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EFFECT OF SALT ON REVERSIBILITY OF GLUCOCORTICOID RECEPTOR BINDING*

NANCY S. KRIEGER,† JOHN L. MIDDLEBROOK‡ and LEWIS ARONOW§
Department of Pharmacology, Stanford University School of Medicine, Stanford, California 94305, U.S.A.

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SUMMARY

Previous studies with [^{3}H]-triamcinolone acetonide indicated that the initial glucocorticoid-receptor interaction in the cytoplasm of a fibroblast cell is an essentially irreversible reaction. The reaction has now been shown reversible at 0°, but observation of reversibility depends upon the salt concentration. When the binding reaction is studied *in vitro* at low ionic strength (0.05 M KCl), the association rate constant at 0° is 6.0×10^5 M^{-1} min^{-1}, the dissociation rate constant is 2.4×10^{-3} min^{-1} and the equilibrium constant is 2.5×10^8 M^{-1}. Bound receptor examined in hypotonic extracts of cytosol obtained from cells prebound with steroid at 37° demonstrates an exchange of about 50% of the binding with an unlabeled chase (non-radioactive TA at 10^{-5} M) over a period of 4 days at 0°, yielding a dissociation rate constant of 1.2×10^{-4} min^{-1}. Increasing the ionic strength of the incubation to 0.15 M or higher masks the chase effect by an effect of salt alone, such that the rate of degradation of the bound complex approaches and equals the rate of dissociation seen in the presence of a chase. Under these conditions no rebinding occurs and there is therefore no chase effect over and above the apparent degradation rate. The unbound receptor is less stable in high salt than at 0.05 M KCl, largely accounting for this phenomenon. The nuclear extractable form of the receptor complex cannot be shown to rebind steroid under any salt condition, and therefore seems to be a further modified form of the steroid–receptor complex.

INTRODUCTION

Mouse fibroblasts (strain L929) growing *in vitro* are target cells for an antianabolic effect of glucocorticoids, resulting in growth inhibition. These cells contain a specific binding component for triamcinolone acetonide¶ (TA), a potent synthetic glucocorticoid [1]. Binding occurs with high affinity and the receptor–steroid complex can be shown to exist in three distinct cellular forms: a cytoplasmic form, a nuclear extractable form, and a nuclear residual form. The subcellular distribution of these glucocorticoid–receptor complexes can be altered depending upon experimental conditions [2].

Earlier work from this laboratory found the binding reaction in the soluble fraction to have an association rate constant at $0° = 8.0 \times 10^5$ M^{-1} min^{-1} when studied under *in vitro* conditions at isotonic salt concentration, and the reaction was apparently irreversible [3]. The studies reported here demonstrate that the reversibility of the binding of triamcinolone acetonide to its cytoplasmic receptor is at least partly governed by the ionic strength of the incubation conditions. In solutions of low ionic strength, the initial binding reaction is seen to be reversible.

MATERIALS AND METHODS

Chemicals. [1,2,4,-^{3}H]-triamcinolone acetonide (4.0 Ci/mmol) was purchased from Schwarz Bioresearch, Inc., Orangeburg, New York or from New England Nuclear (18.9 Ci/mmol), Boston, Massachusetts. 11α-cortisol was donated by the Squibb Institute for Medical Research, 11β-cortisol was purchased from Mann Research Laboratories, Inc., New York. Norit A charcoal and Dextran T70 were obtained from Pfanstiehl Laboratories, Inc., Waukegan, Illinois and Pharmacia Fine Chemicals, Uppsala, Sweden, respectively. All other chemicals were reagent grade and were used without further purification.

Cell culture. Suspension cultures of L929 fibroblast cells were maintained in basal medium [4] modified as described previously [5]. Cultures were kept at 37° with constant stirring in an atmosphere of humidified air.

Incubation of cells. Cells were harvested from suspension cultures by centrifugation at 600 ***g*** for 10 min at 0°. For steroid binding to intact cells, the cells were resuspended to approximately 2×10^7 cells/ml in growth medium without serum and incubated with 10^{-8} M [^{3}H]-triamcinolone acetonide at 37° for 40 min. All steroids were prepared at 100 times the final

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† Predoctoral fellow supported by Public Health Service Training Grant 5 T01 GM 00322.

‡ Postdoctoral Fellow of the Arthritis Foundation. Present address: U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, Maryland 21701.

§ To whom correspondence should be addressed.

¶ The trivial names for steroids used are: triamcinolone acetonide, 9α-fluoro-11β,16α,17,21-tetrahydroxyl-1,4-pregnadiene-3,20-dione 16,17-acetonide; 11α-cortisol, 11α,17,21-trihydroxy-4-pregnene-3,20-dione; and 11β-cortisol, 11β,17-21-trihydroxy-4-pregnene-3,20-dione.

concentration desired in 10% ethanol. When competing steroids (11α- and 11β-cortisol) were used, they were added to the cell suspension immediately before the [^{3}H] TA.

Cell fractionation. The cell suspension was harvested by centrifugation at 600 *g* for 10 min at 0–4° and washed in 8–10 vol. of a cold balanced salt solution. The washed cells were resuspended in 1.5 vol. of hypotonic solution (0.01 M Tris buffer at pH = 7.35 and 0.1 mM EDTA) for 5 min, and then homogenized with 15 strokes of a tight-fitting pestle in a Dounce-type glass homogenizer. The broken cell suspension was centrifuged at 105,000 *g* for 1 h. The 105,000 *g* supernatant (0.9–1.2 mg protein N/ml) is defined as the fraction containing the cytoplasmic steroid receptor. Hackney *et al.*, have shown that over 90% of the cytoplasmic binding obtained with [^{3}H] TA under these conditions is specific (1). This hypotonic supernatant contains, by chloride analysis, about 0.03 M Cl^- derived from the cellular cytoplasm.

To obtain a nuclear extract, the 10,000 *g* pellet from TA-bound cells was washed once with hypotonic buffer, resuspended in 6 vol. of extraction buffer (0.3 M KCl, 0.01 M Tris, pH 7.35) and incubated for 10 min at 0° before recentrifugation at 10,000 *g* for 10 min. The supernatant obtained was then spun at 105,000 *g* for 1 h and the resulting supernatant, which contains 0.1–0.2 mg protein N/ml, defines the nuclear extract.

Measurement of binding capacity. 105,000 *g* supernatant was obtained from cells not previously incubated with steroid, and was then incubated with 10^{-8} M [^{3}H] TA in the presence of 10^{-5} M 11α-cortisol or 11β-cortisol and 250 mM Hepes* buffer, pH 7.35, for 21 h at 0°. Specific binding is determined by subtracting the bound radioactivity observed in the presence of 11β-cortisol from that observed in the presence of 11α-cortisol.

Dialysis. Dialysis of samples was carried out in preboiled tubing at 0° for 4 h. Approximately 100 vol. of dialysis buffer containing the desired salt concentration were used per sample vol., and the buffer solution was changed every hour.

Assay for bound radioactivity and protein. The Dextran-coated charcoal adsorption technique was employed for all binding assays [2,6]. Samples were added to 10 ml of a scintillation solution prepared according to Carey and Goldstein [7] and counted in a Packard Tri-Carb liquid scintillation spectrophotometer, Model 3310. Quenching was determined by use of internal standards. Protein determinations were performed according to the method of Oyama and Eagle [8].

RESULTS

Reversibility of binding; kinetics of chase effect. A 105,000 *g* hypotonic supernatant from [^{3}H] TA bound cells was incubated at 0° and the loss of binding over a period of 4 days was measured either in the presence or absence of 10^{-5} M cold TA (Fig. 1). In addition, one sample of hypotonic supernatant was raised to 0.3 M KCl prior to incubation, and a sample of nuclear extractable receptor was studied under the same conditions.

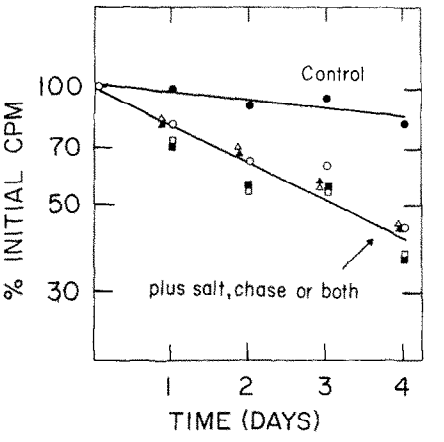

Fig. 1. *Reversible binding of steroid–receptor complex.* Cells were incubated with [^{3}H] TA as described in Methods, and cell fractions obtained. Samples were divided in half and either 0.10% ethanol (solid symbols) or 10^{-5} M unlabeled TA (open symbols) was added. The fractions were incubated at 0° for 4 days. At intervals samples were taken and the amount of bound complex remaining was determined by the charcoal assay. Each value represents the average of duplicate samples. Symbols are: 105,000 *g* bound supernatant (○); bound supernatant raised to 0.3 M KCl (□); and nuclear extract (△).

Only under hypotonic salt conditions was a chase effect seen. That is, in the presence of ethanol alone (vehicle), binding remained approximately constant at 100% of its initial level, while in the presence of excess cold TA an apparent decline in bound receptor was seen. At high salt concentration, the rate of loss of bound receptor was identical in the presence or absence of a chase. If dissociation is occurring, it must be taking place at the same rate as an inactivation or alteration of the binding component itself, since rebinding with free steroid does not seem to occur. This seems to hold true for both the nuclear extractable receptor complex and hypotonic supernatant receptor raised to 0.3 M KCl. It has been shown previously that this loss of binding cannot be accounted for by any significant metabolism of TA by fibroblast cell fractions [1].

Kinetics of chase effect after dialysis. In order to determine if the effect of salt on nuclear extractable receptor could be altered by dialyzing away the salt, as well as studying the effects of free steroid in the system, samples were dialyzed against hypotonic buffer immediately after isolation. Then either vehicle, 10^{-3} M cold TA or 10^{-6} M [^{3}H] TA were added at 1/100 vol. and the loss of binding was followed as before. Under these conditions, no chase effect was

* Hepes buffer is N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid.

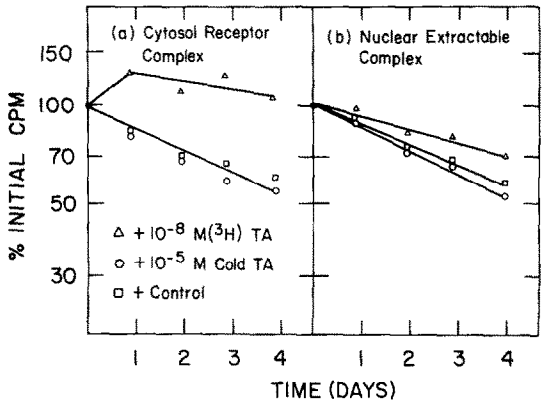

Fig. 2. *Kinetics of chase effect and reversible binding of steroid receptor complex after dialysis.* Samples of 105,000 *g* bound supernatant or nuclear extract were dialyzed for 4 h against hypotonic buffer at 0°, and then each was divided into 3 equal parts. To these, 10^{-8} M [^{3}H]TA (△), 10^{-5} M cold TA (0) or 0.10% ethanol (□) was added and all samples were incubated at 0° for 4 days. Aliquots were removed at various intervals and the amount of bound complex remaining was determined using the charcoal assay. a. Cytosol receptor. b. Nuclear extract.

seen even with untreated hypotonic bound supernatant unless [^{3}H] TA was added back (Fig. 2a). This indicates that there is an optimal concentration of free TA (already present in undialyzed cytosol extracts) in order for the dissociated receptor to rebind and maintain a steady-state level. Hypotonic cytosol extracts prepared from prebound cells as described in Methods contain about 2.5×10^{-9} M free TA. Dialysis for 4 h at 0° against 3 changes of 100-fold vol. of buffer decreases the free TA concentration to 1/10th the initial concentration while the KCl concentration comes to equilibrium with the external dialysis bath solution. In several experiments with cytoplasmic receptor complex, the rebinding upon addition of [^{3}H] TA rose somewhat above initial control values, but only part of that was specific. It appears that removal of the salt does not allow demonstration of reversible binding to nuclear extract (Fig. 2b).

Salt effect on exchange of TA. A dose response curve was generated to show the effect of increasing salt concentration on the reversibility of binding in the cytoplasmic fraction (Fig. 3). There is a peak in stability of the bound receptor between 0.05–0.10 M KCl as measured by the ability to show a substantial chase effect over 4 days incubation at 0°.

Essentially superimposable results were obtained when unbound hypotonic supernatant was dialyzed for 4 h at 0° against buffer containing different concentrations of KCl, after which remaining specific binding capacity of each sample was measured. Controls for comparison included measurements of initial binding capacity and binding capacity of samples kept at 0° for 4 h without dialysis. Again, maximum stabilization was observed at a low concentration of salt (0.05 M), followed by a steady decrease in binding ability with increasing salt concentrations above 0.10 M. The ordinary preparation of hypotonic extract (see Methods) results in a solution approximately 0.03 M in chloride concentration; this salt comes from the cellular cytoplasm. It is noteworthy that the unbound receptor (and to a lesser extent, the bound receptor) is unstable if the salt is totally removed by dialysis (Fig. 3).

The data shown were all derived using KCl, but other experiments have demonstrated that almost any monovalent or divalent cation (of equivalent ionic strength) can be used. As a result of these findings subsequent dialyses were done against 0.05 M KCl buffer unless otherwise stated.

Comparison of cytoplasmic receptor and nuclear extractable receptor. Bound cytoplasmic receptor was raised to 0.3 M KCl for 1 h or simply kept at 0° for 1 h. Both samples were then dialyzed against 0.05 M KCl before measuring the loss of binding in the presence and absence of a chase over 4 days at 0° This was compared with nuclear extract that was dialyzed against 0.05 M KCl immediately after its preparation, so that both forms of the bound receptors were actually in the presence of high salt for approximately 1 h. The results (Fig. 4) show that the differential response of these two binding components, in terms of their ability to exhibit a chase effect, cannot be accounted for by a 1 h exposure to high salt at 0°. Also of interest is the difference in part a and

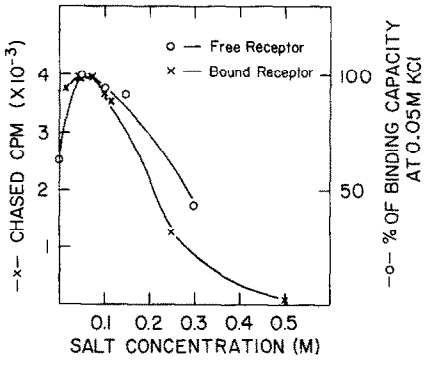

Fig. 3. *Effect of salt concentration on bound and free receptor.* 105,000 *g* bound supernatant (×) was obtained and aliquots were adjusted to various salt concentrations by the addition of a constant vol. of concentrated KCl solutions. At each salt concentration, the samples were divided and either 10^{-5} M unlabeled TA or 0.10% ethanol was added. Binding was then assayed as usual at 0 time and after 96 h incubation at 0°. Results are the average of duplicate samples and are given as 'chased c.p.m.', which is defined as the difference between the incubation sample with vehicle only and the sample containing excess cold TA, at each salt concentration.

To determine the salt effect on unbound receptor (O), aliquots of cell cytosol were raised to the appropriate salt concentration by 4 h dialysis against buffer at each desired ionic strength, and a specific binding capacity assay was then begun (see Materials and Methods). The ordinate in this case is defined as the percent specific binding of each sample (duplicates) compared to the control at 0.05 M KCl.

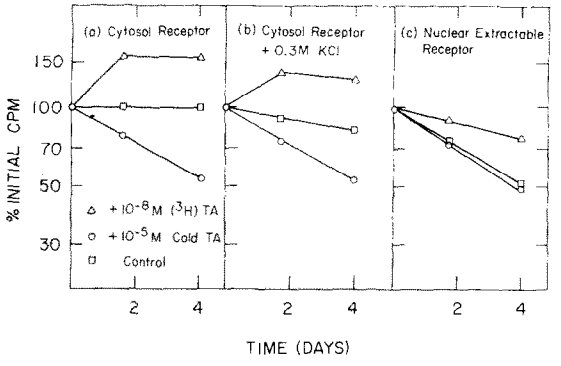

Fig. 4. *Comparison of cytoplasmic and nuclear extractable receptor.* a. 105,000 *g* TA-bound hypotonic cell supernatant was dialyzed 4 h at 0° against 0.05 M KCl-Tris buffer. Aliquots were then incubated in the presence of either 10^{-8} M [^{3}H]TA (△), 0.10% ethanol (□), or 10^{-5} M unlabeled TA (○) at 0°. The decrease in binding was then measured over 4 days. b. Bound supernatant was raised to 0.3 M KCl and held for 1 h at 0° before dialysis. Subsequent treatment followed the procedure outlined as in part a. c. Same procedure as part a, except that 105,000 *g* nuclear extract was used. Each point represents the mean and standard error of at least duplicate samples assayed for bound c.p.m./mg protein N (see Materials and Methods).

c of this figure compared with Figs. 2a and 2b, where the only change in the experimental procedures was the use of 0.05 M KCl buffer for dialysis as opposed to salt-free buffer. The data provides further evidence for stabilization of the bound receptor by 0.05 M KCl, as well as some indication that a small percentage of the binding in the nuclear extract fraction may be reversible.

Reversibility of the salt effect on receptor binding. [^{3}H] TA bound supernatant and unbound supernatant were raised to 0.3 M KCl for periods of time up to 24 h, then dialyzed against 0.05 M KCl and assayed for reversible binding (chase effect over 4 days at 0°), or for remaining specific binding capacity, respectively (Fig. 5). Controls were done to take into account possible effects of the duration of dialysis or incubation time before the determinations were begun.

In both cases the loss of reversibility of binding due to the presence of high salt is relatively slow. For the unbound receptor, the figure shows that specific binding capacity decreases with a half-time of 6–8 h in the presence of 0.3 M KCl, while control values (samples kept at 0.05 M KCl), even after 24 h incubation at 0° were essentially equivalent to initial values for binding capacity. Treatment of bound supernatant shows a similar time course on the ability to observe a chase effect. Controls, where no salt was added, maintained their ability to show a substantial chase effect, even after 24 hours of pre-incubation. But in the presence of 0.3 M KCl, the capacity to demonstrate chaseable bound counts relative to control values also decreases with a half-time of approximately 6 h.

DISCUSSION

While other studies [9,10] have shown that glucocorticoid–receptor binding systems are reversible, the experiments described in this paper demonstrate that detection of reversibility of the binding of [^{3}H] TA to the specific glucocorticoid-receptor of mouse fibroblasts is very much dependent on the ionic strength of the system. If receptor obtained from the cytosol of cells pre-bound with [^{3}H] TA is studied, then a chase effect (indicative of reversible binding) can be demonstrated readily in solutions of low ionic strength. At higher salt concentrations, dissociation and inactivation of the newly generated unbound receptor seem to be occurring simultaneously and no separate chase effect is discernible. We have shown (Fig. 3) a pronounced salt effect on the stability of the unbound receptor. It seems likely, then, that failure to observe reversibility in solutions of high ionic strength fundamentally is explainable on the basis of an ionic strength-sensitive unbound receptor macromolecule, which degrades rapidly at higher ionic strength. This fact, combined with a slow forward binding reaction rate (about 6.0×10^5 M^{-1} min^{-1}, see below), can account for our results. Maximum stability of the unbound receptor is seen at about 0.05 M KCl, and binding activity is rapidly lost both at lower ionic strength, and if the ionic strength goes above about 0.10 M KCl.

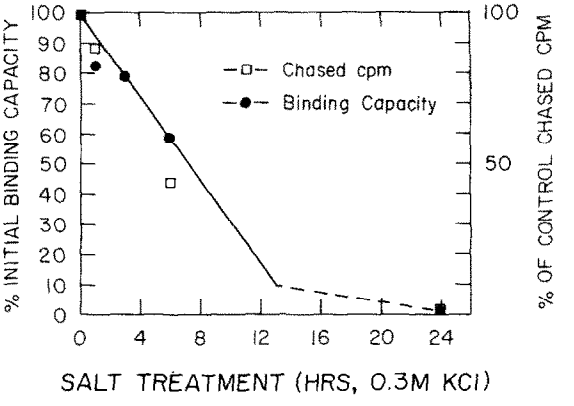

Fig. 5. *Time course of reversibility of salt effect on bound and free receptor.* Unbound 105,000 *g* supernatant (●) was either kept at low ionic strength or raised to 0.3 M KCl and incubated at 0° for various lengths of time. Samples were then dialyzed for 4 h with 0.05 M KCl before measuring specific binding capacity. Values given are the combined results of 3 experiments in which specifically bound c.p.m./mg protein N was assayed as usual (see Materials and Methods). [^{3}H] TA bound 105,000 *g* supernatant (□) was similarly salt treated. After dialysis, samples were divided and incubated at 0° in the presence of either 0.1% ethanol or 10^{-5} M unlabeled TA, and the loss of binding after 90 h was assayed. Chased cpm is defined as before (see Fig. 3), and the ordinate in this case is a measure of the percent chased counts relative to control values where no salt was added to the incubation. Controls for both sets of experiments were samples to which equivalent volumes of hypotonic buffer were added, and were held at 0° for 6 or 24 h before dialysis and assay.

We have also demonstrated that the nuclear extractable form of the receptor cannot be shown to re-bind steroid either in high or low salt solutions, although the loss of bound radioactive steroid follows similar kinetics as the rate of loss of bound steroid from receptors studied from cytosol extracts. The observations are consistent with the notion that activation of the initial, reversible steroid–receptor complex generates a species with altered properties, one of which is the inability to re-bind steroid following dissociation. Although the nuclear extractable form of the receptor is isolated using 0.3 M KCl, studying the complex in solutions of 0.05 M KCl does not alter its properties in this respect. In comparison, exposing bound cytosol receptor to 0.3 M KCl at 0° for an equivalent period of time before dialyzing to low ionic strength does not affect its properties, in that reversibility of binding is still readily shown in the solution of low ionic strength.

The ionic environment of hormone receptor molecules has been shown to be of critical importance in several steroid hormone systems. A prime example is the estrogen receptor system [11]. High salt concentration promotes the dissociation of the bound hormone–receptor complex from an 8S form at low or physiological ionic strength to a 4S form, as measured by sucrose gradients. Similar findings are observed with some glucocorticoid systems [9]. In addition, more rapid inactivation of the binding component and decreased ability to bind glucocorticoids as the salt concentration is increased up to 0.4 M KCl has been observed in thymocytes [12] and liver [13]. Kalimi *et al.*, [14] have shown that hepatic cytosol contains a receptor for glucocorticoids; treatment of the bound complex at 20° converts it to an "activated" form able to bind to DNA, an effect which was enhanced by 0.15 M NaCl. All of these studies are consistent with current models for steroid hormone binding mechanisms which postulate conformational changes or other physical alterations such as loss of subunits of the complex subsequent to hormone binding [15,16].

If the binding reaction is studied *in vitro*, rather than studying the properties of steroid–receptor complex isolated from cytosol or nuclear fractions of cells exposed to [^{3}H] TA, then association rate constants and equilibrium constants can be determined. Such a study was done by Pratt and Ishii [3] and Pratt *et al.* [16], but only under isotonic salt conditions (0.15 M NaCl, plus small amounts of K^+ and Mg^{2+}). Those two studies report association rate constants of [^{3}H] TA with fibroblast receptors at 0° of 8×10^5 M^{-1} min^{-1} and 6.7×10^5 M^{-1} min^{-1}. We repeated these studies at an optimal ionic strength for stabilizing the unbound receptor, 0.05 M KCl, and obtained a similar K_a of 6.0×10^5 M^{-1} min^{-1} (data not shown). Using the binding reaction velocities and known steroid concentrations from our association rate data, a Lineweaver–Burk plot can be done and an equilibrium constant derived, 2.5×10^8 M^{-1}. The calculated dissociation constant (using the relationship $K_s = k_a/k_d$) is therefore 2.4×10^{-3} min^{-1}.

Studies done with prebound cytosol receptor in extracts of whole cells treated with [^{3}H] TA show reversibility (i.e. a chase effect) only under conditions of low ionic strength. Based on the half-life of the chase effect on bound receptors at 0° (Figs. 1, 2 or 4), a dissociation rate constant can be calculated of 1.2×10^{-4} min^{-1} (i.e. $t_{1/2} = 4$ days). This directly measured K_d is some 20-fold lower than the value calculated in the association rate experiment described above, 2.4×10^{-3} min^{-1}. Once again the data is consistent with a model that postulates a change of the initial, readily reversible steroid–receptor complex to an altered form in the cellular environment which dissociates more slowly. It seems apparent that studies of the *in vitro* binding reaction can lead to markedly different estimates of dissociation constants, depending on ionic strength and probably temperature conditions as well.

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