Interaction of 17β-Estradiol and Its Specific Uterine Receptor. Evidence for Complex Kinetic and Equilibrium Behavior*

Barbara M. Sanborn, B. Ramanath Rao, and Stanley G. Korenman†

ABSTRACT: Investigation of the association of [^{3}H]17β-estradiol (E_2) with a soluble receptor obtained from immature rabbit uteri and of the dissociation of the complex revealed complicated kinetics. Association at 0, 9, 20, and 37° gave second-order rate constants of 4.6, 12, 22, and 76 × 10^5 M^{-1} sec^{-1} using Dextran-coated charcoal to adsorb unbound E_2. Dissociation, measured as a first-order process in the presence of an excess of unlabeled E_2, was biphasic at all temperatures with rate constants of 1.8, 5.5, 36, and 1360 × 10^{-5} sec^{-1} for the first phase and 4.8, 6.0, 27, and 850 × 10^{-6} sec^{-1} for the second phase at 0, 9, 20, and 37°, respectively. The same biphasic pattern was observed in the presence of a variety of salts. Scatchard plots constructed from data obtained after equilibration at 0° suggest positive cooperativity at low concentrations of E_2 (0.08 nM), a linear portion (K_{ass} = 0.73 × 10^{10} M^{-1}, n = 0.81 nmole/g of cytosol protein), and a small nonsaturable component at high E_2 concentrations (6 nM). Thermodynamic considerations point to a high energy of activation (12 kcal/mole).

Since the discovery of a specific estrogen binding protein in uterine cytosol (Jensen and Jacobsen, 1960; Noteboom and Gorski, 1965; Toft and Gorski, 1966), many attempts have been made to characterize the receptor and relate the binding phenomenon to physiological events. The receptor has been shown to be specific for estrogens, the relative binding approximately paralleling the physiological potency (Korenman, 1968, 1969; Puca and Bresciani, 1969). Upon entering the cell, 17β-estradiol (E_2)[1] apparently binds to the cytosol receptor and migrates to the nucleus in a temperature-dependent process (Jensen *et al.*, 1968; Shyamala and Gorski, 1969).

The nature of the interaction between E_2 and its specific receptor(s) is consistent with the concept that the steroid binds reversibly by noncovalent forces; in addition, intact E_2 can be recovered from both cytosol and nuclear steroid–receptor complexes (Jensen, 1962; Maurer and Chalkley, 1967).

During the course of these studies it was natural to assume that the E_2–receptor interaction was a simple bimolecular reaction with second order association and first-order dissociation constants. This report constitutes kinetic and equilibrium experiments describing the E_2–cytosol interaction.

We found that the simple model did not adequately characterize the reaction. In particular, dissociation took place in two first-order phases and cooperativity was noted in the equilibrium studies. Other investigations of the E_2–receptor complex have produced data which are similarly interpretable (Best-Belpomme *et al.*, 1970).

Materials and Methods

Materials. Chemicals were reagent grade. [6,7-^{3}H]17β-Estradiol (40 Ci/mmole, New England Nuclear lot 500-036) and 17β-estradiol were used without further purification.

Uteri were removed from immature rabbits, immediately frozen in Dry Ice, and stored at −20°. The frozen tissue was homogenized with cold buffer A (0.01 M Tris-HCl–1 mM ETDA–0.25 M sucrose, pH 8.0) for 30 sec in a Waring blender. The homogenate was centrifuged at 2000*g* for 15 min and the resulting supernatant solution was centrifuged at 100,000*g* for 90 min (Spinco L-2). The fat-free cytosol was stored in 1-ml aliquots in liquid N_2 and was stable for several months as judged by binding capacity.

Measurement of Bound Estradiol. Bound E_2 was measured as previously described (Korenman, 1968). Cold buffer B (0.01 M Tris-HCl–1 mM EDTA, pH 8.0; 1 ml) containing 0.5% activated charcoal (Norit A, Matheson Coleman & Bell) and 0.05% Dextran D (Mann) was added to the incubation mixture (0.35 ml). The suspension was mixed, incubated at 0° for 15 min, and centrifuged at 4° for 10 min at 1000*g* (International PR6, 253 rotor). The supernatant solution was decanted into a scintillation vial containing 10 ml of toluene scintillation solution (Liquifluor, New England Nuclear), mixed thoroughly, and counted (Packard 4322 spectrometer). No sample by sample variation in quenching was observed when the total amount of buffer, E_2, or protein was changed. Extraction of E_2 was quantitative. Reproducibility between identical samples was better than ±2% at 4000 cpm.

Results

Validation of the Charcoal Adsorption Procedure. Incubation of the charcoal suspension with [^{3}H]E_2 in the absence of binding protein resulted in adsorption of 99% of the counts employed.

The charcoal suspension rapidly removed unbound E_2 from solution while having no significant effect on the amount of bound hormone during the 15-min incubation time employed (Korenman *et al.*, 1970).

Additional support for the efficacy of the charcoal system for separating bound from free steriod came from the comparison of estrogen–receptor complex and charcoal-treated complex by sucrose density ultracentrifugation (Figure 1). The

* From the Department of Internal Medicine, University of Iowa, Iowa City, Iowa 52240, and from the Department of Obstetrics and Gynecology, Washington University, St. Louis, Missouri 63110. *Received June 1, 1971.* This work was supported in part by the Research and Education Service of the Veterans Administration Hospital, Iowa City, Iowa, and by Grant 11545 of the American Cancer Society.

† To whom reprint requests should be sent.

[1] Abbreviations used are: E_2, 17β-estradiol; [^{3}H]E_2, [6,7-^{3}H]17β-estradiol.

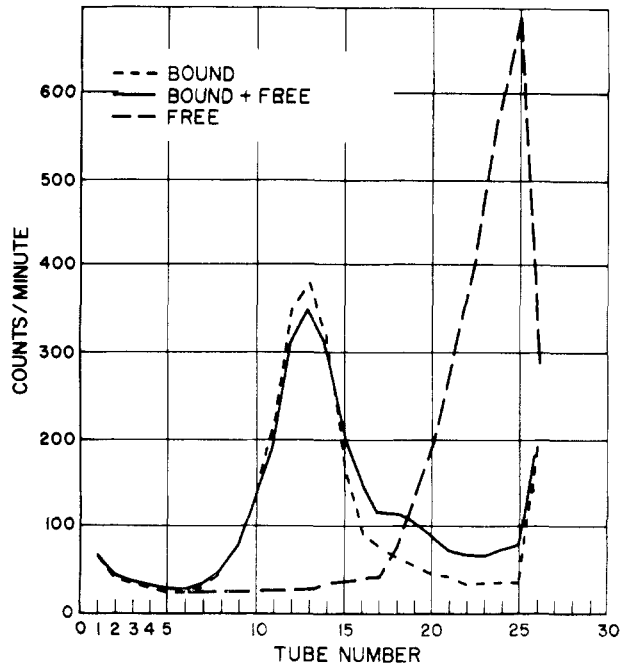


FIGURE 1: The effect of charcoal treatment on 17β-estradiol binding by rabbit uterine cytosol. After incubation of uterine cytosol with slight excess of [^{3}H]E_2, an aliquot was reacted with activated charcoal (5 mg) for 10 min at 0° and centrifuged. Charcoal-incubated (- - -) and -unincubated (——) steroid-cytosol complex and free [^{3}H]E_2 (– – –) were applied to 5–20% sucrose gradients and centrifuged for 15 hr at 39,000 rpm. The radioactivity in fractions of 10 drops was assayed by extraction into toluene–phosphor and counting in a liquid scintillation spectrometer.

location and concentration of bound hormones in the major peak (8 S) was the same in both cases, but a small amount of [^{3}H]E_2 bound to a lower molecular weight material was eliminated by the charcoal in addition to excess free steroid.

Binding Capacity. A binding curve was constructed by measuring the amount of bound [^{3}H]E_2 at equilibrium in mixtures containing a fixed amount of [^{3}H]E_2 and increasing amounts of unlabeled E_2. When the data are displayed in a Scatchard plot

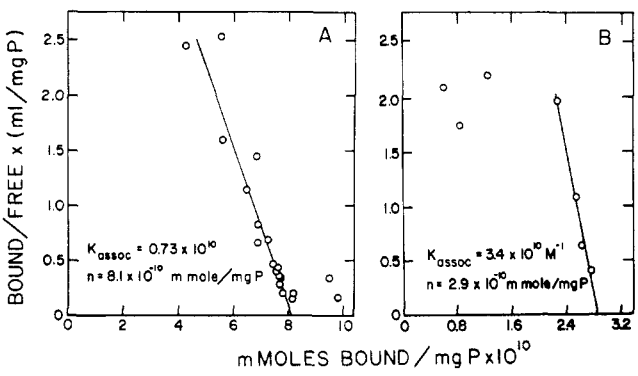


FIGURE 2: (A) A Scatchard plot of binding data obtained after incubation of 0.45 mg of cytosol protein, 0.25–0.38 pmole of [^{3}H]E_2, and increasing amounts of unlabeled E_2 for 18 hr at 0° in a total of 0.35 ml (buffer B). Each point represents the mean of four determinations. The data were obtained in two experiments, the second of which utilized the same cytosol after storage for 2 months in liquid nitrogen. (B) A Scatchard plot under similar conditions using a different protein preparation. Each point represents the average of two determinations.

TABLE I: Reversible Dissociation of the Estradiol–Cytosol Complex at 20 and 37°.

	Incubation Conditions		
Group	Temp (°C)	Time	% Cpm Bound[a]
I	0	16 hr	
	20	30 min	90
II	0	16 hr	
	20	30 min	
	0	24 hr	100
III	0	16 hr	
	37	15 min	28
IV	0	16 hr	
	37	15 min	
	0	24 hr	40

[a] Compared to incubation mixture kept at 0° for the same amount of time, all samples in triplicate.

(Scatchard, 1949), a K_{ass} of 0.73 × 10^{10} M^{-1} and a concentration of binding sites of 0.81 nmole/g of supernatant protein (Layne, 1957) was found (Figure 2A). Other Scatchard plots gave K_{ass} varying from 0.3 to 3 × 10^{10} M^{-1} for various preparations.

On examination of Figure 2, several features may be noted. The linearity of the Scatchard plots suggests a preponderance of independent sites with similar K_{ass}. However, at low concentrations, increasing E_2 does not result in a fall in the ratio of bound: free steroid (Figure 2B). This suggests positive cooperativity such that binding of E_2 at one site promotes further binding to other sites. The chemical nature of such a cooperative effect is as yet unknown. At very high concentrations of E_2, the appearance of a much less potent binding component occurs (Figure 2A).

Dissociation Reaction. Initially data were analyzed on the basis of the reaction equation

$$E + R \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ER \qquad (1)$$

where E refers to E_2 and R to the specific receptor protein. The process of dissociation of the [^{3}H]E_2–receptor complex was rendered irreversible with respect to ^{3}H by dilution, with a 40- to 10^3-fold excess of unlabeled E_2 in a negligible volume, of mixtures brought to equilibrium by preincubation for 18 hr at 0°. The tubes were then incubated at the appropriate temperature.

Decreases in bound [^{3}H]E_2 at elevated temperatures were the result of three processes: establishment of a new equilibrium concentration of bound hormone at the new temperature, temperature-dependent instability of the E_2–receptor complex, and dissociation of [^{3}H]E_2 rendered irreversible as decribed above. That the first process is largely reversible is shown in Table I. Reincubation of E_2–receptor complex at 0° reversed the effects of incubation at 20°, but at 37° an additional, irreversible process had taken place.

The establishment of a new equilibrium and the temperature-dependent instability of the hormone–receptor complex are obvious upon examination of the "blank" (no unlabeled E_2 added) reactions at 20 and 37° (Figures 6, 7, and 10). Com-

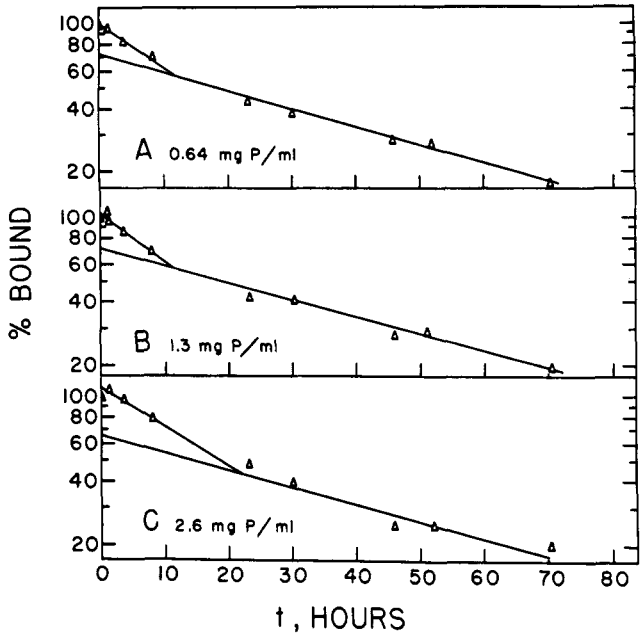


FIGURE 3: The irreversible dissociation at 0° of [^{3}H]E_2 from the [^{3}H]E_2–receptor complex as determined by addition of a large excess of E_2. The remaining bound [^{3}H]E_2 at each time is measured by charcoal adsorption as described in the test. The data are plotted as a first-order reaction obeying the formulation: [^{3}H]E_2 − R → [^{3}H]E_2 + R. Each point is the mean of 4 determinations. Lines are least-squares lines. Incubation conditions prior to unlabeled E_2 addition were 18 hr at 0°; [^{3}H]E_2 concentration: 7.1 nM; protein concentration: A (0.64 mg/ml), B (1.3 mg/ml), C (2.6 mg/ml).

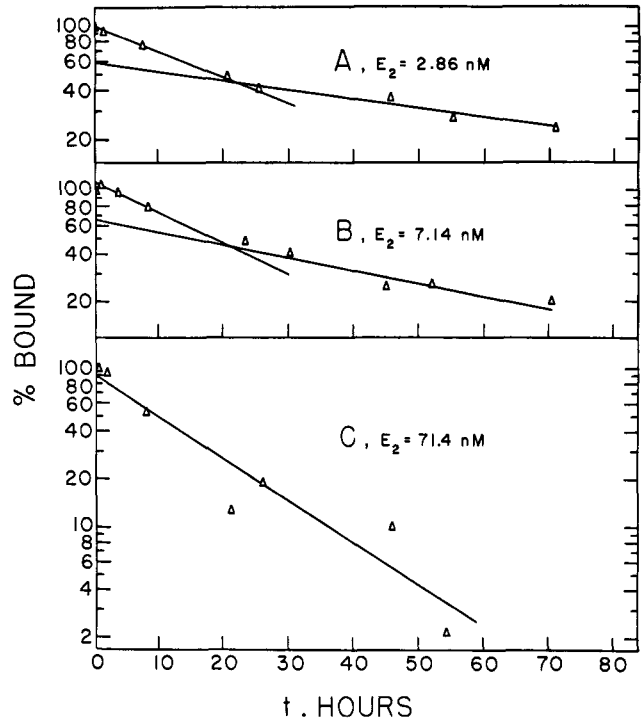


FIGURE 4: The dissociation of estrogen from [^{3}H]E_2–receptor complex at 0° performed as described in Figure 3 with 1.28 mg of protein/ml and several [^{3}H]E_2 concentrations: A (2.86 nM), B (7.14 nM), C (71.4 nM).

plex instability made a significant contribution to the total dissociation reaction only at 37°; rate constants at this temperature were corrected for the blank reaction.

Temperature-dependent adherence of the E_2–cytosol complex to the test tube walls (Clark and Gorski, 1969) could not account for the blank reaction at 37°. That was demonstrated by comparing the fall of counts bound during incubation at 37° with counts bound to the glass surface of the test tube. At all times of incubation (up to 1 hr) the number of counts adherent to glass as measured by extractability by three washes with phosphor solution was <3% of those added. In the same time period, the characteristic fall of bound counts had occurred.

When plotted as a first-order reaction the irreversible dissociation of the [^{3}H]E_2–receptor complex at 0° was biphasic and independent of protein concentrations over a fourfold range (Figure 3). From Figure 4, it can be seen that at concentrations of [^{3}H]E_2 which are in the range required for saturation of the primary binding site as judged by the Scatchard plot (lines A and B), the reaction was again biphasic and independent of E_2 concentration. A tenfold increase in [^{3}H]E_2 concentration resulted in an apparent first-order dissociation reaction with considerable scatter in the final stages where the counts bound approached background values (line C).

In accord with the biphasic first-order plot, the dissociation reaction can be considered in terms of two components

$$^3\text{H[ER]} \underset{k_{-2}}{\overset{k_{-1}}{\rightleftharpoons}} [^3\text{H}]\text{E} + \text{R} \qquad (2)$$

The rate constant for the slow reaction, k_{-2}, can be determined from the second phase of the reaction. The contribution of this reaction to the total dissociation can be subtracted, permitting the calculation of the rate constant for the faster process, k_{-1} (Frost and Pearson, 1971). Table II summarizes the results; the constants calculated from data pooled from all of the experiments except the one using 71.5 nM E_2 are the same as those determined separately within experimental error.

TABLE II: Dissociation Rate Constants Calculated from the First-Order Plots of Log $[ER]_t/[ER]_0$ *vs.* Time.

Temp (°C)	E_2 (nM)	P (mg/ml)	k_{-1},[a] 10^{-5} sec^{-1}	k_{-2},[b] 10^{-6} sec^{-1}
0	7.14	0.64	3.7	5.1 ±0.3
	7.14	1.28	1.8	4.8 ± 0.6
	7.14	2.56	3.6	4.2 ± 0.4
	2.86	1.28	2.1	4.0 ± 0.4
	71.40	1.28	k_{app} = 2.2 ± 0.2[c]	
9	7.14	1.28	5.5	6.0 ± 0.1
20	7.14	1.28	36	27
	7.14	1.28	k_{app} = 15 ± 0.5	
	7.14	1.28	k_{app} = 13 ± 0.2	
37	7.14	1.28	1360[d]	850 ± 6[d]

[a] Calculated from the early dissociation data after corrections for the contribution of the slower reaction were applied (Frost and Pearson, 1971). [b] Calculated from the least-squares line fitted to the data obtained at long incubation times. Errors are probable errors. [c] Apparent k_{-1} values from linear first-order plots, *i.e.*, the second component was missing. [d] Corrected for spontaneous loss of binding as determined from the blank reaction.

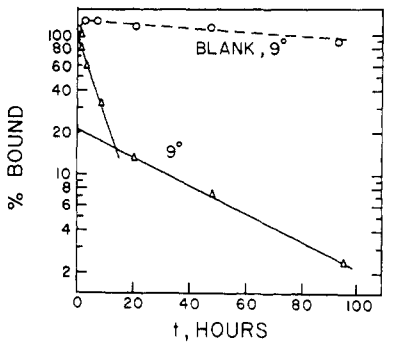


FIGURE 5: The dissociation of estrogen from [^{3}H]E_2–receptor complex performed as described in Figure 3 as a function of temperature (solid lines). The dashed line is the blank reaction at 9° after the addition of a comparable amount of ethanol. Temperatures were held constant within ±0.5 deg using an Eberbach water-bath shaker and a Sargent water-bath cooler. Incubation conditions: 13 hr at 0°; [^{3}H]E_2, 7.14 mM; 1.28 mg of protein/ml.

When studied at 9, 20, and 37°, biphasic dissociation curves were also seen whether addition of unlabeled E_2 took place before or after equilibration at that temperature (Figures 5, 6, and 7).

Biphasic dissociation reactions also were observed when calf endometrium cytosol was employed, when unlabeled estrone was employed instead of E_2, and when incubation took place in the presence of 0.3 M KCl (20°). The rate constants were independent of [^{3}H]E_2 and protein concentration at 20° as well.

Dissociation rate constants obtained at all four temperatures are given in Table II. The influence of temperature on dissociation is readily apparent.

Association Reaction. Association was studied extensively at 0°; under second-order conditions the rate constants appeared to be independent of both protein and E_2 concentration as can be seen in Figure 8. The reaction has progressed to 50–80% completion by 20 min. At 20° the association rate constant was again independent of E_2 and protein concentration. Figure 9 shows data obtained at 0, 9, 20, and 37°. It was possible to calculate an association rate constant k_1 from the linear portions of the second-order plots. The results are listed in Table III. At 37° the reverse reaction made a significant contribution even to the initial rate; in this case, k_1 was calculated from the differential equation

$$\frac{d[ER]}{dt} = k_1[E][R] - k_{-1}[ER] \qquad (3)$$

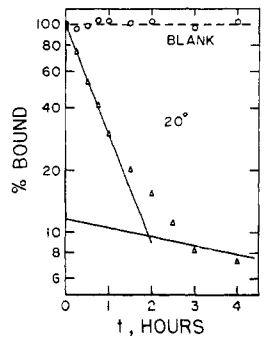
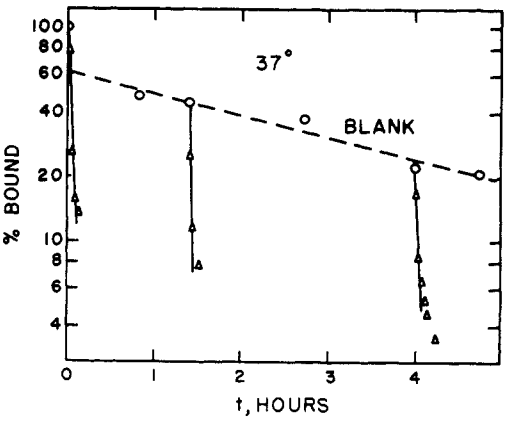


FIGURE 6: Dissociation of the E_2–receptor complex at 20° after incubation under the conditions described in Figure 5.



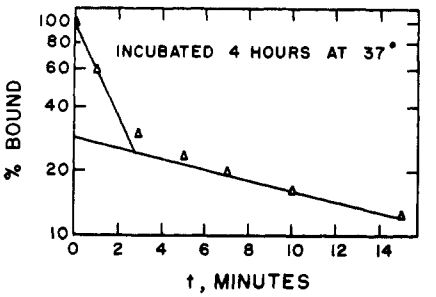


FIGURE 7: The dissociation of the estrogen–receptor complex at 37° under the incubation conditions described in Figure 5. The dashed line represents the blank reaction. Dissociation initiated after 0, 1.5, and 4 hr at 37° is represented with solid lines. The reaction initiated after incubation for 4 hr at 37° is shown in the lower graph with a time scale of minutes.

by substituting the association data and k_{-1} determined from dissociation experiments.

Thermodynamic Considerations. Thermodynamic parameters may be calculated from the kinetic data presented in Tables II and III. The results must be viewed as approximations, since the rate constants are derived from equations which only approximately describe the system under consideration.

A plot of log k_1 (association rate constant) *vs.* reciprocal

TABLE III: Association Rate Constants Determined from the Initial Linear Portions of Second-Order Plots.

Temp (°C)	$[E]_0$ (nM)	$[R]_0$ (nM)[a]	k_1 (10^5 M^{-1} sec^{-1})
0	0.54	1.09	7.5 ± 0.04[b]
	1.07	1.09	4.6 ± 0.1
	1.72	1.09	3.8 ± 0.2
	1.07	2.18	3.8 ± 0.2
	1.07	3.27	5.8 ± 0.3
9	1.07	1.09	12 ± 1
20	1.07	1.09	22 ± 1
	1.07	1.09	21 ± 2
37	0.43	0.33	76 (139)[c]

[a] Based on data from the Scatchard plot at 0°. [b] Determined from the slope of the least-squares line plus and minus probable error. [c] Value in parentheses is the rate constant which best fits the data as calculated from the differential equation taking into account the reverse reaction as described in the text.

TABLE IV: Thermodynamic Parameters Derived from Kinetic Constants.

$1/T$ (K) ($\times 10^3$)	k_1/k_{-1} (10^{10} M^{-1})	$k/_1k_{-2}$ (10^{10} M^{-1})	K_{ass} (10^{10} M^{-1})[a]
3.66	2.2	11	0.73
3.55	2.2	20	
3.41	0.58	7.9	0.43
3.22	0.10	1.6	
	$\Delta H^\circ = -18$ kcal/mole	$\Delta H^\circ = -18$ kcal/mole	
	$\Delta G^\circ = -13$ kcal/mole	$\Delta G^\circ_{293} = -14$ kcal/mole	
	$\Delta S^\circ = -18$ cal (deg^{-1} mole^{-1})	$\Delta S^\circ = -12$ cal (deg^{-1} mole^{-1})	

[a] Determined by Scatchard plots.

temperature ($1/T$) gives a straight line and an activation energy of 12 kcal mole^{-1}.

Using either the fast dissociation rate constant (k_{-1}) or the slower one (k_{-2}), association constants k_1/k_{-1} and k_1/k_{-2} can be compared with the constant obtained from Scatchard plots of equilibrium data (Table IV). Both at 0 and 20°, the ratio k_1/k_{-1} most closely approximates K_{ass}.

Plots of either log k_1/k_{-1} or log k_1/k_{-2} *vs.* $1/T$ give essentially the same slope and $\Delta H = -18$ kcal/mole. Entropy changes are large and negative (−18 and −12 cal (deg mole), respectively) and are probably the result of increased conformational restrictions imposed on the receptor molecule by the binding of the steroid. Similar negative entropy changes accompany the binding of cortisol to transcortin (Westphal, 1970).

Effect of Cations on the Kinetics. It has been shown that the

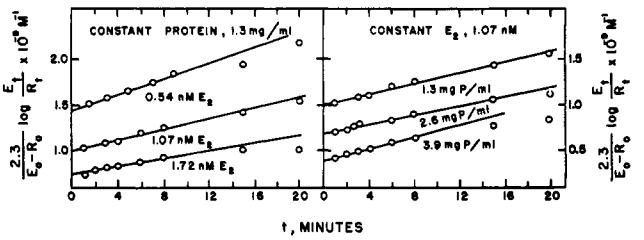


FIGURE 8: The association of [^{3}H]E_2 with cytosol receptor under second order conditions at 0°, varying the concentration of E_2 and protein separately. The reaction is plotted according to the equation

$$\{2.3/([E]_0 - [R]_0)\} \log ([E]_t/[R]_t) = k_1 t + \{2.3/([E]_0 - [R]_0)\} \log ([E]_0 - [R]_0)$$

(where E refers to [^{3}H]E_2 concentration, R to receptor binding site concentration as determined from the Scatchard plot, t to time, and the subscripts 0 and t to time 0 and t, respectively. Lines are least square lines using points obtained in the first 8 min. Lines at constant protein concentration, 1.28 mg/ml (site concentration 1.09 nM); [^{3}H]E_2: 0.54, 1.07, 1.72 nM. Lines at [^{3}H]E_2 concentration, 1.07 nM; protein concentration: 1.28, 2.57, 3.86 mg per ml.

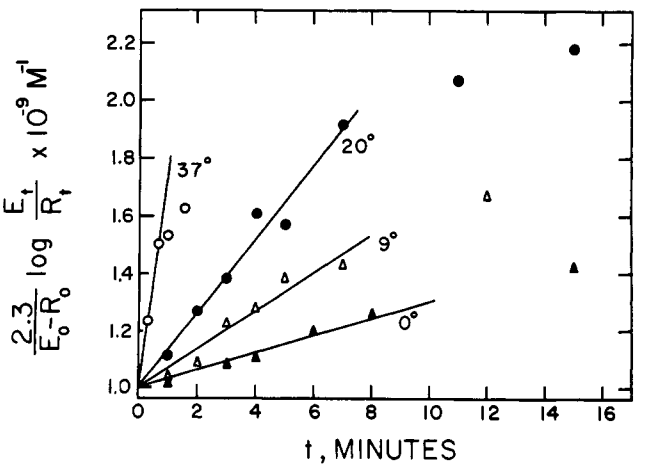


FIGURE 9: The association of [^{3}H]E_2 (1.07 nM) with cytosol receptor (1.09 nM binding sites) at 0, 9, 20, and 37°, plotted as described in Figure 8.

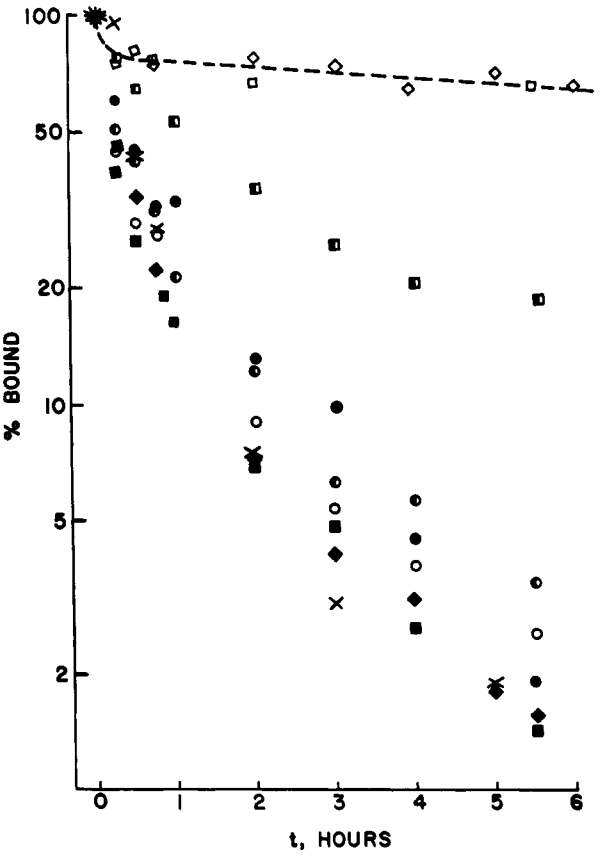


FIGURE 10: The dissociation of the [^{3}H]E_2–receptor complex at 20° performed as described in Figure 6 after preincubation at 0° in 0.01 M Tris-Cl (pH 8.0) plus the following components: [□, blank, none], [◊, blank, 1 mM EDTA], [■, none], [◆, 1 mM EDTA], [○, 1 mM EDTA, 10 mM KCl], [×, 1 mM EDTA, 0.3 M KCl], [●, 10 mM $CaCl_2$], [◐, 10 mM $MgCl_2$], and [◧, 1 mM $ZnCl_2$].

TABLE V: Association Constants for Estradiol–Receptor Interactions.

K_A (10^{10} M^{-1})$_n$	n	Method	Source	Reference
0.14	0.03 pmole/uterus	Density gradients	Immature rat cytosol, 10°	Toft and Gorski (1966)
0.13–0.15	0.32–0.65 nmole/g P	Gel filtration	Calf, nuclear complex, 4°	Puca and Bresciani (1969)
0.35		Gel filtration	Calf, cytosol, 4°	Puca *et al.* (1970)
0.005	2000/nucleus	Centrifugation	Calf endometrial chromatin, 38°	Maurer and Chalkley (1967)
0.02–0.03	0.9 pmole/uterus	Glass, nuclear pellets	Immature rat cytosol, 25°	Clark and Gorski (1969)
0.73	0.3 nmole/g P	Protamine	Mature rat cytosol, 4°	Steggles and King (1970)
0.17	0.15 nmole/g P	Density gradients		
0.11	0.84 pmole/uterus	Centrifugation	Rat uteri, particulate fraction, 37°	Shyamala and Gorski (1969)
4.3		Centrifugation	Immature rat uteri, nuclear myofibrillar fraction	Noteboom and Gorski (1965)
1		Carbon–Dextran	Calf uterine cytosol, 4°	Alberga *et al.* (1970)
10,000		Carbon–Dextran	Calf uterine chromatin nonhistone protein, 37°	
0.67	4.5 nmoles/g P/1	Equilibrium dialysis	Calf endometrial cytosol, 4°	Erdos *et al.* (1969)
0.67	5.2 nmoles/g P/1	Gel filtration		
1.4	0.43 nmole/g P/1	Equilibrium dialysis	Calf endometrial cytosol, 4°	Erdos *et al.* (1970)
0.88	0.39 nmole/g P/1	Hydroxylapatite		
100	0.03 nmole/g P	Carbon–Dextran	Human uterus, 37°	Hähnel (1971)
0.55	2.44 nmoles/g P			
0.34	0.02 nmole/g wet wt	Intravenous infusion	Mature rat uterus, 40°	DeHertogh *et al.* (1971)
0.31	0.03 nmole/g wet wt		Endometrium, 40°	
0.73	0.81 nmole/g P	Carbon–Dextran	Immature rabbit cytosol, 0°	This paper
0.80	0.36 nmole/g P	Carbon–Dextran	Calf endometrial cytosol, 0°	
1.8	0.44 nmole/g P	Carbon–Dextran	Calf myometrial cytosol, 0°	

state of the cytoplasmic estrogen receptor is dependent on the ionic strength and nature of the buffer (Erdos, 1968; Rochefort and Baulieu, 1968; Korenman and Rao, 1968; Brecher *et al.*, 1970). Specifically, 0.3 M KCl reversibly dissociates the 8S receptor into 4S particles; aging in 0.3 M KCl prevents reassociation of the subunits (Vonderhaar *et al.*, 1970).

TABLE VI: Comparison of Kinetic Constants.

Temp (°C)	k_1 (M^{-1} sec^{-1})	k_{-1} (sec^{-1})	k_{-2} (sec^{-1})	Reference
0	7×10^5	1.2×10^{-6}	2.1×10^{-7}	Best-
10	2.7×10^6	6.7×10^{-6}	3.0×10^{-6}	Belpomme
18	5.7×10^6	2×10^{-4}	5×10^{-5}	*et al.* (1970)[a]
0	$1.5–10 \times 10^5$		3.6×10^{-7}	Alberga *et al.* (1970)
0	3.8×10^5	1.8×10^{-5}	4.8×10^{-6}	This paper
9	1.2×10^6	5.5×10^{-5}	6.0×10^{-6}	
20	2.2×10^6	3.6×10^{-4}	2.7×10^{-5}	
37	1.4×10^7	1.4×10^{-2}	8.5×10^{-4}	

[a] Data quoted refers to determinations using crude cytosol, 1.93 mg of P/ml.

In addition, calcium ions appear to stabilize the 4S particle (DeSombre *et al.*, 1969).

Incubation of the receptor and [^{3}H]E_2 overnight at 0° in 0.01 M Tris-Cl (pH 8.0) containing an additional component had various effects on the amount of complex formed compared to that formed in 0.01 M buffer: 0.001 M EDTA (106%), 0.001 M $ZnCl_2$ (27%), 0.01 M KCl (114%), 0.01 M $MgCl_2$ (70%), 0.01 M $CaCl_2$ (73%), and 0.3 M KCl (47%). These data are not in agreement with the report (Emanuel and Oakey, 1969), that 5 mM Zn^{2+}, Ca^{2+}, Mg^{2+}, and K^+ enhance binding. Despite their varied effects on the equilibrium concentration of steroid–receptor complex, the salts had little effect on the shape of the dissociation curve (Figure 10). The two phase patterns therefore probably do not reflect different dissociation rates from the various forms of the receptor.

In contrast to the findings of Vonderhaar *et al.* (1970), association under the conditions of Figure 8 was markedly slower at 20° in the presence of 0.3 M KCl and leveled off at 10% of the amount bound in 0.01 M buffer.

Discussion

While binding to the cytosol receptor is one of the earliest events observed after treatment with estrogens *in vivo*, the role of binding with respect to the sequence of molecular events following such stimulation is unknown. The existence of appropriately specific cytoplasmic binding substances in relatively high concentration in steroid hormone target organs

provides strong indirect support for the thesis that hormone binding is an essential first step in steroid hormone action.

A pure unsaturated receptor preparation is desirable both for kinetic and mechanistic studies. Efforts at purification have been frustrated by the tendency of the receptor to aggregate. Most purified material must be prepared in the presence of E_2 (Brecher *et al.*, 1970). Smaller, stable subunits of the 8S receptor have been purified in the absence of steroid (Brecher *et al.*, 1970; Puca *et al.*, 1970) but their physiological role is not clear.

For these reasons we undertook evaluation of the E_2 receptor reaction in crude cytosol. Because of extensive data regarding ionic concentration-induced changes in sedimentation velocity, studies were undertaken under a variety of salt conditions.

The association constant obtained using charcoal–Dextran compares favorably with those reported by numerous authors using a variety of tissues, tissue fractions, and experimental techniques (Table V). Using hydroxylapatite columns to separate bound from free E_2, Erdos *et al.*, have also found evidence for cooperative effects at low concentrations of steroid (Best-Belpomme *et al.*, 1970; Erdos *et al.*, 1969).

Uterine cytosol bound a maximum of 8.1×10^{-10} mmole of E_2/mg of protein or 1.2×10^{-8} mmole of E_2/g of rabbit uterus. Using a DNA content of 1.14 mg/g of uterus and a rabbit DNA content of 5.3 pg/nucleus (Vendrely, 1955), there would be approximately 34,000 binding sites for E_2 per rabbit uterine cell.

If binding to the cytosol receptor has physiological significance, it would be reasonable to expect that fluctuations in plasma E_2 levels should result in large changes in the amount of E_2 bound. DeHertogh *et al.* (1971) have calculated that the E_2 level in adult rat plasma fluctuates from 10 to 30 pg per ml. These levels of E_2 correspond to 0.3–0.8 K_{dis} in the present system. Therefore the cytosol receptor sites would fluctuate between 20 and 40% saturation and thus provide a substantial intracellular reflection of the extracellular hormonal environment.

The kinetics of association and dissociation as measured by the charcoal–Dextran method show the same qualitative characteristics as those measured by others although some quantitative differences exist (Table VI). This comparison substantiates the contention of all groups that the complex kinetics observed are not artifacts introduced by the separation methods. The fact that the same kinetic characteristics persist after partial purification (Best-Belpomme *et al.*, 1970) suggests that they may be true characteristics of the receptor–steroid complex and not perturbations induced by other components of the cytosol. On the other hand, it is precisely the influence of other cellular components on the nature and stability of the estrogen–receptor complex *in situ* which may give a clue as to its physiological significance.

Both the Scatchard plots and the dissociation curves suggest the presence of at least two interdependent binding sites with similar affinities. The complex kinetics are consistent with the concept that steroid binding has an effect on the affinity and association state of subunits comprising the receptor (Vonderhaar *et al.*, 1970). Subtle differences in the E_2–receptor interaction may be important in effecting the variety of observed physiological responses of the uterus to estradiol.

Acknowledgments

The authors thank Mrs. Leslie Carpenter and Mr. Patrick Callahan for able technical assistance.

References

Alberga, A., Jung, I., Massol, N., Raynaud, J. P., Raynaud-Jammet, C., Rochefort, H., Truong, H., and Baulieu, E. E. (1970), Schering Workshop on Steroid Hormone Receptors, Raspé, G., Ed., New York, N. Y., Pergamon Press (in press).

Best-Belpomme, M., Fries, J., and Erdos, T. (1970), *Eur. J. Biochem. 17*, 425.

Brecher, P. I., Chabaud, J. P., Colucci, V., DeSombre, E. R., Flesher, J. W., Gupta, G. N., Hurst, D. J., Ikeda, M., Jacobsen, H. I., Jensen, E. V., Jungblut, P. W., Kawashima, T., Kyser, K. A., Neumann, H. G., Numata, M., Puca, G. A., Saha, N., Smith, S., and Suzuki, T. (1970), Schering Workshop on Steroid Hormone Receptors, Respé, G., Ed., New York, N. Y., Pergamon Press (in press).

Clark, J. H., and Gorski, J. (1969), *Biochim. Biophys. Acta 192*, 508.

DeHertogh, R., Ekka, E., Vanderheyden, I., and Hoet, J. J. (1971), *Endocrinology 88*, 165.

DeSombre, E. R., Puca, G. A., and Jensen, E. V. (1969), *Proc. Nat. Acad. Sci. U. S. 64*, 148.

Emanuel, M. B., and Oakey, R. E. (1969), *Nature (London) 223*, 66.

Erdos, T. (1968), *Biochem. Biophys. Res. Commun. 32*, 338.

Erdos, T., Bessada, R., and Fries, J. (1969), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 5*, 161.

Erdos, T., Best-Belpomme, M., and Bessada, R. (1970), *Anal. Biochem. 37*, 244.

Frost, A. A., and Pearson, R. G. (1971), Kinetics and Mechanism, New York, N. Y., Wiley, p 163.

Hähnel, R. (1971), *Steroids 17*, 105.

Jensen, E. V. (1962), *Rec. Progr. Hormone Res. 18*, 387.

Jensen, E. V., and Jacobson, H. I. (1960), *in* Biological Activities of Steroids in Relation to Cancer, Pincus, G., and Vollmer, E. P., Ed., New York, N. Y., Academic Press, p 161.

Jensen, E. V., Suzuki, T., Kawashima, T., Stumpf, W. E., Jungblut, P. W., and DeSombre, E. R. (1968), *Proc. Nat. Acad. Sci. U. S. 59*, 632.

Korenman, S. G. (1968), *J. Clin. Endocrinol. 28*, 127.

Korenman, S. G. (1969), *Steroids 13*, 163.

Korenman, S. G., and Rao, B. R. (1968), *Proc. Nat. Acad. Sci. U. S. 61*, 1028.

Korenman, S. G., Tulchinsky, D., and Eaton, L. W., Jr. (1970), *Acta Endocrinol. Suppl. 147*, 291.

Layne, E. (1957), *Methods Enzymol. III*, 448.

Maurer, H. R., and Chalkley, G. R. (1967), *J. Mol. Biol. 27*, 431.

Noteboom, W. D., and Gorski, J. (1965), *Arch. Biochem. Biophys. 111*, 559.

Puca, G. A., and Bresciani, F. (1969), *Nature (London) 223*, 745.

Puca, G. A., Nola, E., Sica, V., and Bresciani, F. (1970), *in* Schering Workshop on Steroid Hormone Receptors, Raspé, G., Ed., New York, N. Y., Pergamon Press (in press).

Rochefort, H., and Baulieu, E. E. (1968), *C. R. Acad. Sci. Ser. D 267*, 662.

Scatchard, G. (1949), *Ann. N. Y. Acad. Sci. 51*, 660.

Shyamala, G., and Gorski, J. (1969), *J. Biol. Chem. 244*, 1097.

Steggles, A. W., and King, R. J. B. (1970), *Biochem. J. 118*, 695.

Toft, D., and Gorski, J. (1966), *Proc. Nat. Acad. Sci. U. S. 55*, 1574.

Vendrely, R. (1955), *in* The Nucleic Acids, Chargaff, E., Ed.,

Academic Press, Inc., p 155.
Vonderhaar, B. K., Kim, U. H., and Mueller, G. C. (1970), *Biochim. Biophys. Acta 215*, 125.
Westphal, U. (1970), *Acta Endocrinol., Suppl. 147*, 16.

Ribonuclease U_1. Physical and Chemical Characterization of the Purified Enzyme*

William C. Kenney† and Charles A. Dekker‡

ABSTRACT: An improved procedure is described for the formation and purification of an extracellular ribonuclease (RNase U_1) from *Ustilago sphaerogena*. Conditions favorable for growth of the organism and induction of the enzyme by RNA have been established, permitting isolation of suitable amounts of RNase U_1 for characterization of its physical and chemical properties. DEAE-cellulose and ammonium sulfate were used to concentrate the enzyme from the extracellular medium. Acetone fractionation was essential to remove an unidentified viscous component of the extracellular medium which interfered with other means of purification. Repetition of DEAE-cellulose chromatography and ammonium sulfate precipitation gave a preparation which was found to be homogeneous in discontinuous gel electrophoresis and in ultracentrifugation studies; yield, 40 mg of enzyme from 40 l. of culture medium. At pH 7.2 and μ 0.01 this ribonuclease has an ultraviolet absorption maximum at 277 mμ and a minimum at 251 mμ. The A_{280}:A_{260} ratio is 2.5 and a 0.1% solution has an absorbance of 1.71 at 280 mμ. The specific activity of RNase U_1 is 300,000 units/mg of anhydrous protein. The amino acid composition of RNase U_1 is Lys_3, His_2, Arg_2, Asp_{15}, Thr_9, Ser_{13}, Glu_6, Pro_4, Gly_{15}, Ala_5, half-Cys_4, Val_6, Ile_2, Leu_1, Tyr_{12}, and Phe_4 for a total of 103 residues. The amide content of this enzyme is 12 ± 1 and the nitrogen content is 16.2%. The molecular weight is 10,998 based on the above amino acid composition. The amino-terminal residue is half-cystine. This enzyme has a sedimentation coefficient of 1.9 S. From sedimentation equilibrium data a molecular weight of 11,100 was calculated. RNase U_1 has many properties in common with RNase T_1 of *Aspergillus oryzae*. Both enzymes are stable to heat and acid conditions and are inactivated by iodoacetic acid at pH 5.5 in a similar manner. Moreover, the specific activity, specificity, mode of action, and molecular weight of these two enzymes are very similar.

In the search for highly specific ribonucleases, several genera of fungi have been examined (Sato and Egami, 1957; Tanaka, 1961; Glitz and Dekker, 1964a,b; Rushizky *et al.*, 1964; Tatarskaya *et al.*, 1964). In this paper, we are concerned with an RNA-induced extracellular RNase (EC 2.7.7.26, ribonucleate guaninenucleotido-2′-transferase (cyclizing), *Ustilago sphaerogena*) which was shown by Glitz and Dekker (1964b) to be specific for guanylyl residues in RNA. In keeping with the nomenclature of similar RNases from related organisms, this enzyme from *U. sphaerogena* is referred to as RNase U_1.[1] To produce adequate quantities of this enzyme for determination of structure and active site, we have reexamined the conditions for optimum growth, induction, and purification on a large scale. The method described, which makes extensive use of DEAE-cellulose for concentration of the enzyme (Uchida, 1965) and fractionation, permits the preparation of 40 mg of highly purified enzyme from 40 l. of culture medium. This has enabled us to study some of the physical and chemical properties of the enzyme, including the amino acid composition, and to compare them with the corresponding properties of RNase T_1.

Experimental Section

Materials. Yeast sodium ribonucleate was obtained from Schwarz BioResearch, Inc. DEAE-cellulose, a product of Bio-Rad Laboratories (Cellex D), had a capacity of 0.7 ± 0.1 mequiv/g. Bovine serum albumin (BSA)[2] and tris(hydroxymethyl)aminomethane were products of the Sigma Chemical Co. HCl was used to adjust the buffers to the desired pH. Na_2EDTA was obtained from Matheson Coleman & Bell. Iodoacetic acid (Eastman Organic Chemicals) was twice recrystallized before use. For dialysis, cellulose casing (Union Carbide Corp.), $^{18}/_{32}$-in. diameter, was used. Whenever possible, reagent grade chemicals were employed.

Methods

Assay for RNase Activity. The assay for RNase activity was performed by measuring the absorbance at 260 mμ of perchloric acid soluble products obtained from the digestion of high molecular weight RNA according to the method of

* From the Department of Biochemistry, University of California, Berkeley, California 94720. *Received August 9, 1971.* Supported in part by a grant (GB-4018) from the National Science Foundation and departmental grants (USPH TI GM 31-09, USPHS AM 8845, and USPHS AM 10109). A preliminary report of this work was given at the Pacific Slope Biochemical Conference, Davis, Calif., June 1967.

† Submitted in partial fulfillment of the requirements for the Ph.D. degree in biochemistry at the University of California, Berkeley. Present address: Department of Biochemistry and Biophysics, University of California Medical Center, San Francisco, Calif.

‡ To whom to address correspondence.

[1] The subscript, 1, is used to denote the major extracellular RNase. Other nucleases from the extracellular medium have been observed with distinct chromatographic properties and specificities.

[2] Abbreviations used are: BSA, bovine serum albumin; CM-RNase U_1, carboxymethylated ribonuclease U_1.