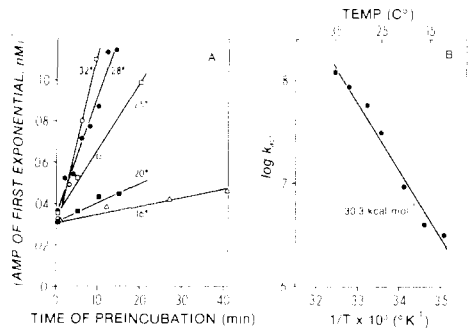

FIGURE 6: The effect of temperature on the rate of receptor activation. Cytosol was prepared in buffer TD and equilibrated with 8 nM [^{3}H]estradiol at 0 °C for 1 h. Aliquots were incubated at the indicated temperatures for the times cited and then cooled. The remaining experimental conditions and calculations were identical with Figure 5 (A). The apparent second-order rate constant, k_{act}, is plotted by the Arrhenius relationship described in Figure 4 (B).

vation energy of receptor activation in buffer TD was independent of the receptor concentration. At a receptor concentration of 1.0–1.6 nM, the Arrhenius activation energy was also 30 kcal mol^{-1}.

Discussion

The data reported here demonstrate that the biphasic kinetics of [^{3}H]estradiol dissociation from the receptor can be used to measure the factors that influence receptor activation, the rate of receptor activation, and the thermodynamic relationship between estradiol dissociation and receptor activation.

The KCl concentration has specific effects on the parameters of the biphasic [^{3}H]estradiol dissociation kinetics. The magnitude of the fast exponential component is inversely proportional to the initial receptor concentration, while the dissociation rate constants k_{-1} and k_{-2} are not significantly changed, when values observed in buffer TD/0.4 M KCl are compared with buffer TD or buffer TD/0.15 M KCl (Figure 1). The dependence of the amplitude of the fast component of the [^{3}H]estradiol dissociation process on receptor concentration is consistent with our previous analysis of estrogen receptor structure and the receptor activation process (Notides & Nielsen, 1974, 1975; Notides et al., 1975, 1976; Weichman & Notides, 1977). In the high salt concentration, the two monomeric units (that may or may not be identical) that compose the activated estrogen receptor are initially dissociated into the monomeric, nonactivated state; therefore, the rate of activation is dependent upon the rate of favorable molecular collisions and associations between the monomeric units to form the 5S dimer. At low receptor concentration, the rate of dimer formation would be relatively slow; i.e., the half-time of the reaction is longer. This is borne out by the [^{3}H]estradiol dissociation curve in high salt, which showed a large magnitude for the fast component; conversely, high receptor concentration showed a small magnitude for the fast component. It appears that dissociation of the 5S monomer from an inhibitory complex by buffer TD/0.4 M KCl may be necessary, but insufficient, for receptor activation. If dissociation were sufficient for activation, as has been suggested (Sato et al., 1978), then the transition from the lower to the higher affinity state of the receptor would be independent of the receptor concentration in 0.4 M KCl, and the [^{3}H]estradiol dissociation curves would be superimposable.

In buffer TD or buffer TD/0.15 M KCl the [^{3}H]estradiol dissociation kinetics showed that the magnitude of the fast exponential component was independent of the initial receptor concentration. In an ideal second-order reaction, the half-time depends strictly upon reactant concentration; i.e., the lower the concentrations of the reactants, the slower the rate. However, as we previously demonstrated by centrifugation analysis, the 4S to 5S transformation of the receptor from the rat uterus in low ionic strength buffers is a complex second-order reaction: as the cytosol is diluted, an increase in the rate of formation of the 5S dimer is observed, suggesting that the nonactivated receptor is in either a dead-end conformation with itself, in an inhibitory complex with other macromolecules at high protein concentrations, or complexed with a specific inhibitor of receptor activation; the exact mechanism remains to be resolved (Notides et al., 1975).

Nevertheless, the overall reaction is second order and not first order (Figure 5), with the apparent second-order rate constant increasing with a decrease in the initial receptor concentration, while the half-time changes less (Table III). Thus, the predissociation of the 4S receptor from an inhibitory complex followed by the formation of the 5S dimer, in buffer TD or buffer TD/0.15 M KCl, results in a minimal change in the half-time of receptor activation; i.e., the rate of formation of the 5S dimer remains fairly constant over the range of receptor concentrations studied.

The ammonium sulfate precipitation of the receptor and its subsequent redissolution in buffer TD induce a diminution of the nonactivated, and an enhancement of the activated, state of the receptor. Sucrose gradient analysis confirmed the presence of predominantly the 5S receptor state. The mechanism of receptor activation by ammonium sulfate may be similar to that induced by a high KCl concentration. When the nonactivated receptor in 0.4 M KCl is diluted to a lower ionic strenth ($\leq$0.1 M KCl), the 4*S* to 5*S* receptor transformation is induced; however, if the dilution is accomplished by addinig 0.4 M KCl, the 4S receptor predominates. This suggests that receptor activation is initially dissociation of the 4S receptor, followed by dimerization to the 5S receptor, in the lower ionic strength buffer (Notides, 1978). The temperature-induced receptor transformation has been previously recognized (De Sombre et al., 1975) as being stimulated more at pH 8.5 than at pH 7.0. The decrease in the magnitude of the fast exponential component of the biphasic [^{3}H]estradiol dissociation curve as the pH increases coincides with stimulation of the rate of receptor activation (Table II).

In the presence of buffer TD/0.4 M NaSCN the appearance of only the fast exponential component, in conjunction with sucrose gradient analysis which revealed only a 4S form of the receptor, indicates that 0.4 M NaSCN retarded the formation of the 5S receptor dimer. Therefore, both the [^{3}H]estradiol dissociation kinetics and the sedimentation characteristics substantiate the presence of only the nonactivated form of the receptor.

In the presence of buffer TD/0.1 or 0.2 M NaSCN the relationship between the [^{3}H]estradiol dissociation kinetics and the sedimentation behavior of the receptor is more complex. The [^{3}H]estradiol dissociation displayed a single exponential component with rate constants between the k_{-1} and k_{-2} values of the biphasic dissociation curve observed in buffer TD, while sucrose gradient analysis showed 4S and some 5S form of the receptor (apparently incongruous with the presence, in reduced quantity, of the active 5S form of the receptor).

We suggest that, in 0.1 and 0.2 M NaSCN, the monomer–dimer equilibrium constant of the receptor shifts toward the 4S monomer, less than in the presence of 0.4 M NaSCN, but still effectively reducing the quantity and half-time of the 5S receptor state. As a consequence of the reduced half-time

of the 5S receptor state, much less [^{3}H]estradiol will dissociate from the receptor while it is in the 5S state. Thus, the [^{3}H]estradiol dissociation curve in 0.1 M and 0.2 M NaSCN is predominantly from the nonactivated 4S receptor and appears as a monophasic dissociation curve with a change in slope; the less 5S receptor generated, the smaller the change in slope.

The Arrhenius plot of the dissociation rate constants yields an energy of activation of 15.0 kcal mol^{-1} for k_{-1} and 29.9 kcal mol^{-1} for k_{-2} (Figure 4). The dissociation of the [^{3}H]estradiol from the nonactivated, as compared with the activated, receptor requires less energy and suggests that the conformational changes brought about by receptor activation result in "tighter" estradiol binding.

Two pathways are available to the nonactivated [^{3}H]estradiol receptor: (1) the [^{3}H]estradiol could dissociate, leading to a nonactivated and unligated receptor (k_{-1}), and (2) the estradiol–receptor complex could transform to the activated state (k_{act}). As the temperature increased at which the [^{3}H]estradiol dissociation was assayed, the magnitude of the fast exponential component increased in buffer TD or buffer TD/0.15 M KCl. The increased temperature preferentially stimulated the estradiol dissociation from the nonactivated receptor rather than the transformation of the nonactivated receptor to the activated receptor (Table I). The Arrhenius plot of the rate constants of receptor activation (k_{act}) indicates that the Arrhenius energy of activation was 30.3 kcal mol^{-1}, which is higher than the Arrhenius energy of activation required for estradiol dissociation (k_{-1}) from the nonactivated receptor, 15.0 kcal mol^{-1} (Figure 6). The Arrhenius energy of activation for the rat estrogen receptor, measured as the rate of transformation from the 4S to 5S form of the receptor by sucrose gradient analysis, was high, 21.3 kcal mol^{-1} (Notides et al., 1975), but less than reported here for the calf estrogen receptor.

Previously, the activated state of the estrogen receptor was distinguished from the nonactivated state by an increase in the sedimentation coefficient (Jensen et al., 1968; Notides & Nielsen, 1974) or by an increase in the binding to isolated nuclei, chromatin, or DNA (Jensen & De Sombre, 1973). This report demonstrates that the biphasic [^{3}H]estradiol dissociation kinetics provides a new and sensitive criterion for measuring the transition of the receptor from the nonactive to the active state.

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