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KINETICS OF PROGESTERONE BINDING TO THE CHICK OVIDUCT RECEPTOR PROTEIN

PHILIP E. HANSEN, ARON JOHNSON, WILLIAM T. SCHRADER* and BERT W. O'MALLEY
Department of Cell Biology, Baylor College of Medicine, Texas Medical Center, Houston, Texas 77030, U.S.A.

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SUMMARY

Kinetic parameters have been determined for the binding of progesterone to receptor proteins of the chick oviduct. Association and dissociation rate constants and the equilibrium constant have been determined as a function of temperature and ionic strength. Both the association and dissociation rate constants vary with temperature by about 2 orders of magnitude. However the equilibrium constant for the binding reaction does not change substantially over the temperature interval from 0 to 24°. The half-life of the complex under pseudo first-order conditions decreases from about 10 h at 0° to about 3 min at body temperature of the animal (42°). Arrhenius plots of the dissociation rate constant information show a linear plot over the temperature range studied indicating that the aggregation and the conformational changes accompanying this temperature range do not cause any apparent change in the hormone binding site.

On the other hand glycerol has a considerable stabilizing effect on the receptors increasing the half-life at 24° from about 24 min to a matter of about 100 min as the glycerol concentration is increased to 40%. The hormone binding site can be disrupted irreversibly by high salt or urea concentrations in excess of one molar. Ionic strength over the range from 0–0.5 M has essentially no effect on any of the parameters measured. The half-life of the complexes decreases by only about 10–15% over this interval.

The possible perturbation of the hormone binding sites accompanying binding of the proteins to ion exchange resins was studied. There is no detectable change in any of the kinetic parameters when the receptor protein is adsorbed to either DEAE cellulose or phosphocellulose. The hormone binding characteristics of the protein remain the same for up to 100 hours; only a single class of hormone binding site could be detected. Variations in half-life accompanying dissociation of hormone off the receptors was shown to be due merely to the presence of non specific binding in the preparation. The study concludes that the conformation of the hormone binding site is not strongly perturbed by the environment of the protein.

INTRODUCTION

Our laboratory has been studying the mechanism of progesterone action in chick oviduct cells [1, 2]. As progesterone molecules enter the cell, they are bound to receptor proteins at specific binding sites to form hormone–receptor complexes [3, 4]. These complexes then translocate from the cytoplasm to the nucleus where they interact with chromatin [5–7] and exert an effect upon the genetic expression of the cell [8, 9].

In an attempt to understand this mechanism on a molecular level, we have purified the receptor proteins from cytosol preparations of chick [10] and hen [11] oviducts and have characterized their interaction with nuclear constituents *via in vitro* experiments [12, 13]. Recently we have reported that the progesterone receptor functions as a dimer comprised of two 4S components [14, 15]. Both components have been shown to bind hormone. In addition to its role in allowing nuclear binding to take place, we found that the hormone also causes a de-stabilization of the dimer and subsequent production of monomeric units upon interaction of the dimer with nuclear "acceptor" sites. Thus, it has become important to re-examine more fully the kinetics of hormone binding to the various receptor forms.

In previous experiments, the kinetic parameters of binding (K_d, k_a, and k_d) for the two receptor components A and B were determined at 0°C [16] and were found to be similar for both subunits and constant during the steps of the purification process. We have therefore used cytosol as a model of the more purified receptor in further kinetic studies of the hormone–receptor binding mechanism.

In the present communication we report upon several aspects of our studies of receptor binding kinetics. The effects of changing the temperature, pH, and the ionic strength are shown. Second, possible stabilization of the receptor–hormone complex is investigated. Next, the effect of aging upon the stability of the receptor is reported. Finally, the effect of adsorption of the receptors to ion-exchange resins is studied as a model for binding of the complexes to the genome. This information may also shed some light upon the question of whether or not the binding

*To whom correspondence should be sent.

site on the receptor undergoes a conformational change upon binding.

EXPERIMENTAL

Reagents

All chemicals were reagent grade from J. T. Baker except as noted. Tris buffer and ammonium sulfate were from Schwarz–Mann (Orangeburg, New York, U.S.A.). Spectrafluor was obtained from Amersham–Searle (Arlington Heights, Illinois, U.S.A.). Triton X-100 was purchased from Rohm and Haas Co. (Houston, Texas, U.S.A.). Non-radioactive steroids were from Steraloids (Pauling, New York, U.S.A.). Radioactive [1,2-$^3H_2$]-progesterone (50.1 Ci/mmol) was purchased from New England Nuclear (Boston, Mass., U.S.A.).

Animals and tissues

Female White Leghorn chicks 7–30 days of age were given Purina feed and water *ad lib.* Each received 5 mg per day of diethylstilbestrol in sesame oil by subcutaneous injection for 13–21 days. The chicks were then killed, and the oviducts (magnum portion) were removed and washed in 0.9% NaCl at 0°C.

Buffers

All experiments were performed in Buffer A (0.01 M Tris–HCl, 0.001 M Na_2EDTA, 0.012 M thioglycerol pH 7.4) containing additions of KCl or glycerol as noted in the text.

Temperature

All experiments were conducted at 0°C, or as noted in the text.

Receptor preparation

Cytosol. Crude cytoplasmic extracts of oviducts were prepared as described previously [17]. Ice-cold oviducts were dissected clean and homogenized with a Teflon–glass homogenizer (Glenco Instruments, Houston, Texas). This homogenate was then centrifuged at 20,000 ***g*** for 10 min in a Sorvall HB-4 rotor. The fatty layer was aspirated from the top, and the supernatant fraction was centrifuged at 150,000 ***g*** for 1 h in a Spinco SW-50.1 rotor. The fatty layer was again aspirated from the top, and the supernatant (cytosol) fraction was maintained at 0°C. Preparations were made fresh each day. This cytosol had a total protein content of about 20 mg/ml and contained progesterone receptors with essentially no endogenous progesterone.

Partial purification by ammonium sulfate precipitation. Saturated ammonium sulfate in Buffer A was added dropwise with stirring to cytosol to bring the final ammonium sulfate concentration to 30% saturation. This was allowed to stand for 30 min at 0°C, and was then centrifuged at 10,000 rev./min for 20 min in a Sorvall HB-4 rotor. The supernatant was discarded. The pellet was washed with Buffer A containing 50% ammonium sulfate and was then redissolved in Buffer A at two-thirds of the original cytosol volume. Previous experiments have shown that this procedure provides a 30-fold purification of the receptor compared to cytosol with a yield of about 70% [16].

Radioactivity counting

Tritium was counted using a Beckman LS-233 scintillation spectrometer. 1.0 ml aqueous extracts were counted by adding 5.0 ml of a toluene-based PPO-POPOP fluor (Amersham–Searle Spectrafluor) containing 33% Triton X-100 by vol. Counting efficiency was 33%.

Hormone binding assay

A modified form of the charcoal absorption technique of Korenman was employed [18] utilizing a suspension of 0.5% washed, powdered charcoal and 0.05% Dextran T-70 in Buffer A. The mixture was stirred at 0°C for 24 h before use. Receptor samples (0.5 ml) to be assayed were put into ice cold 12 × 75 mm glass tubes. Aliquots (0.5 ml) of the charcoal suspension were added to each and briefly vortexed. Preliminary experiments showed that greater than 95% of the free [^{3}H]-progesterone was removed during a 2 min contact time. For these reasons, the vortexed samples were allowed to stand in the presence of the charcoal for 2 min at 0°C. To remove the charcoal, the samples were centrifuged in a Sorvall GLC-1 at 2000 rev./min at 0°C for 5 min. The 1 ml supernatant fractions were decanted into vials for counting.

Measurement of binding constants

Equilibrium constants. These were determined by the method of Scatchard as described previously [19].

Rate constant for dissociation. 1.0 ml Cytosol was labeled by adding 10 μl [^{3}H]-progesterone (1.8 μM) and was allowed to stand at 0° for 2 h. 19 ml Buffer A was added, and the mixture allowed to reach the proper temperature. 2.0 μl Non-radioactive progesterone (3.2 mM) was then added with stirring. At measured intervals, aliquots (0.5 ml) were removed and assayed by the charcoal hormone binding method. A semilog plot of per cent bound [^{3}H]-progesterone *vs* time produced a linear graph whose slope was k_d.

Rate constant for association. The method of Best-Belpomme *et al.* [20] and Schrader and O'Malley [16] was used. 2.0 ml cytosol and 28 ml Buffer A were mixed and allowed to equilibrate at the proper temperature. 20 μl of [^{3}H]-progesterone (1.8 μM) was then added with stirring. At measured times thereafter, aliquots (0.5 ml) were removed and subjected to the charcoal hormone binding assay to determine the quantity of bound hormone at that time. Plotting bound hormone *vs* time elapsed produced an association curve from the equation in Ref. 16. The concentration of available binding sites

(2×10^{-9} M) was determined by a Scatchard plot using cytosol. Using this value, linear transformations of the association curves were constructed whose slope was k_a.

Arrhenius plot. Log $1/k_d$ vs $1/T$ gave a linear plot whose slope provided a measure of the energy of dissociation, E_{diss}.

Ion exchange resin techniques

DEAE-cellulose and phosphocellulose were washed and equilibrated in Buffer A until a constant pH and a constant ionic conductivity were reached [21].

Hormone-binding kinetics of receptors bound to DEAE cellulose. A slurry of DEAE-cellulose in Buffer A (1 ml packed resin plus 1 ml Buffer A) was prepared by adding Buffer A to equilibrated DEAE-cellulose such that the total vol. was twice that of the packed vol. of DEAE-cellulose.

(a) Dissociation rate constant determination. 1.0 ml Cytosol was labeled by adding 10 μl [^{3}H]-progesterone (1.8 μM) and allowed to stand at 0°C for 2 h. Ten ml of DEAE slurry and 4 ml Buffer A were added with mixing. The mixture was allowed to equilibrate to the proper temperatures (about 5 min). Ten μl of non-radioactive progesterone (3.2 mM in ethanol) was then added and mixed by vortexing. At measured times thereafter, 700 μl aliquots were removed, placed in ice-cold 12 × 75 mm glass tubes, and centrifuged at 2500 rev./min for 5 min in a GLC-1 centrifuge at 0°C. The supernatant was decanted into a vial for counting. The increase in radioactivity in the supernatant with time represented the rate of loss of labeled hormone from the receptor, i.e., the dissociation rate. Rate constant determinations were made from semilogarithmic plots as described above.

(b) Association rate constant determination. Twenty ml of the DEAE slurry, 18 ml Buffer A, and 2 ml cytosol were combined with stirring and allowed to equilibrate to the proper temperature for 5 min. 20 μl of [^{3}H]-progesterone (1.8 μM) was then added and mixed by vortexing. At measured times thereafter, 700 μl aliquots were removed, placed in ice-cold 12 × 75 mm glass tubes and centrifuged at 2500 rev./min for 5 min in a GLC-1 centrifuge at 0°C. The supernatant fractions were decanted and counted for tritium. To obtain the association curve, the difference between the initial radioactivity in the supernatant and that in later samples was plotted against the reaction time.

Hormone-binding kinetics of receptors bound to phosphocellulose. (a) Dissociation rate constant determination. Eight and a half ml cytosol labeled with [^{3}H]-progesterone was precipitated utilizing 30% saturation ammonium sulfate. The washed pellet was redissolved in 5 ml Buffer A and was applied to a 5 ml column and again washed with Buffer A to remove any free hormone. The resin from the column was then resuspended in 20 ml Buffer A. Fifty μl non-radioactive progesterone (3.2 mM) was added with stirring. At measured intervals thereafter, 700 μl aliquots were removed, put into cold 12 × 75 glass tubes and centrifuged at 2500 rev./min for 5 min in a GLC-1 centrifuge at 0°C. The supernatant fractions were decanted into vials for counting. The increase in radioactivity in the supernatant occurring with time was plotted to obtain the dissociation rate constant for receptors bound to phosphocellulose.

Partial purification of receptor—DEAE-cellulose chromatography

Cytosol was first labeled with [^{3}H]-progesterone and then partially purified using 30% ammonium sulfate precipitation. The redissolved pellet was applied to a 5 ml DEAE-cellulose column. The column was washed with 5 ml Buffer A. Receptor A subunits were eluted stepwise with 0.15 M KCl in Buffer A [5]. In a similar manner, the receptor B subunit fraction was eluted with 0.30 M KCl in Buffer A [5]. As reported previously [16], this procedure yielded the two 4*s* subunits purified about 300-fold.

RESULTS

Despite considerable progress in purifying the progesterone receptor activity, we had not undertaken a rigorous study of the kinetics of hormone binding in this system. We had already determined the rate constants and equilibrium constants at 0°C. However, a range of conditions was needed to shed more light on the nature of the binding sites.

For the binding assay, the charcoal-Dextran method was chosen because of its simplicity and ease of handling large number of samples quickly and reproducibly. Although this method has received criticism in the past due to a deleterious effect of charcoal on the receptors themselves, it was felt that in these experiments the cytosol provided sufficient protection of the receptor binding sites to allow its use. This conclusion was substantiated by comparing this method with an ammonium sulfate precipitation method used previously. The ammonium sulfate method has been shown to be less subject to artifacts due to receptor destruction [16]. In experiments not shown, Scatchard plots were done on cytosol preparations at 0°C by both techniques. Both gave the same value for K_d and N_{max} of 2×10^{-9} M and 5×10^{-9} M, respectively. Thus, in all further hormone-binding studies we used the charcoal-Dextran assay.

We first studied the effects of temperature on the association rate constant. The results of these experiments are shown in Fig. 1a. Other studies [13] have shown that the receptor without hormone bound is unstable at 37°C. Therefore we studied the range from 0 to 23°C. As expected, the figure shows clearly that the rate of association increased with increasing temperature during the early course of the reaction. From the plots, k_a values were determined at three temperatures as shown in Table 1.

Table 1. Progesterone-binding kinetics of chick oviduct receptor protein

Parameter		Rate constants[a] Association $10^{-7} \times k_a$ (M^{-1} s^{-1})	Dissociation $10^4 \times k_d$ (s^{-1})	Half-Life from k_d (min)	Equilibrium constant[b] from Rates $10^9 \times k_d/k_a$ (M)	Scatchard $10^9 \times K_d$ (M)
Temperature	0°C	0.077	0.21	540	0.027	5.09
	15	7.9	1.4	82	0.0018	5.53
	24	405	4.9	24	0.00012	—
	31	—	17	6.6	—	—
	42	—	385	3.0	—	—
KCl	0 M	0.077	4.2	27	—	5.09
	0.15	—	4.5	25	—	4.73
	0.3	—	4.8	24	—	5.23
	0.4	0.022	—	—	—	—
	0.5	—	5.6	21	—	4.4
Glycerol	0%	—	4.8	24	—	—
	5	—	4.1	28	—	—
	10	—	3.1	37	—	—
	20	—	2.6	45	—	—
	25	—	1.3	87	—	—
	30	—	0.73	110	—	—
Resins	CYTO	0.077	0.20	570	0.026	—
	DEAE	0.075	0.19	600	0.026	—
	PC	—	0.27	420	—	—

[a] k_a from slopes of Figs. 1 and 7. k_d from slopes of Figs. 2, 6 and 7.
[b] K_d from Scatchard plot slopes of Fig. 5.

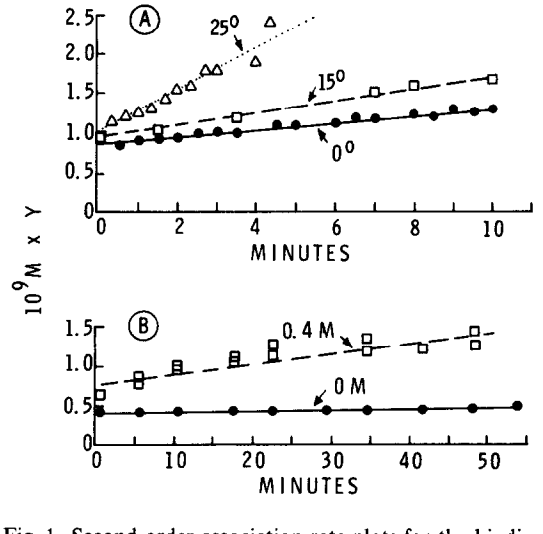

Fig. 1. Second order association rate plots for the binding of radioactive progesterone to chick oviduct cytosol receptor proteins. The parameter Y is a logarithmic function of the bound and unbound progesterone concentrations defined as follows:

$$Y = (1/T - S)\ln T - X/S - X \text{ for } T > S$$

where T = total concentration of hormone; S = total concentration of receptor sites; X = concentration of receptor–hormone complexes at time t. Binding to the receptors was determined as outlined in Methods by using the Dextran charcoal method. The association rate constants, k_a, are determined from the slope of each line. Panel A—association rate constants as a function of incubation temperature. Cytosol was prepared as described in Methods in Buffer A without KCl. Incubations carried out at 0° (●——●), 15° (□– – –□) or 25° (△···△). Panel B—association rate constants as a function of KCl molarity. Cytosol was brought to indicated KCl molarity with Buffer A containing 1.0 M KCl and incubated at 0° either with KCl (●——●) or 0.4 M KCl (□– – –□). Each point was the mean of triplicate determinations and each experiment was repeated 3 times.

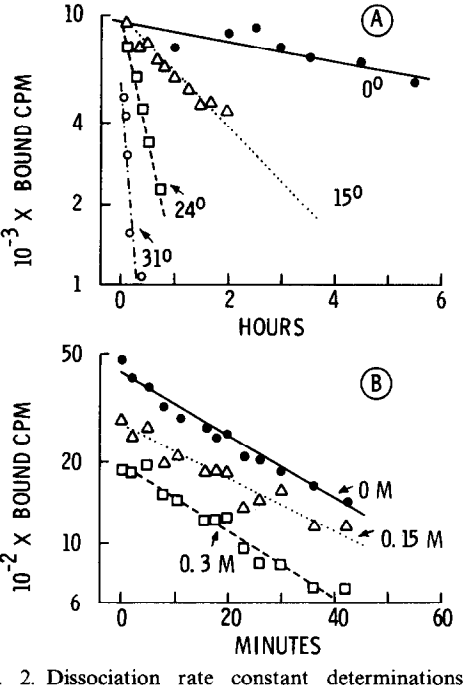

Fig. 2. Dissociation rate constant determinations for labeled progesterone–receptor complexes. Semilogarithmic plot of labeled progesterone retained vs time of incubation under pseudo first order dissociation conditions as described in Methods. Receptor-bound labeled progesterone was determined in aliquots at the times indicated. Panel A—effect of temperature on dissociation rate. Cytosol incubated in Buffer A without KCl at 0° (●——●); 15° (△– – –△); 24° (□– – –□); and 31° (○·–·–○). Half-life for each temperature was determined from the slopes of the lines. From these values, dissociation rate constants were calculated from the relationship $k_d = 0.693/t_{1/2}$. Panel B—effect of KCl molarity on dissociation rates. Incubation of cytosol complexes at 24° in Buffer A without KCl (●——●; 0.15 M KCl (△···△); or 0.3 M KCl (□– – –□). Each point was the mean of triplicate determinations and each experiment was repeated 3 times.

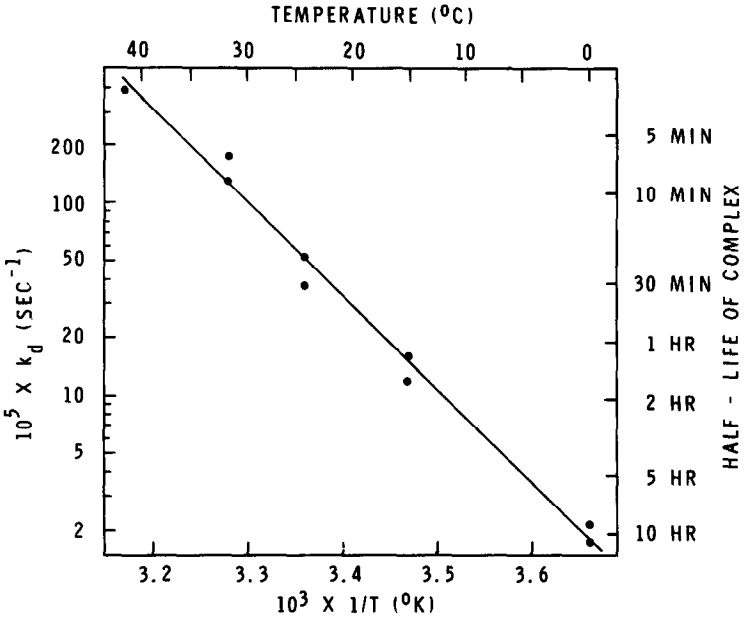

Fig. 3. Arrhenius plot of log K_d vs $1/T$ for the incubation temperatures used in Fig. 2. Additional data points for plots not shown in Fig. 2 were determined by the same protocol.

Pseudo first order dissociation rate constants were determined as described in Fig. 2. These values were plotted as a function of reciprocal of absolute temperature. For convenience the calculated half-life and temperature in degrees centigrade are also indicated on the figure. The slope corresponds to the activation energy for dissociation, $-E_a/R$. Each point was the mean of triplicate determinations and each experiment was repeated twice.

Next, the effect of ionic strength upon the association rate constant was studied. Figure 1b shows that ionic strength does not exert a major effect on the hormone binding site. The time scale is changed in Fig. 1b; there is a reproducible difference in association rate due to KCl, but the effect is very small compared to the effect of temperature. This supports the results of previous experiments which have shown that there was very little change in conformation or electrostatic properties of the receptor with changing KCl concentrations.

Companion studies were done of the dissociation reaction as shown in Fig. 2. The process was made pseudo-first order by adding a large excess of non-radioactive progesterone to the sample containing receptors which had previously been labeled with [^{3}H]-progesterone. Measurement of the decrease in labeled hormone bound to the receptors as a function of time allowed calculation of the dissociation half-life from which the dissociation rate constant was computed. Values of k_d and half-life at the temperatures studied are shown in Table 1. Plotting log k_d vs $1/T$ produced an Arrhenius plot shown in Fig. 3. In this linear transformation, the slope represents the energy of dissociation, E_{diss}. This was equal to 23 kcal/mol which is consistent with the hypothesis that the binding process involves a conformational change. Of interest is the fact that the plot is linear with no discontinuities. Experiments have shown that the hormone–receptor complex undergoes a change in aggregation state upon warming above 0°C. The fact that this changed state does not affect the binding parameters is shown by the linear Arrhenius plot. This implies that the hormone binding sites are not perturbed measurably by the mechanism responsible for aggregation or dimerization of the receptor subunits.

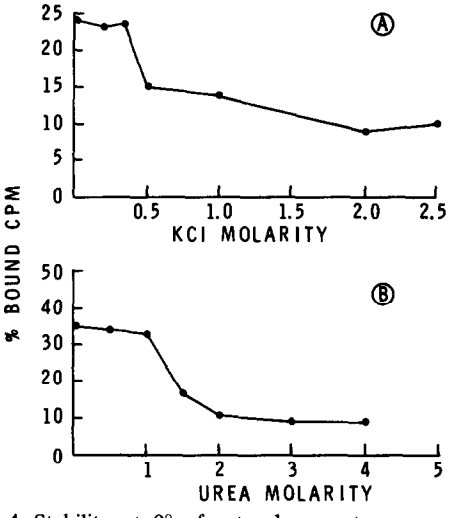

Fig. 4. Stability at 0° of cytosol progesterone receptor complexes in KCl and urea. Labeled progesterone receptor complexes in Buffer A were exposed to either KCl (Panel A) or urea (Panel B) at the indicated molarities as described in Methods. Bound hormone remaining after incubation was determined by gel filtration on 5 ml G-75 columns. The per cent of the radioactivity chromatographing in the void volume of the column was an index of the amount of receptor binding activity remaining in the samples. Each point was the mean of triplicate determinations and each experiment was repeated twice.

The effect of changing ionic strength upon the dissociation rate constant was small, as seen in Fig. 2b. This, too, is to be expected since the receptor is only slightly affected by changing KCl concentrations.

The effect of KCl and urea upon receptor stability were studied. Results of these studies are shown in Fig. 4a and 4b. Dilution of cytosol hormone–receptor complexes with these agents to the indicated molarity was followed by incubation at 0°C for 3 h. The preparations were then assayed immediately for bound hormone. It can be seen from these figures that the receptor complexes were 50% dissociated in 1.0 M KCl (Fig. 4a) or 1.5 M urea (Fig. 4b). Subsequent studies of these effects have shown that the receptors are not permanently denatured by these treatments; gradient dialysis to remove 3.0 M urea over a 24 h period results in a recovery of progesterone-binding capacity of 90–100%.

Next, we looked at the effect of changing the temperature and the ionic strength upon the equilibrium constant, K_d. As shown graphically in Scatchard plots in Fig. 5a and 5b, neither changing the temperature nor the KCl concentration had any significant effect upon K_d. The value of K_d was near 5×10^{-9} M. The graphs show no evidence of co-operativity. This implies only one hormone binding site class per receptor molecule. In addition, K_d shows that, at physiological salt conditions (0.15 M), the binding of progesterone to its receptor molecule is very tight.

Two other important results may be derived from this experiment. The only apparent change that

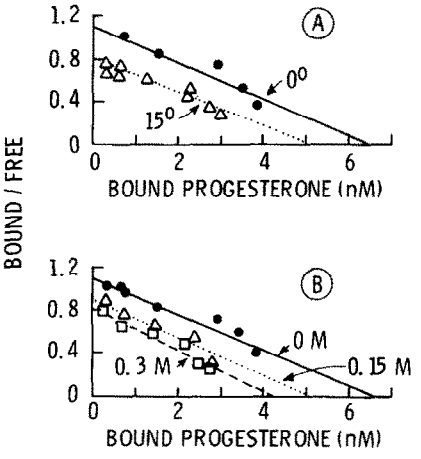

Fig. 5. Scatchard plots of progesterone binding to cytosol receptors as determined by charcoal-Dextran assay. Cytosol and varying concentrations of labeled progesterone were incubated as described in Methods for 16 h. Panel A, effect of temperature on dissociation equilibrium constant. Equilibrium constant K_d was determined from the reciprocal slopes of the plots. Intercepts on X-axis were indices of receptor concentration in the assay. Panel A—effect of temperature on equilibrium constant in Buffer A without KCl. Incubations were at 0° (●——●) and 15° (△···△). Panel B—effect of KCl molarity on equilibrium constant at 0°. Incubations were in Buffer A only without KCl (●——●); 0.15 M KCl (△···△); and 0.3 M KCl (□——□). Each point was the mean of triplicate determinations and each experiment was repeated 3 times.

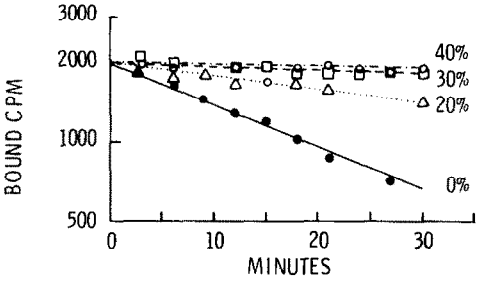

Fig. 6. Effect of glycerol on half-life of progesterone receptor complexes. Pseudo first order dissociation of progesterone receptor complexes as a function of glycerol concentration. Labeled progesterone receptor complexes were incubated in glycerol at the indicated concentrations at 25°C and handled as described in Fig. 2. Progesterone receptor binding was determined by the charcoal assay on aliquots removed to the indicated times. (●——●), no glycerol added; (△···△), 20% glycerol (v/v); (□——□), 30% glycerol; (○—·—○), 40% glycerol. Each point was the mean of triplicate determinations and each experiment was repeated twice.

occurs in the receptor with lowered temperature is that hormone dissociates from it more slowly. Thus, studies of the hormone–receptor interaction at 0°C where the receptor is more stable merely slow the rate of this dissociation process. In addition, since the receptor changes its aggregation state from 6*s* and 8*s* to 4*s* over the temperature range used in the experiments [24], it appears that no change in the binding site kinetics occurs with the changing aggregation state. Thus, interactions between receptor molecules do not affect the binding site dramatically.

Other workers have reported that the addition of glycerol to receptor preparations caused a change in the stability of the receptor [21–23]. We studied this glycerol effect; the half-life of pseudo first-order dissociation was measured following addition of excess non-radioactive progesterone. The results are shown graphically in Fig. 6, and the computed half-lives are shown in Table 1. It is evident that the addition of glycerol to cytosol causes a substantial stabilization of the receptor toward dissociation of its hormone.

In data not shown, we looked to see whether a similar stabilization of more purified receptor preparations could be achieved by this method. The dissociation half-life ($t_{1/2}$) of receptors purified about 500-fold by DEAE-cellulose chromatography was measured with and without added glycerol under the same conditions used for the cytosol study. The study indicated that both of the partially-purified receptor subunit forms eluted from DEAE are stabilized by added glycerol. Thus the glycerol effect appears to act directly on the subunits and is independent of subunit–subunit interactions.

We next studied the relationship between the hormone binding site and the receptor's role in nuclear binding. Buller and co-workers in our laboratory have previously shown the requirement of bound hormone for receptor binding to nuclei [13]. Jaffe *et al.* have reported different hormone binding rate kinetics

when the receptor is bound to chromatin [24]. We therefore attempted to measure the rate kinetics of hormone binding when the receptors were bound to ion-exchange resins having binding properties similar to those of DNA and chromatin. For this purpose phosphocellulose and DEAE-cellulose were used. These represent examples of receptor interactions mediated by negative and positive charges, respectively.

In the association rate study, the receptor without hormone was adsorbed onto a slurry of DEAE-cellulose. Labeled hormone was then added and the rate of binding measured as described in the methods section. The results are shown graphically in Fig. 7a. The association rate appears to be the same whether or not the receptor is adsorbed to DEAE-cellulose. In an experiment not shown, another identical curve was obtained for the association reaction in the presence of DEAE-cellulose, but at a KCl concentration of 0.4 M at which the receptor does not bind to the resin. Thus the interaction of the receptor with DEAE-cellulose causes a negligible change in the hormone binding site. Similar association rate experiments on phosphocellulose were not done. The uncomplexed receptor in cytosol exists as a 6S dimer which does not bind to phosphocellulose [14]. After labeling with hormone, there is a time- and salt-dependent dissociation to 4S monomers. These monomers are able to bind to the resin, permitting dissociation studies to be done.

Although the association rate experiment shows that it is unlikely that any electrostatic interactions have an effect upon hormone–receptor binding, we completed the study by measuring the dissociation rate from DEAE-cellulose and phosphocellulose. Receptors in cytosol were complexed with [^{3}H]-progesterone and then adsorbed to the ion exchange resin. Excess non-radioactive progesterone was added. At intervals, 700 μl aliquots of the supernatant fraction were taken and assayed for radioactivity released. The loss of ^{3}H from receptors with time is shown in Fig. 7b. This figure shows that binding to ion exchange resins produces no change in kinetics, implying that the hormone binding site dissociation is also not affected by electrostatic interactions.

We noted in previous long-term studies of receptor stabilization that after many hours the dissociation rate plot began to show a change in slope. Similar results have been shown by Sanborn [25], and others. The theoretical studies of Rodbard [26] predict that this flattening of the curve is an artifact due to the technique used for measurement caused by the existence of a large source of non-specific binding.

In order to study these effects, the experiments of Fig. 8 were done. The receptors in the cytosol dissociated in the presence of excess non-radioactive progesterone showed a biphasic curve with about 25% appearing to have a longer half-life. This could be due to progesterone no longer being in excess either due to its metabolism or its adsorption to other proteins.

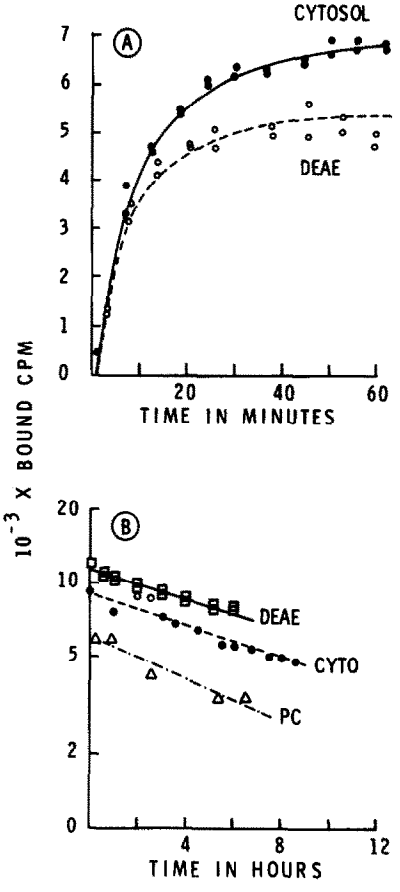

Fig. 7. Effect of receptor binding to ion exchange resins on kinetics of progesterone interaction with the proteins. Panel A—association curve for binding of tritiated progesterone to cytosol receptors either free in solution (●——●) or when receptors were first bound to DEAE cellulose as described in Methods (○---○). Association carried out as described in Fig. 1. Aliquots of cytosol or DEAE slurry were removed at the indicated times and assayed for progesterone binding activity. Panel B—half-life of progesterone receptor complexes under pseudo first order dissociation conditions when receptor is bound to ion exchange resins or free in solution. Incubation conditions were as described in Methods and Fig. 2. Aliquots of cytosol were assayed for binding by the charcoal assay at the indicated times. In the case of the receptors bound to DEAE or phosphocellulose the aliquots were centrifuged and radioactive progesterone which had been released into the supernatant fraction was measured directly by scintillation counting. Cytosol alone, (●——●); receptor bound to DEAE cellulose, (□——□); receptor bound to phosphocellulose, (△-·-△). Each point was the mean of triplicate determinations and each experiment was repeated twice.

In experiments not shown, we found that simply adding more non-radioactive progesterone had no effect upon the dissociation curve. To study this problem further, cytosol was labeled with [^{3}H]-progesterone and then incubated in the presence of excess non-radioactive progesterone for 66 h at 0°. The receptors were then concentrated with ammonium sulfate. The concentrated, redissolved pellet was treated with more non-radioactive progesterone and the dissociation

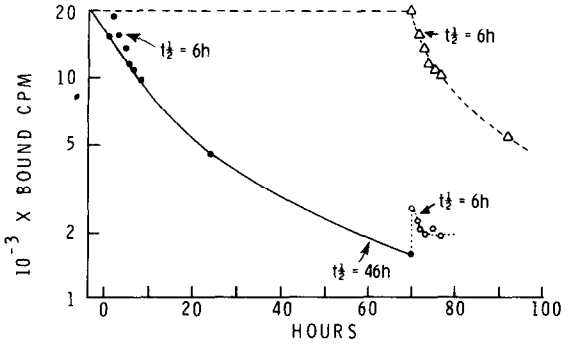

Fig. 8. Effect of storage on dissociation rates for progesterone receptor complexes. Semilogarithmic plot under pseudo first order dissociation conditions for receptors stored under a variety of conditions as described in Fig. 2. Cytosol receptors labeled with progesterone stored at 5°C and assayed for bound hormone by the charcoal assay at indicated times (●——●). The remaining cytosol preparation was stored under pseudo first order dissociation conditions for 70 h and was then concentrated 3-fold by precipitation with ammonium sulfate as described in the text (O···O). The resuspended receptors were again subjected to pseudo first order dissociation conditions by addition of non-radioactive progesterone. Cytosol also was stored with hormone bound but without added progesterone until 70 h had elapsed. At $t = 70$ h, excess non-radioactive progesterone was added and bound hormone again determined as a function of time (△---△). Each point was the mean of triplicate determinations and each experiment was repeated 3 times.

rate measured. As shown in Fig. 8, the plot began again with a sharp slope approaching that of the original cytosol.

Another possible reason for the biphasic dissociation curve is that the hormone binding sites on the receptor were destroyed or altered with time. To look at this, the receptors in a cytosol sample were labeled with [^{3}H]-progesterone and allowed to stand at 0° without any excess non-radioactive progesterone for 72 h. As shown in Fig. 8, this was sufficient time for the dissociation of hormone from receptor to level off. Excess non-radioactive progesterone was then added and the dissociation rate measured. As shown in Fig. 8, the half-life again was found to be 6 h. Next, we aged unlabeled cytosol until the time that the companion dissociation curve leveled off. The cytosol was then labeled with [^{3}H]-progesterone. Excess non-radioactive progesterone was added and the dissociation rate measured. The half-life was near that found in previous dissociation experiments.

These experiments show that the hormone binding sites on the receptor molecule remain intact and active over time both with and without bound hormone. We conclude that all of the progesterone binding sites are the same, and are quite stable over time at 0°C.

Furthermore, these experiments strongly suggest that the apparent biphasic nature of the steroid–receptor dissociation curves reported in the past probably reflect artifacts of assay techniques rather than true site heterogeneity.

DISCUSSION

Both the association and dissociation rates are markedly temperature-dependent. In contrast, the dissociation equilibrium constant, K_d, remains fairly constant over a wide range of temperatures. In addition, the Arrhenius plot is linear with no multi-phasic character. These facts show that the nature of the hormone-binding site does not vary appreciably with temperature. Thus, the results of hormone-binding experiments at 0° where the receptor is more stable are also valid at physiological temperatures.

The concentration of KCl seems to have little effect upon any of the kinetic parameters measured. This result implies that forces other than electrostatic ones are most important in the receptor–hormone interaction studied. The hydrophobicity of the steroid and the tightness of binding would tend to favor a binding site lipophilic in nature. The steroid presumably may be deeply buried in the receptor protein interior. The fact that some small effect of salt is seen could indicate that the electrostatic forces are not altogether absent, however. In fact, this lack of KCl effect is unusual since other laboratories have reported that receptor binding does change with salt conditions [27].

Additions of high concentrations of glycerol cause stabilization of the receptor–hormone complex toward dissociation. This effect is noted in partially purified as well as cytosol preparations. A possible explanation is that the added glycerol increases the viscosity of the solution and therefore decreases the rate of diffusion thus making re-association of the hormone to the receptor more likely after a partial dissociation occurs. A direct effect of glycerol on the site cannot be ruled out, however.

DEAE-cellulose has been used by others as a model for chromatin binding. From our association rate studies on DEAE-cellulose, it is evident that bound hormone is not necessary for the receptor to stick to the resin. However, the interaction of the receptor with the resin does not occlude or perturb the hormone binding site. The measured association rate is the same whether the receptors are bound to DEAE-cellulose or not. Since it has been shown in other studies that bound hormone is required for the receptor to bind to chromatin [6], our studies imply that the chromatin binding site is different from the DEAE-cellulose binding site. In addition, since binding to the charged DEAE-cellulose does not change the association rate, the hormone-binding site is not perturbed by the electrostatic effects and may therefore be somewhat remote from the DEAE-cellulose binding sites.

Binding to phosphocellulose was also shown to have little effect upon the dissociation rate. Thus, the hormone binding site is not perturbed by the electrostatic interaction of the receptor with this ion-exchange resin either. As in the case of binding to DEAE-cellulose, this implies that the hormone bind-

ing site is somewhat remote from the phosphocellulose binding site. The distribution of charged regions and the hormone binding site indicated by these ion-exchange resin experiments is consistent with a separation of these regions over the surface of the molecule.

Recent unpublished studies in this lab have shown that the electrostatic profile of the receptor as shown by its elution from ion-exchange columns with KCl gradients is the same both with and without bound hormones. Since bound hormone is required for both DNA and chromatin binding, the hormone-binding site is either more closely linked to these functional sites or its perturbations which affect these functional regions are more subtle.

Our studies of the effects of aging the receptor preparation have produced some important results. First, the majority of the receptor sites behave identically under experimental condition. Any heterogeneity of the half-life of dissociation may be attributed to experimental artifacts in the assay due to a large source of non-specific binding. In addition, aging the receptor either with or without hormone present does not change the measured kinetic parameters for the hormone-binding reaction. These results imply that the hormone site is not easily perturbed and presumably remains in the same state in the cell at all times. Those manipulations of the receptor which do seem to change hormone-binding characteristics, such as Ca^{2+}-activated proteases [22], must involve major changes in the hormone site.

In the light of these results, it is tempting to speculate that the hormone-induced nuclear binding sites may be formed through a conformational change and may function through some sort of steric recognition of the proper nuclear site. This hormone-induced conformational change in receptor functional sites from an allosteric locus is an attractive model for future studies involving purified preparations, and is consistent with an allosteric site model of hormone action.

Finally, the data of Table 1 show an important discrepancy, which may indicate that the mechanism of hormone binding to its receptor protein is considerably more complicated than presently thought. The equilibrium constant at 0° determined by Scatchard plot (5×10^{-9} M) differs by two orders of magnitude from the value obtained from the ratio of the rate constants (0.027×10^{-9} M). The charcoal assay was used for all of the determinations, and hence the same sort of measurement errors ought to be present in both values. If the pathway of the binding reaction were adequately described by the rate equations, both methods should yield equivalent values. The same equilibrium constant by Scatchard plot method has been obtained by other assays; furthermore, this value reflects the physiologic concentrations of progesterone active in the chick. Finally, the equilibrium method is by definition independent of the path of reaction. Hence, it is most likely that the rate equations are in error.

Since the association rate constant varies with temperature more drastically than that for dissociation, it is reasonable to speculate that the association pathway rate equation is more in error. We may conclude from this that the data indicate a reaction more complicated than simply $R + H \rightleftarrows RH$.

Two possibilities are worth more careful attention: First, receptor proteins may exist in a non-binding conformation in equilibrium with a small amount of a receptor form able to interact with the hormone. Then hormone or temperature may shift the equilibrium to the right in favor of binding:

$$R \text{ inactive} \rightleftharpoons R \text{ active}$$

$$R \text{ active} + H \rightleftharpoons RH$$

This mechanism was first proposed by Tomkins and co-workers [28] for glucocorticoids.

Second, there may be two types of hormone site on the molecule, one called an "entry" site and one a strong binding site. Occupancy of the entry site by hormone may facilitate the occupancy of the strong site. If the two sites are of grossly different affinities, co-operativity may have been overlooked in the past. Rate kinetics of the strong site may be considerably altered by conditions of the binding reaction. For example, association and dissociation studies here were done in the presence of a large excess of hormone. This could result in continuous occupancy of the entry sites, facilitating the binding reaction or dissociation. Such a concentration dependence for rates may be indicated in the data of Fig. 8.

In any event, it has not yet been possible to study the hormone-binding reaction rigorously enough to determine whether any of these more complex schemes are active. Clearly, however, there is reason to doubt that the reaction is presently well-characterized.

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