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The Kinetics of Specific Glucocorticoid Binding in Rat Thymus Cytosol: Evidence for the Existence of Multiple Binding States

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SUMMARY

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The 27,000 × *g* supernatant prepared from rat thymocytes contains a steroid-binding protein that binds glucocorticoids in a specific, high-affinity manner. In this study the kinetics of binding of dexamethasone has been examined in order to provide an explanation for the very slow rate at which the binding equilibrium is achieved. The second-order rate constant of association of 6×10^5 M^{-1} min^{-1} is much lower than that expected for a diffusion-limited process. Kinetic evidence is presented in support of the proposal that the apparent slow rate of binding is due to the initial rapid formation of a weak binding complex (dissociation constant = 1.75×10^{-7} M) followed by the production of a stronger complex. The first-order rate constant of formation for the second binding state is calculated to be 0.137 min^{-1}, and the measured rate constant of dissociation is 4.6×10^{-3} min^{-1}. On extended incubation a third, very tight binding state is produced.

INTRODUCTION

High-affinity glucocorticoid-binding molecules have been demonstrated in several model systems utilized for the study of the antianabolic effects of this class of steroid hormones, including fibroblasts (1), thymocytes (2), lymphoma cells in culture (3), the P1798 mouse lymphosarcoma (4), and human leukemic blast cells (5). At present there is no direct evidence demonstrating that the high-affinity binding represents specific association of the steroid with a receptor responsible for initiating the hormone response. However, there are characteristics of the binding that strongly suggest this may be the case. Extensive studies in a variety of target cells have demonstrated a good correlation between the physiological potency of many active steroids and their binding activity, e.g., in hepatoma cells in culture (6), pituitary tumor cells (7), mouse mammary tumors (8), fetal lung (9), thymocytes (10), and fibroblasts (11). One of the observations strongly supportive of the proposal that the binding may represent an association

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with the receptor is the demonstration that steroid-resistant cells often contain a very low level of binding activity (3, 11, 12). High-affinity, stereospecific binding appears, at this time, to be necessary for the hormone response, although it is clear that the presence of binding itself does not dictate that a cell will respond (13).

In the studies of glucocorticoid binding that have been conducted, it has been assumed that the binding represented a bimolecular reaction with simple kinetics described by the equation

$$S + R \rightleftarrows RS$$

where S refers to the steroid and R to the specific binding protein. In previous studies of specific glucocorticoid binding in L929 mouse fibroblasts (11) we observed that the potent drug triamcinolone acetonide bound very slowly, with a rate constant of association of 8.0×10^5 M^{-1} min^{-1} at 0°. Others have reported that the binding of dexamethasone to other specific binding proteins reaches equilibrium very slowly. Reported rate constants of association for this steroid at 0–4° range from 4×10^5 M^{-1} min^{-1} for thymocytes (10) to 4×10^6 M^{-1} min^{-1} for HTC cells (14) and mouse mammary tumor (8).

There are several possible mechanisms that lead to slow reactions of this type. In this paper we present evidence supporting a more complex kinetic model for the steroid binding reaction. We propose that the steroid associates with the soluble binding component to form a weak complex that is transformed on continued incubation to a second complex with a slow rate constant of dissociation according to the reaction equation

$$\underset{\text{fast}}{S + R \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} (RS) \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} RS'} \quad \text{slow}$$

On extended incubation an even tighter third complex is produced.

MATERIALS AND METHODS

Chemicals

[1,2,4-^{3}H]Dexamethasone[3] (10 Ci/mmole) was purchased from Schwarz/Mann. Nonradioactive dexamethasone and triamcinolone acetonide were obtained from Sigma Chemical Company.

Methods

Cell fractionation. Male Sprague-Dawley rats weighing 100 g each were bilaterally adrenalectomized by the dorsal route and maintained on 0.9% NaCl for 3–7 days before experimental use. Although we could not find the appropriate pharmacokinetic data for the rat, 3 days should be sufficient time for complete elimination of endogenous glucocorticoid. As a reference, the plasma half-life for cortisol in the human being is 80–90 min (15). Animals were killed by decapitation, and the thymus glands were immediately excised and placed in iced Earle's solution. All subsequent procedures were performed in the cold. The glands were minced with scissors and disrupted by pressing them twice through two layers of cheesecloth. The resulting suspension of intact thymocytes was centrifuged at low speed, and the cell pellet was suspended in 1.5 volume of a hypotonic solution of 0.01 M Tris buffer at pH 7.35 and 0.1 mM EDTA for 5 min and homogenized with 15 strokes of a tightly fitting pestle in a Dounce-type glass homogenizer. Both the cell membrane and the nuclei are ruptured by this procedure. After homogenization, 0.1 volume of hypertonic buffer (1.43 M NaCl, 0.11 M KCl, 0.033 M $MgCl_2$, and 0.11 M Tris, pH 7.35) was added to bring the broken cell suspension to isotonicity. The suspension was immediately centrifuged at 27,000 × g for 15 min. The 27,000 × g supernatant defines the soluble fraction of the cell as that term is employed in this paper.

Incubation with steroid and binding assay. Each 0.5-ml incubation contained the soluble fraction (400–600 μg of protein nitrogen) and radioactive dexamethasone added from a stock solution dissolved in 10% ethanol. Nonspecific binding was determined by incubating identical aliquots of the mixture with 20 μM nonradioactive triamcinolone acetonide or 50 μM dexa-

[3] The trivial names for steroids used are: dexamethasone, 9α-fluoro-16α-methyl-11β,17α,21-trihydroxypregna-1,4-diene-3,20-dione; triamcinolone acetonide, 9α-fluoro-11β,16α,17α,21-tetrahydroxypregna-1,4-diene-3,20-dione 16,17-acetonide.

methasone. The appropriate amount of vehicle solution was added to the control incubations, and the final concentration of ethanol was never more than 0.5%. The binding is not affected by concentrations of ethanol as high as 2%. All incubations were carried out in an ice bath.

The bound steroid was separated from the free compound by passage through 1 × 22 cm columns of Sephadex G-25 with an elution buffer of 0.01 M Tris (pH 7.35) and 0.04 M KCl. The flow rate was maintained such that the macromolecular peak was eluted within 6 min. The columns were run at 4°. Under these conditions it was determined that a small amount of binding takes place during the time when the sample enters the column. This binding is equivalent to a 1-min incubation in ice, and this reaction is included in the computation of data obtained in the short-time binding studies presented in this paper. One-milliliter samples were collected from the columns, and the macromolecular peak was identified by the blue color resulting from the addition of 2 drops of blue dextran (Pharmacia) to each sample prior to application to the column. The amount of bound steroid was assayed by combining the macromolecular peak fractions and determining the radioactivity content. The concentration of free dexamethasone was determined by subtracting the bound radioactivity from the total radioactivity present in each incubation.

Assays for radioactivity and protein. The macromolecular peak material (0.5 ml) was added to 10 ml of scintillation solution prepared according to Bray (16) and counted in a Packard Tri-Carb liquid scintillation spectrometer, model 3310. Appropriate quench corrections were carried out according to a quench correction curve determined with internal standard. Protein determinations were carried out according to the method of Oyama and Eagle (17), utilizing bovine serum albumin (Armour, protein standard solution) as a reference standard.

RESULTS

Time for Binding Equilibrium

Soluble fractions prepared from thymocytes obtained from normal and adrenalectomized rats were incubated at 0° with 70 nM tritium-labeled dexamethasone in the presence and absence of nonradioactive triamcinolone acetonide. The amount of steroid bound at various times was assayed (Fig. 1A). At this concentration of dexamethasone the nonspecific binding comprised 20% of the total binding, and this was subtracted to yield the specifically bound dexamethasone plotted on the ordinate. The soluble fraction from adrenalectomized animals had a slightly higher specific binding activity than that from normal rats, and all subsequent experiments were carried out with adrenalectomized preparations.

The time course of binding for subsaturating concentrations of dexamethasone is shown in Fig. 1B. At both the high and the low concentration maximum binding was achieved at about 4.5 hr at 0°. The amount of binding decreased thereafter as the bound complex became inactivated. The basis of this inactivation is not known; however, several investigators have suggested that it represents a denaturation of the binding protein and is not the result of enzymatic degradation (4, 18, 19). It has been well demonstrated (10) that the rate of inactivation of the glucocorticoid-binding component is significantly slowed when it is bound by dexamethasone. We have determined that the half-time for inactivation of the unoccupied thymocyte binding protein in this buffer system in the presence of EDTA is 4 ± 0.6 hr at 0°.[4]

Determination of Apparent Dissociation Constant

The soluble preparation was incubated at 0° for 4.5 hr with various concentrations of [^{3}H]dexamethasone at constant specific activity in the presence and absence of competing triamcinolone acetonide. At the end of the incubation the binding was determined in each sample (Fig. 2). Specific binding, determined by subtracting the nonspecific binding value from the total binding, is represented by the dashed line. The inset in Fig. 2 presents a Scatchard plot of the binding. The linear Scatchard plot suggests a single class of high-affinity

[4] C. J. Nielsen and W. B. Pratt, unpublished observations.

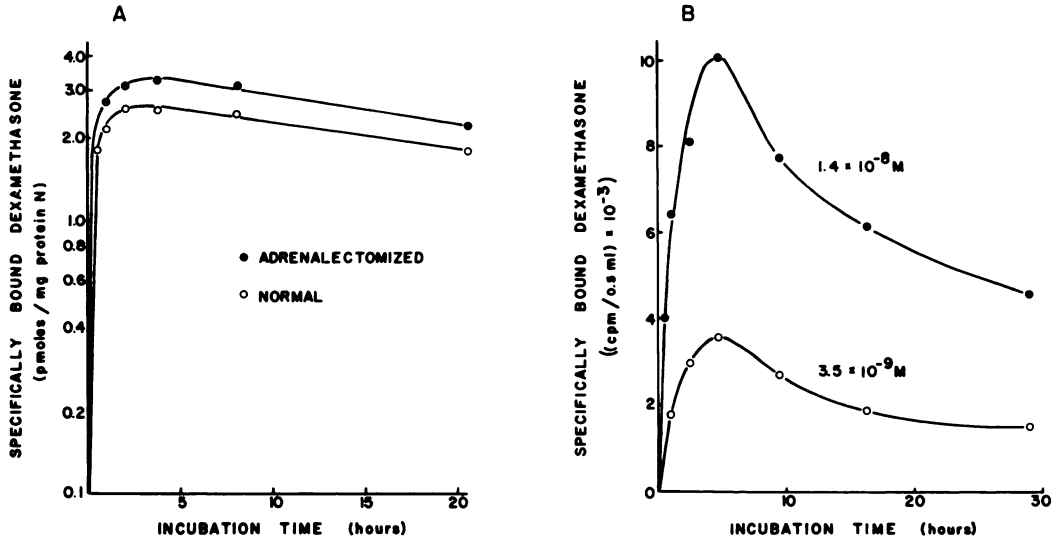

FIG. 1. *Time course of binding of dexamethasone to thymocyte binding protein*

A. Soluble fractions were prepared from normal (○) and adrenalectomized (●) rats and incubated at 0° with 70 nM [^{3}H]dexamethasone in the presence and absence of 40 μM nonradioactive triamcinolone acetonide. At the indicated times the amount of bound steroid was determined on 0.5-ml aliquots of each incubation. The nonspecific binding (binding in the presence of the unlabeled competing steroid) was subtracted from the total binding at each time point. The results are expressed as picomoles of dexamethasone specifically bound per milligram of protein nitrogen in the incubation mixture.

B. Incubation was carried out as described in Fig. 1A with subsaturating concentrations of [^{3}H]dexamethasone. The results are expressed as the counts per minute specifically bound per 0.5-ml assay. ●, 14 nM dexamethasone; ○, 3.5 nM.

binding sites. The K_D determined from one-half saturation was 8.8 nM, and from the slope of the Scatchard plot it was 13 nM. The dissociation constant determined from a Scatchard plot of a second experiment was 10 nM.

As has been pointed out by Bell and Munck (10), the determination of the dissociation constant by this approach is complicated by the fact that the unoccupied receptor is being rapidly inactivated during the time when the binding reaction takes place.

Determination of Rate Constant of Association

It has been demonstrated that the association of glucocorticoids with the binding protein follows second-order kinetics (11). In the experiment presented in Fig. 3, aliquots of the soluble preparation were incubated with various concentrations of radioactive dexamethasone for 8 min and the binding was assayed. The raw data are presented in the inset and demonstrate the linearity of binding. The total specific binding capacity of the preparation was determined by incubating the soluble fractions with a saturating concentration of [^{3}H]dexamethasone until maximum binding was achieved. The figure presents the fraction of binding component bound vs. the concentration of unbound dexamethasone. A second-order rate constant of association determined from the slope of this plot was 6.0 $\times 10^5$ M^{-1} min^{-1}. This value must necessarily be a slightly high estimate because of the inactivation of the unbound form of the binding component during the 4-hr incubation employed to assay the total amount of binding protein in the preparation.

Rates of Dissociation of Dexamethasone from Complex with Binding Protein

In order to determine the characteristics of dexamethasone dissociation from the bound complex, the soluble preparation was first incubated at 0° with 70 nM [^{3}H]dexamethasone for 4.5 hr. At that time the incubation was divided, and 50 μM

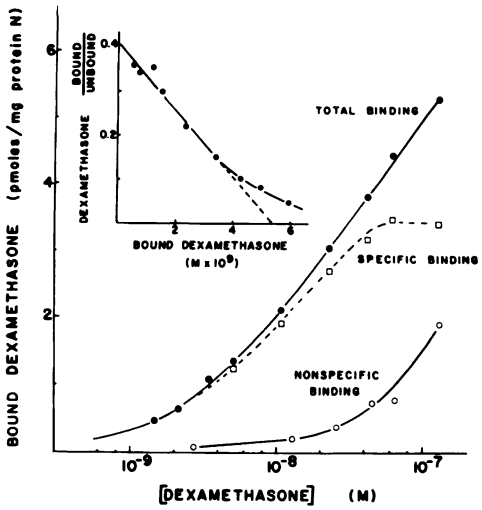

FIG. 2. *Binding of dexamethasone at various steroid concentrations*

Incubations (0.5 ml) containing soluble fraction and various concentrations of [^{3}H]dexamethasone in the presence of nonradioactive triamcinolone acetonide or vehicle were maintained at 0°, and binding was assayed after 4.5 hr. Results are expressed as picomoles of dexamethasone bound per milligram of protein nitrogen as a function of concentration of the radioactive steroid. ●, binding in the presence of vehicle; ○, binding in the presence of triamcinolone acetonide; □, specific binding (- - -). The inset presents a Scatchard plot of binding in the presence of vehicle. The straight line was determined by the method of least squares.

nonradioactive dexamethasone or vehicle was added to each portion. The effect of this chase is shown in Fig. 4. Initially there was a rapid decrease in binding, followed by a very slow component. The line described by solid squares presents the values for the rapid dissociation after correction for the contribution of the slow component. This line gives $t_{\frac{1}{2}}$ = 150 min and a rate constant of dissociation of 4.6×10^{-3} min^{-1}.

This biphasic curve for dissociation suggested that the formation of the complex with a slow dissociation rate might be time-dependent. Accordingly, the soluble preparation was incubated for 14 hr in the presence of radioactive dexamethasone, and a portion was chased with a 700-fold excess of the nonradioactive drug. As can be seen from the data presented in Fig. 5, there was no measurable chase after 14 hr of incubation with radioactive dexamethasone. This does not mean that there was no dissociation. With this rate of inactivation of the control binding we could not accurately detect a rate of dissociation less than 1×10^{-4} min^{-1}.

The dissociation constant calculated from the second-order rate constant of association and the rapid dissociation observed in Fig. 4 equals 0.8×10^{-8} M. This is in close agreement with the average of the two Scatchard determinations, 1.15×10^{-8} M. The tight binding state demonstrated in Figs. 4 and 5 is not reflected by the dissociation constant determined by Scatchard analysis of equilibrium binding, because the rate of inactivation of the un-

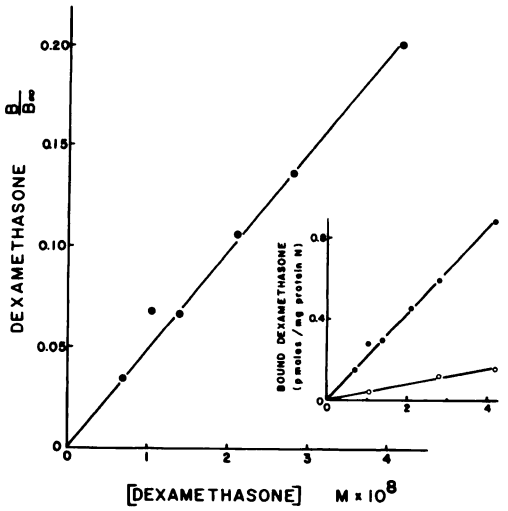

FIG. 3. *Determination of second-order rate constant of association for dexamethasone*

Incubation mixtures (0.5 ml) were prepared as described in the legend to Fig. 2 and incubated for 8 min at 0°. The inset presents the raw data: ●, binding values determined for incubations in the presence of vehicle; ○, binding in the presence of nonradioactive triamcinolone acetonide. The principal graph represents the fraction of binding protein occupied during the 8-min incubation as a function of the free concentration of dexamethasone. The amount of binding protein present was determined by incubating the soluble preparation with a saturating concentration of [^{3}H]dexamethasone in the presence and absence of triamcinolone acetonide until maximum binding was achieved. The maximum specific binding is represented by the expression B_∞. The units for the abscissa of the inset are the same as those for the principal graph.

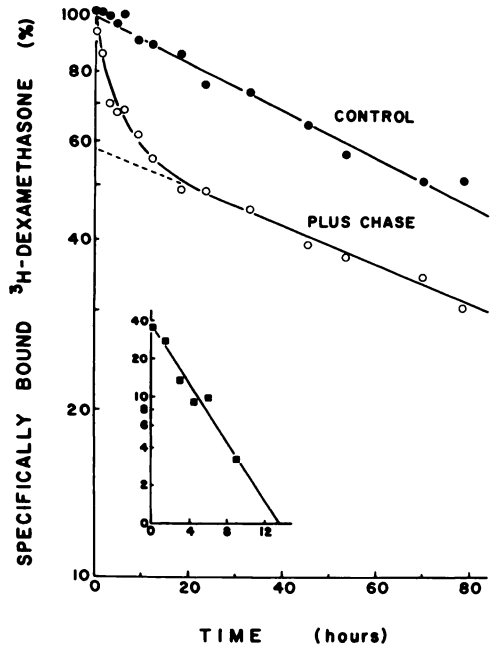

FIG. 4. *Dissociation of dexamethasone from binding protein–steroid complex after 4.5 hr of incubation*

The soluble preparation was incubated with 70 nM [³H]dexamethasone for 4.5 hr at 0°. At this time the incubation was divided in half, and nonradioactive dexamethasone (final concentration, 50 μM) or vehicle was added to each half. The amount of binding was determined on 0.4-ml aliquots of each divided incubation at the indicated times. Nonspecific binding was determined on samples preincubated for 4.5 hr in the presence of a high concentration of nonradioactive dexamethasone. Each value represents the average of duplicate determinations. A linear regression line was determined for specific binding in the samples containing vehicle. The values are percentages of the intercept of the regression line at the time of chase addition. Control (●); plus chase (○). A regression determined for the slow phase of the chase curve was extrapolated to zero time, and the line in the inset (■) represents the data for the rapid component after correction for the slow component.

bound binding protein is fast with respect to the rate of formation of the very tight complex at 0°.

Kinetics of Binding at High Steroid Concentration

The slow rate of formation of the binding that we observed by our column assay procedure is consistent with the suggestion that the steroid may form an even weaker initial complex with the binding protein. We could not demonstrate such a weak interaction directly, as it would dissociate too rapidly to survive the column assay. However, the possibility that an initial rapid weak reaction might exist could be examined by kinetic methods. The model suggested is as follows:

$$\begin{matrix} & \text{fast} & & \text{slow} & & \text{very slow} \\ R + S & \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} & (RS) & \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} & RS' & \underset{k_{-3}}{\overset{k_3}{\rightleftarrows}} & RS'' \end{matrix} \qquad (1)$$

where (RS) represents an initial weak complex rapidly formed between the steroid and the binding protein, and RS'' the tight complex that is formed very slowly. As the third equilibrium product is produced very slowly, its contribution to the binding observed in incubations of only a few minutes can be ignored.

Figure 6 presents two short-term binding curves obtained at dexamethasone concentrations much higher than that at which receptor saturation is reached on long-term incubation (e.g., Fig. 2). If the formation of the RS' complex were the

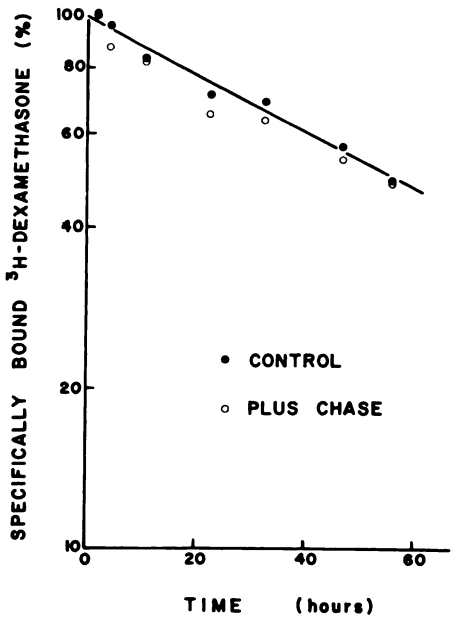

FIG. 5. *Effect of chasing after 14 hr of incubation with radioactive dexamethasone*

See the legend to Fig. 4 for experimental methods.

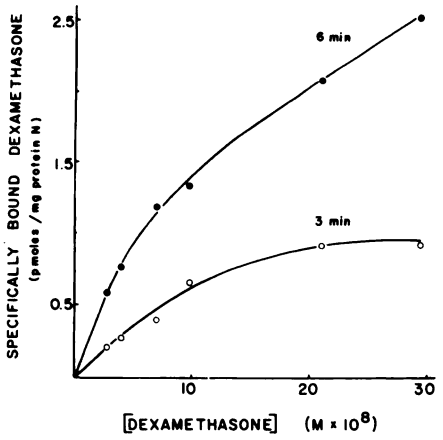

FIG. 6. *Short-time course of binding at very high dexamethasone concentrations*

Incubations (0.5 ml) were carried out with various concentrations of [^{3}H]dexamethasone in the presence and absence of 50 μM nonradioactive dexamethasone for 3 min (○) and 6 min (●) at 0°. The specific binding was determined for each dexamethasone concentration and is presented as picomoles of specifically bound dexamethasone per milligram of protein nitrogen.

result of a simple, single-stage interaction expressed by the equation

$$R + S \rightleftarrows RS' \qquad (2)$$

where S represents the steroid, R the specific binding protein, and RS' the bound complex that we observed after Sephadex G-25 chromatography, then in a short-time course of binding one would expect the binding curve to turn over at very high concentrations of steroid as the binding protein became occupied. In this experiment the total amount of binding protein was determined as described for the experiment of Fig. 3. At the highest point of the 6-min curve the binding protein is 59% occupied. Both curves begin to turn over at 10^{-7} M, and in the 3-min incubation the binding protein is less than 20% occupied at this concentration. If the binding reaction represented a single-step equilibrium, the curve described by the shorter time of incubation should turn over at the same percentage of receptor occupancy as the 6-min curve.

As the 6-min incubation resulted in a curve that achieved approximately twice the binding generated in the 3-min incubation, the rate of binding is linear at each concentration of steroid. According to the two-step model the rate of production of RS' from RS would be determined by the proportion of binding protein that is in the RS form and the rate constant of formation of RS'. This relationship may be expressed by the equation

$$V_i = \left[\frac{[S]}{[S] + K_{Df}} \cdot R_t\right] \cdot k_2 \qquad (3)$$

where V_i represents initial velocity of binding, $[S]$ the free dexamethasone concentration, K_{Df} the dissociation constant for the fast reaction, R_t the total amount of specific binding protein, and k_2 the rate constant of association for the second interaction. In reciprocal form Eq. 3 becomes

$$\frac{1}{V_i} = \frac{1}{k_2} \cdot \frac{[S] + K_{Df}}{R_t \cdot [S]} \qquad (4)$$

and this may be rearranged to

$$\frac{1}{V_i} = \frac{1}{k_2 \cdot R_t} + \frac{K_{Df}}{k_2 \cdot R_t \cdot [S]} \qquad (5)$$

As the maximum rate of formation of RS' from RS is achieved at very high steroid concentrations when all of R is rapidly bound in the RS form, the expression $k_2 \cdot R_t$ is equal to V_{max}. Thus Eq. 5 is equivalent to the Lineweaver-Burk equation, where K_{Df} is equivalent to K_m. A plot of $1/V_i$ vs. $1/[S]$ will yield a line with a slope of $K_{Df}/k_2 \cdot R_t$, where the intercept on the abscissa equals $-1/K_{Df}$. By this analysis, if the binding values achieved at each concentration of dexamethasone in Fig. 6 are reduced to a rate (picomoles specifically bound per minute) and plotted as reciprocal of the binding rate vs. reciprocal of dexamethasone concentration, the intercept on the abscissa should yield a valid estimation of the dissociation constant for the weak interaction. This plot is presented in Fig. 7, and the dissociation constant for the fast reaction calculated from the intercept on the abscissa is 1.75×10^{-7} M.

Calculation of Kinetic Constants for Conversion of RS to RS'

At infinite steroid concentration all of the binding protein would be occupied in the weak form and the maximum velocity of observed binding would be equal to $k_2 \cdot R_t$. As we have measured R_t and as V_{max}

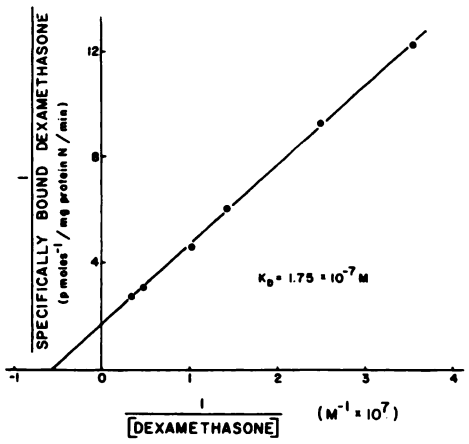

FIG. 7. *Estimation of dissociation constant for weak initial binding*

Each of the binding values presented in Fig. 6 was converted to picomoles bound per minute. The resulting binding rates at each dexamethasone concentration were averaged and are presented here as the reciprocal of the binding rate, in picomoles per milligram of protein nitrogen per minute, vs. the reciprocal of the free dexamethasone concentration. See the text for further details.

can be determined from 1/*y* intercept, we can calculate a first-order rate constant of association for the conversion of the initial weak interaction to a second complex, *RS'*, with slower dissociation. $k_2 = 0.137$ min^{-1}.

The apparent dissociation constant for the binding observed in Fig. 2 (K_D) may be expressed as

$$K_D = \frac{[R]\,[S]}{[RS] + [RS']} \tag{6}$$

and, converting to a reciprocal expression,

$$\frac{1}{K_D} = \frac{[RS]}{[R]\,[S]} + \frac{[RS']}{[R]\,[S]} \tag{7}$$

The association constant for the second reaction (K_{As}) is equal to RS'/RS. Since $[RS'] = K_{As}\,[RS]$, Eq. 7 may be expressed as

$$\frac{1}{K_D} = \frac{[RS]}{[R]\,[S]} + \frac{K_{As}\,[RS]}{[R]\,[S]} \tag{8}$$

Therefore

$$\frac{1}{K_D} = \frac{1}{K_{Df}} + \frac{K_{As}}{K_{Df}} \tag{9}$$

As we know K_D from the Scatchard plots and K_{Df} from the double-reciprocal plot, we can determine the association constant for the second stage of the binding, $K_{As} = 10.7$. As we have an estimate of k_2, from

$$K_{As} = \frac{k_2}{k_{-2}} \tag{10}$$

we can calculate that k_{-2} equals 1.28×10^{-2} min^{-1}. This value is about 2.5 times the measured dissociation constant, and it would determine a dissociation half-time of 54 min as compared to 150 min calculated from the inset in Fig. 4. These values are reasonably close, given the methods involved. Indeed, when K_{As} is determined utilizing the apparent K_D determined by half-saturation (Fig. 2), we calculate a k_{-2} of 7×10^{-3} min^{-1}.

DISCUSSION

The studies of the kinetics of steroid binding in thymic lymphocytes have been carried out in crude preparations at low temperature. The work of Bell and Munck (10) was performed at 0–4° in a cytoplasmic fraction that was prepared by centrifuging ruptured cells at 1500 × *g* for 3 min. The work of Schaumburg (18) was carried out at −5° in a 100,000 × *g* supernatant in which the binding component was partially stabilized with 40% glycerol. The studies presented in this paper utilized a 27,000 × *g* supernatant that clearly contained particulate material. As noted previously, the specific binding capacity of the thymocyte preparation decreases rather rapidly with time ($t_{\frac{1}{2}}$ = 4 hr at 0°). Accordingly, we elected to employ the 27,000 × *g* supernatant, which takes 15 min to prepare, rather than the 100,000 × *g* fraction, which requires an additional hour of centrifugation accompanied by a 20–30% loss of specific binding capacity. As the same slow binding is observed in the high-speed supernatant (18), and as we observed the same biphasic dissociation curve in the 100,000 × *g* thymocyte supernatant as in the 27,000 × *g* preparation, it is unlikely that the different binding states presented in this paper are due to inhomogeneous compartmentalization of the receptor. However, it is entirely possible that the specific steroid-binding component may interact with soluble components of the prep-

aration. Such interactions could explain the complex binding kinetics, and this uncertainty places limits on the interpretations we have made in this paper.

We would like to have performed these binding studies with a preparation that was at least partially purified with respect to the specific binding molecule. We could not, however, obtain any purification of the unoccupied receptor beyond the 100,000 × *g* supernatant. When the 100,000 × *g* supernatant is passed through Sephadex G-100 or adsorbed to an ion-exchange column or precipitated with ammonium sulfate, all specific binding activity is lost. This same observation is true in the L929 mouse fibroblast, where we have achieved a 2100-fold purification of the triamcinolone acetonide-bound receptor (20), but we have been unable to achieve any purification of the unbound receptor. It would also be helpful if we could study the effects of temperature changes on the kinetics of binding. However, the specific binding capacity of either the 27,000 × *g* or the 100,000 × *g* supernatant is very rapidly inactivated at higher temperatures. For example, the $t_{\frac{1}{2}}$ for inactivation of the unoccupied thymocyte receptor at 25° is only 8 min.[4]

The slow rate at which the binding between various glucocorticoids and soluble binding proteins attains equilibrium at 0° is not a general observation that pertains to association of all steroids with intracellular binding proteins. Reported association constants for estradiol with uterine cytosol-binding proteins are approximately two orders of magnitude higher at 0°, and are close to the appropriate range for a diffusion-limited process (21). The glucocorticoid binding reaction may, for some reason, not be diffusion-limited. For example, as Rousseau, Baxter, and Tomkins (6) have suggested, the binding protein could exist in several conformational states, and at any time only a small fraction of it might exist in a form capable of binding the steroid. Alternatively, there might be a weak, diffusion-limited interaction (not observed on direct binding assay) that precedes formation of the tighter complex that we observed. The type of experiment presented in Figs. 6 and 7 suggests that the latter proposal may be correct, although it does not entirely rule out all other possibilities. By this analysis the rate of conversion of RS to RS' would be the slow step in the production of observable binding.

The second-order rate constant of association for several steroids at 0° was measured by Bell and Munck (10). They determined that steroids of widely different potencies all have similar slow rates of association with the soluble rat thymus binding protein. We confirmed this observation with respect to the binding of cortisol, dexamethasone, and triamcinolone acetonide in the 105,000 × *g* supernatant fraction of L929 mouse fibroblasts (22). We also determined, by the same method used in this paper, that all these steroids may initially interact with the L-cell-binding protein in a weak manner. In the high-speed supernatant from L-cells, the apparent dissociation constant for the weak interaction is the same ($0.9–2 \times 10^{-7}$ M) both for cortisol and for the very potent compounds dexamethasone and triamcinolone acetonide (22).

The observation that the dexamethasone binding that we could directly assay has two rates of dissociation is not unique to this system. It is well known that the uterine cytosol estradiol-protein binding complex has a biphasic dissociation curve (21, 23). Biphasic steroid dissociation kinetics has not to our knowledge been reported for glucocorticoid-binding proteins. This may be because the chase experiments done previously were performed after different periods of incubation with the steroid. As presented in Table 1, Schaumburg (18) published a slow rate constant of dissociation for dexamethasone determined by addition of nonradioactive chase after 2–3 days of incubation with radioactive dexamethasone. Bell and Munck (10) reported a more rapid dissociation constant determined by chasing after 1.25 hr of binding. It is not possible from our data to say that we observed only two dissociation rates (Fig. 4), but that possibility is suggested by the straight line obtained after subtraction of the slow component. It is also clear from Table 1 that the three laboratories determined similar, slow rate constants of association.

TABLE 1

Comparison of kinetic constants for dexamethasone binding to soluble glucocorticoid-binding protein from rat thymocytes

Kinetic data determined in two other laboratories and those presented in this paper are compared. The work of Schaumburg (18) was carried out on a soluble preparation stabilized by 40% glycerol at −5°, and the work of Bell and Munck (10) was done at 0–4°. The times noted in parentheses under the dissociation constant refer to the time of incubation with [^{3}H]dexamethasone prior to binding assay, and those under the dissociation rate constants, the incubation time prior to addition of chase.

Reference	K_D	Association rate constant	Dissociation rate constant
	M	$M^{-1}\ min^{-1}$	min^{-1}
Schaumburg (18)	1.9×10^{-9} (6 days)	2.8×10^5	3.7×10^{-4} (2–3 days)
Bell and Munck (10)	1.5×10^{-8}[a] (1.25 hr)	4×10^5	3.1×10^{-3} (1.25 hr)
This paper	1.15×10^{-8} (4.5 hr)	6×10^5	4.6×10^{-3} (4.5 hr) $< 1 \times 10^{-4}$ (14 hr)

[a] Determined by a competitive assay procedure.

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