Specific Binding of Glucocorticoids *in Vitro* in the Soluble Fraction of Mouse Fibroblasts†

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ABSTRACT: The binding of triamcinolone acetonide has been studied in the 105,000*g* supernatant from mouse fibroblasts. Triamcinolone acetonide binds in a specific manner in the 105,000*g* supernatant at 0° to the same extent as in the intact cell. The binding reaction for triamcinolone acetonide has a rate constant of association at 0° of 8.0×10^5 M^{-1} min^{-1}. The binding of both triamcinolone acetonide and the less potent glucoorticoids, dexamethasone and 11β-cortisol, is irreversible at 0°. The soluble fraction from steroid-resistant cells contains only 10% as much of the specific binding component as sensitive cells. The bound complex is degraded slowly at 0° and rapidly at 37°. At 0° the bound complex degrades at a slower rate than the unbound binding component.

Like thymocytes and lymphocytes, fibroblasts are target cells for the antianabolic action of glucocorticoid steroids. In the presence of glucocorticoids, the rate of replication of mouse fibroblasts growing *in vitro* is markedly reduced. We have previously reported the identification of a binding component in strain L 929 mouse fibroblasts which binds triamcinolone acetonide in a manner which is specific for the growth inhibition effect (Hackney *et al.*, 1970). The specific nature of the steroid binding was inferred from the following three experimental observations. (1) Active glucocorticoids competed for the association of radioactive triamcinolone acetonide with the binding component in a manner which reflected their acivity as inhibitors of cell growth. Steroids which do not inhibit cell growth did not inhibit the binding. (2) The binding component saturated at the concentration of triamcinolone acetonide which produced a maximal growth inhibitory response. (3) In a resistant subline of L 929 cells, in which replication is not inhibited by high concentrations (5×10^{-5} M) of potent glucocorticoids, triamcinolone acetonide was bound to the high-affinity-binding component only 10–15% of the extent observed with sensitive cells.

The glucocorticoid binding component is recovered largely in the 105,000*g* supernatant of cells ruptured by hypotonic lysis. The binding complex has an apparent molecular weight of 620,000 as determined by filtration through Sephadex G-200 (Hackney and Pratt, 1971). The exact chemical composition of the binding component is unknown, but it would appear to contain both protein and phospholipid. This is inferred from the fact that the sedimentation coefficient of 5.5 S is considerably smaller than would be expected for a globular protein with an apparent molecular weight of 620,000 as determined by gel filtration and the fact that the binding is sensitive to digestion with phospholipases as well as Pronase. A 2100-fold purification of the binding complex has been achieved, however the yield in such extensive purification is too low (due to extensive inactivation) to permit a great deal of physical characterization of the receptor.

These studies all involved preliminary incubation of intact cells with tritium-labeled triamcinolone acetonide, and subsequent lysis of the cell. We were unable to observe *in vitro* binding by incubating radioactive steroid with the soluble fraction which had previously been submitted to any purification procedures. However, binding could be detected *in vitro* using a crude cytosol fraction, and we have therefore carried out the present study which is concerned with the conditions for and kinetics of specific *in vitro* association of triamcinolone acetonide to the binding component in the soluble fraction from mouse fibroblasts.

Materials and Methods

Materials. Triamcinolone acetonide-*1,2,4-t* (20 Ci/mmole)[1] was purchased from Schwarz BioResearch Inc., Orangeburg, New York. Dexamethasone and fluocinolone acetonide were gifts from Dr. Ralph Dorfman of Syntex Corp. 11α-Cortisol was donated by the Squibb Institute for Medical Research. 11β-Cortisol and nonradioactive triamcinolone acetonide were purchased from Calbiochem, Los Angeles, Calif., and Sigma Chemical Co., St. Louis, Mo., respectively.

Cell Culture. Suspension cultures of L 929 cells were maintained in basal medium (Eagle, 1955) modified as described previously (Gray *et al.*, 1971). Cultures were maintained at 37° with constant stirring in an atmosphere of humidified air. The glucocorticoid-resistant line of L 929 cells was selected and cloned as described by Hackney and Pratt (1971). Dose response experiments for growth inhibition were carried out on monolayer cultures of mouse fibroblasts by procedures described by Pratt and Aronow (1966).

Cell Fractionation. Cells were harvested from suspension culture by centrifugation at 600*g* for 10 min in a refrigerated centrifuge and washed twice by resuspension in four to six

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[1] The trivial names for steroids used are: triamcinolone acetonide, 9α-fluoro-11β,16α,17α,21-tetrahydroxypregna-1,4-diene-3,20-dione 16,17-acetonide; fluocinolone acetonide, 6α,9α-difluoro-11β,16α,17α,21-tetrahydroxypregna-1,4-diene-3,20-dione 16,17-acetonide; dexamethasone, 9α-fluoro-16α-methyl-11β,17α,21-trihydroxypregna-1,4-diene-3,20-dione. 11α-Cortisol and 11β-cortisol refer to 11α,17α,21-trihydroxypregn-4-ene-3,20-dione and 11β,17α,21-trihydroxypregn-4-ene-3,20-dione, respectively.

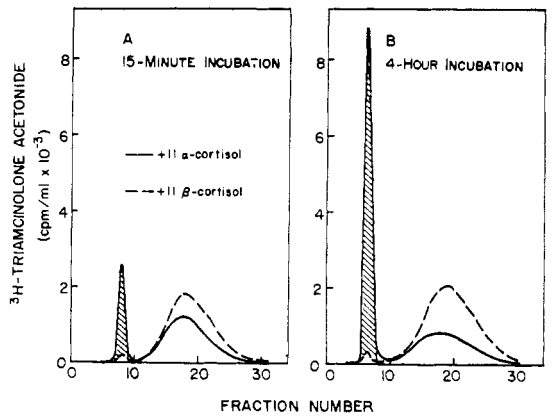

FIGURE 1: Binding of triamcinolone acetonide-*t* in the presence of nonradioactive 11α- or 11β-cortisol in a broken cell preparation of mouse fibroblasts. Washed L cells were ruptured by hypotonic lysis with Dounce homogenization as described in Materials and Methods. The ruptured cell suspension containing 2.6 mg of protein N/ml was incubated at 0° for 15 min (*A*) or 4 hr (B) with 10^{-8} M triamcinolone acetonide-*t* in the presence of 2×10^{-5} M 11α-cortisol (solid line) or 11β-cortisol (dotted line). At the end of the incubation the samples were centrifuged for 3 min at 3000*g* and 1.0 ml of the supernatant was filtered on a small column of Sephadex G-25. The area marked by the slanted lines represents the binding of triamcinolone acetonide-*t* which is competed for by 11β-cortisol but not by 11α-cortisol.

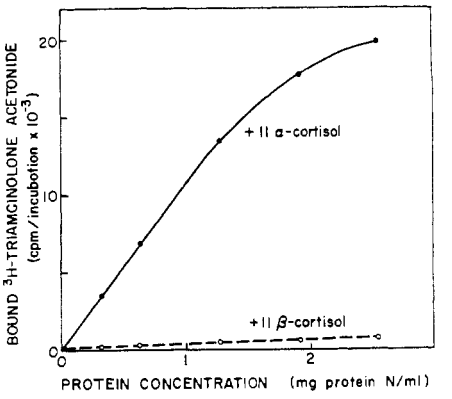

FIGURE 2: Binding of triamcinolone acetonide-*t* (10^{-8} M) in the 105,000*g* supernatant from L cells with increasing concentrations of protein. Aliquots (0.5 ml) of 105,000*g* supernatant containing various concentrations of protein N were incubated with triamcinolone acetonide-*t* (10^{-8} M) and either 11α- or 11β-cortisol at 2×10^{-5} M for 4 hr at 0° and the bound radioactivity in each incubation was assayed.

volumes of cold Earle's saline and centrifugation at 600*g*. The washed cells were suspended in 1.5 volumes 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 tight-fitting pestle in a Dounce-type glass homogenizer. After homogenization, exactly one-tenth 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 broken cell suspension was centrifuged at 600*g* for 10 min, and the supernatant was centrifuged at 10,000*g* for an additional 10 min. The 105,000*g* supernatant defines the soluble fraction of the cell as that term is employed in this paper. In some lengthy experiments sodium azide was added to the 105,000*g* supernatant at a final concentration of 0.02% (w/v) in order to eliminate the chance of bacterial contamination during the course of the experiment.

Incubation with Steroid and Binding Assay. In most of the experiments the soluble fraction was incubated with radioactive triamcinolone acetonide at 10^{-8} M, 20 Ci/mmole. Normally, radioactive steroid was added in a solution of 10% ethanol in water, at a volume of $^1/_{20}$th of the incubation volume. Nonradioactive steroids were added in a 10% solution of ethanol at $^1/_{100}$th of the incubation volume. Control experiments demonstrated that the amount of specific binding of radioactive triamcinolone acetonide was unaffected by a final concentration of ethanol up to 4% of the incubation volume. This is more than four times the highest concentration of ethanol achieved in any of the experiments presented. The incubations were carried out in an ice bath except as noted in the individual descriptions of the experiment. The bound steroid was separated from the free compound by passage through 1×25 cm columns of Sephadex G-25 with an elution buffer of 0.01 M Tris-0.04 M KCl (pH 7.35). The elution period is 6–8 min from the time a 0.5-ml sample is placed on the column until the bound sample is collected. All assays were carried out at 4°. Fractions of approximately 1 ml were collected from the columns, and the macromolecular peak was identified by optical density. The amount of bound steroid was assayed by combining the macromolecular peak fractions and determining the radioactivity content.

Assays for Radioactivity and Protein. The aliquot to be assayed was added to 10 ml of scintillation solution prepared according to Bray (1960), and counted in a Packard Tri-Carb liquid scintillation spectrometer, Model 3310. Appropriate quench corrections were carried out using internal standards where applicable. Protein determinations were carried out according to the method of Oyama and Eagle (1956).

Results

Assay System. A ruptured L cell suspension was incubated with 10^{-8} M tritium-labeled triamcinolone acetonide in the presence of 11α-cortisol or 11β-cortisol. Aliquots (1.0 ml) of the supernatant were passed through a column of Sephadex G-25, and the radioactivity elution patterns determined (Figure 1). 11β-Cortisol is an active growth-inhibitory glucocorticoid, while 11α-cortisol, structurally different only in the orientation of the hydroxyl group at position 11, is inactive as a growth inhibitor. 11β-Cortisol competes for binding to specific receptors whereas the 11α compound is inactive (Hackney *et al.*, 1970). The difference between the binding observed in the presence of the active and inactive cortisol isomers (Figure 1, area marked by the slanted lines) represents binding specific for the growth inhibitory effect. The specific binding increases with time whereas the nonspecific binding (dotted line in fractions 7–9) is complete by 15 min. The macromolecular peak was eluted from this column in fractions 7–9. In the rest of the experiments to be presented these macromolecular fractions are combined and the binding values are expressed in terms of total bound radioactivity or in terms of specific activity on the basis of protein N. There is essentially no contamination of bound radioactivity with free steroid when the binding is assayed by this method.

The amount of triamcinolone acetonide bound to the complex at 4 hr increases linearly with respect to the concentration of 105,000*g* fraction until approximately 1.5 mg of protein N is present per ml of incubation (Figure 2). The binding curve turns over because the free steroid concentration is reduced and eventually becomes limiting at high binding site concentrations. For example, in the sample containing 2.5 mg of

TABLE I: Relative Amount of Binding After 4 hr at 0° in the Intact Cell, in a Broken Cell Preparation, and in a 105,000*g* Supernatant.[a]

Cell Fraction	Sp Act. of Binding (cpm/μg of Protein N)[b]	
	Plus 11α-Cortisol	Plus 11β-Cortisol
Intact cells	19.2	0.7
Broken cells[c]	14.9	0.5
105,000*g* supernatant[c]	19.1	0.8

[a] For incubation of intact cells, washed L cells were suspended in replicate in medium 199 with 25 mM Hepes buffer at a cell density of 4×10^6 cells/ml and incubated for 4 hr at 0° with 10^{-8} M triamcinolone acetonide-*t* and either 11α- or 11β-cortisol at 2×10^{-5} M. At the end of the incubation each 2.0 ml incubation was centrifuged at 2500*g*, the supernatant was discarded, and the cells were ruptured by lysis in hypotonic Tris buffer containing the same steroids. The ruptured cells were centrifuged at 10,000*g* for 10 min and the supernatant was filtered on small Sephadex G-25 columns. A broken cell preparation and a 105,000*g* supernatant were prepared from washed L cells in the usual manner and portions were incubated at 0° for 4 hr with the same steroids as above. The bound radioactivity was determined in the 10,000*g* supernatant of the broken cell preparation. [b] The specific activity of the intact cell and broken cell incubations has been corrected for the protein N which is contributed to the 10,000*g* supernatant by the 105,000*g* pellet. Each value represents the average of duplicate incubations. [c] The amount of broken cell or soluble fraction is equivalent to that derived from the number of cells present in the incubation of intact cells. The soluble fraction contained 1.8 mg of protein N/ml of incubation.

protein N per ml, approximately 30% of the total drug is in the bound form and its free concentration is reduced to approximately 7×10^{-9} M.

As shown in Figure 3, maximal association of triamcinolone acetonide with the binding complex takes place between pH 6.9 and 7.4. The binding of steroid seen in the presence of 11β-cortisol is unaltered by pH in the range studied.

Effect of Temperature on Specific Binding in the Soluble Fraction. A 105,000*g* supernatant was prepared and incubated at three temperatures with 10^{-8} M triamcinolone acetonide in the presence of 2×10^{-5} M 11α-cortisol or 11β-cortisol and the specific binding was determined at various times (Figure 4). Although the rate of association is slower at the lower temperatures, the total amount of binding recovered at the later time intervals is much greater at 0° than at 22 or 37°. Rapid loss of binding was observed at the higher temperature; for this reason the rest of the experiments presented in this paper were carried out in an ice bath.

Efficiency of Binding at 0° in the 105,000g Supernatant Compared to that in Intact Cells. As presented in Table I, the 105,000*g* supernatant *in vitro* binds the same amount of radioactive triamcinolone acetonide as the 105,000*g* supernatant prepared from cells which were incubated with steroid while intact. This is not the case if the binding in the soluble fraction and the intact cell are carried out at 37°. The steroid equili-

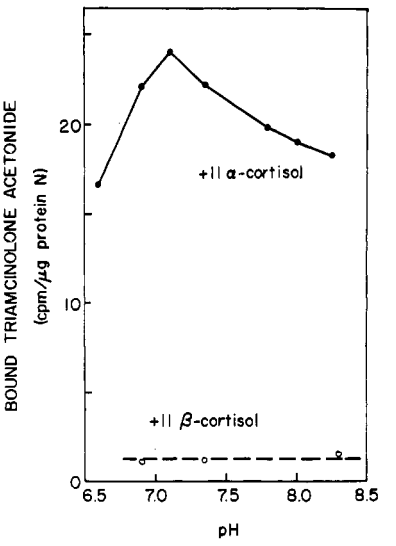

FIGURE 3: Alteration of specific binding of triamcinolone acetonide-*t* in the soluble fraction of mouse fibroblasts with varying pH. Samples of a 105,000*g* supernatant (0.5 ml), each containing 575 μg of protein N, were incubated with triamcinolone acetonide-*t* (10^{-8} M) and either 11α-cortisol or 11β-cortisol at 2×10^{-5} M for 3 hr at 0°. The binding in each fraction was then assayed by passage through a Sephadex G-25 column.

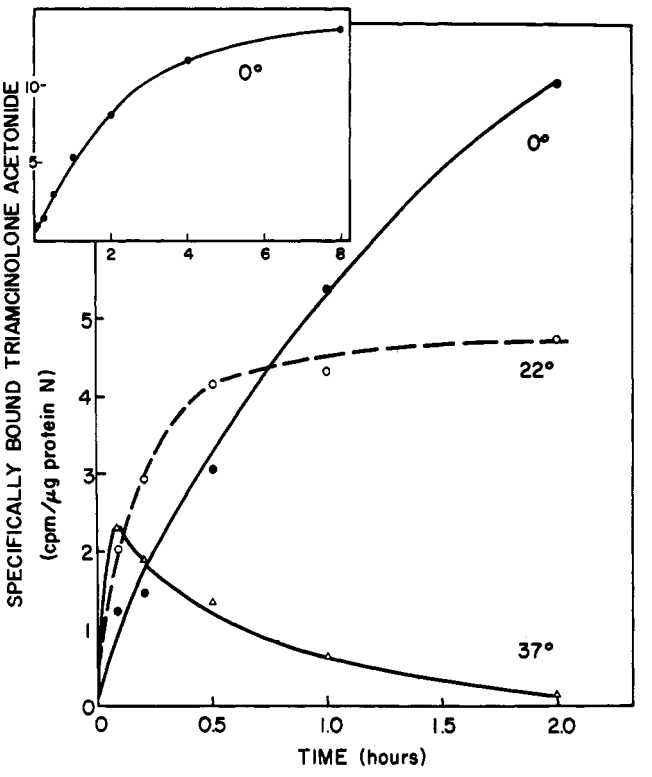

FIGURE 4: Time and temperature dependence of specific binding of triamcinolone acetonide in the soluble fraction of mouse fibroblasts. A 105,000*g* supernatant fraction was prepared in the Tris buffer (pH 7.35) containing 50 mM Hepes buffer. The supernatant was incubated at the indicated temperatures with 10^{-8} M triamcinolone acetonide-*t* and 2×10^{-5} M nonradioactive 11α- or 11β-cortisol. At the indicated times, 0.5-ml aliquots were removed from the incubations and the amount of bound radioactivity was determined by passage through the Sephadex G-25 columns. The values in the figure represent the cpm bound per μg of protein N minus the binding in an identical 1-hr incubation at each temperature in the presence of 2×10^{-5} M 11β-cortisol.

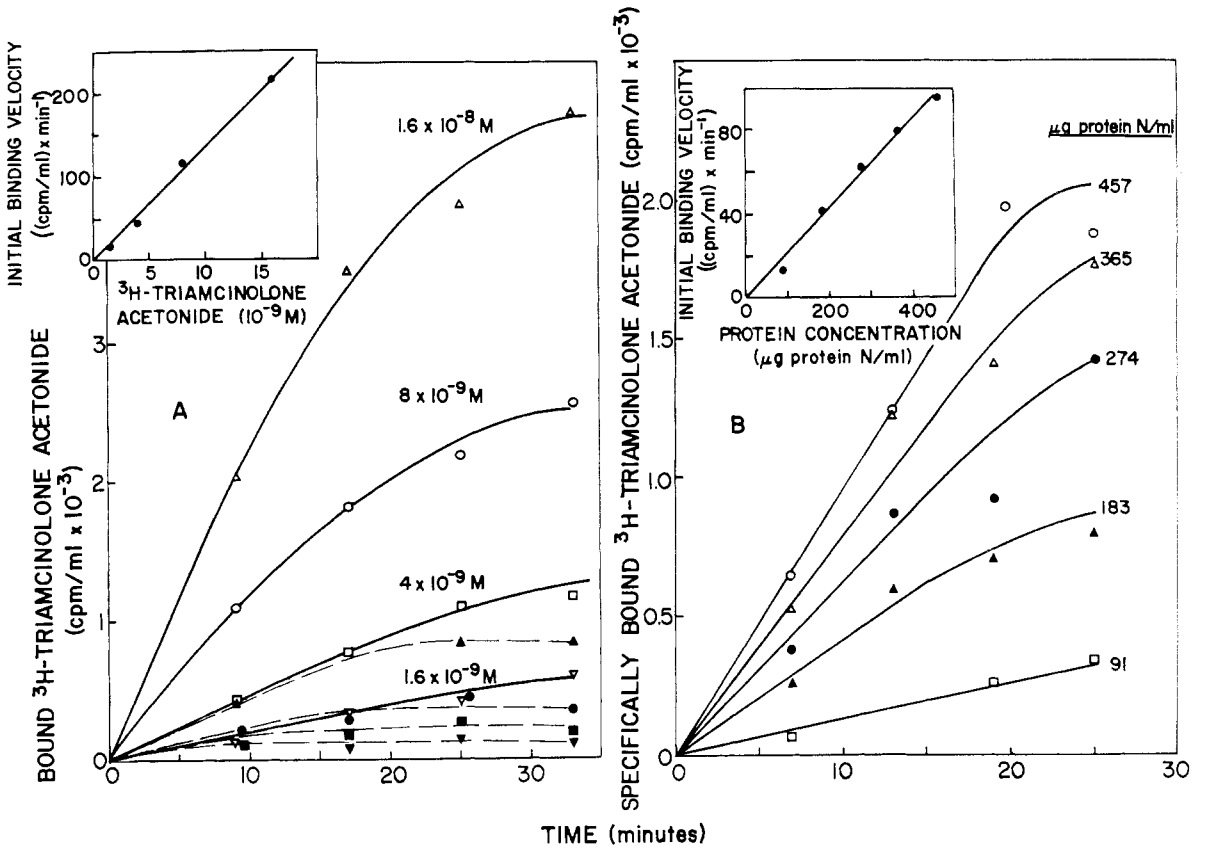

FIGURE 5: Rate of association of triamcinolone acetonide with the binding component in the soluble fraction at 0°. (A) The dependence of the rate of association on triamcinolone acetonide concentration. Replicate aliquots of a 105,000*g* supernatant fraction from L cells were incubated with varying concentrations of triamcinolone acetonide-*t* in the presence of 2×10^{-5} M 11α- or 11β-cortisol. At the indicated times, 1.0-ml aliquots were assayed for bound radioactivity in the usual manner. The total amount of binding component was determined by continuing the incubations containing 1.6×10^{-8} M triamcinolone acetonide for 24 hr. At this point the binding was assayed. The incubations contained 478 μg of protein N/ml. The values in the figure present the cpm bound per ml of incubation. The open symbols connected by the solid line represent the binding in the presence of 11α-cortisol. The solid symbols connected by dashed lines represent the binding in the presence of 11β-cortisol. (△) 1.6×10^{-8} M triamcinolone acetonide. (○) 8.0×10^{-9} M, (□) 4.0×10^{-9} M, (▽) 1.6×10^{-9} M. The inset presents the initial velocity of specific binding plotted against steroid concentration. The specific binding was determined by subtracting the binding found in the presence of 11β-cortisol from that found in the presence of 11α-cortisol. (B) The dependence of the rate of association on protein concentration. The 105,000*g* supernatant was diluted with reconstituted hypotonic–hypertonic buffer to yield several samples of different protein concentration. Binding assays were performed at various times as in part A, after incubation with 8×10^{-9} M triamcinolone acetonide-*t*. Each value represents the amount of specific binding determined as in part A in terms of cpm per ml of incubation. The concentration of protein in each incubation in terms of μg of protein N per ml are as follows: (○) 457, (△) 365, (●) 274, (▲) 183, and (□) 91. The inset presents the initial velocity of binding plotted against the concentration of protein N per ml of incubation.

brates with the intact cell within a few minutes at 37° and the amount specifically bound remains constant for at least 6 hr.

Determination of the Rate Constant of Association for Triamcinolone Acetonide. The rate of association of triamcinolone acetonide with the binding component in the 105,000*g* supernatant fraction was determined for various concentrations of steroid with a constant amount of supernatant (Figure 5A). The rates of association were determined in the presence of either 11α-cortisol or 11β-cortisol and both sets of data are presented in the figure in order to demonstrate the relatively large contribution to the total binding which is made by nonspecific association at early times in incubation. Part B of Figure 5 presents the rate of association of triamcinolone acetonide-*t* (8×10^{-9} M) with the binding component at various concentrations of supernatant. In this case the binding in the presence of 11β-cortisol has been subtracted from that in the presence of 11α-cortisol to yield an approximation of specific binding at each point.

The following procedure (Moore, 1962) was used to determine the order of the reaction. The initial velocity of binding (V_0) was assumed to be related to the steroid concentration (S) and the concentration of the binding component (BC) in the following manner: $V_0 = k_{ass}(S)^m(BC)^n$. The number of each species involved in the formation of the bound complex is defined by m and n and the rate constant of association is represented by k_{ass}. If only one steroid molecule associates with a single unit of the binding component, then at a constant concentration of the binding component a plot of V_0 *vs.* the concentration of the steroid should be linear (insert Figure 5A). Similarly, the corresponding plot of V_0 *vs.* the concentration of the soluble fraction should also be linear (insert Figure 5B). As both of these relationships are linear, m and n are equal to 1 and it is possible to calculate a second-order rate constant of association for triamcinolone acetonide in each experiment. The rate of association is equal to the slope of the curve presented in the insert divided by the concentration of (BC) for the experiment in part A or (S) for that presented in part B. In order to calculate the molarity of the binding component at any concentration of supernatant protein we determined the total specific binding of steroid after 24 hr at which time the binding reaction is complete. The conversion of this value to a molar concentration of

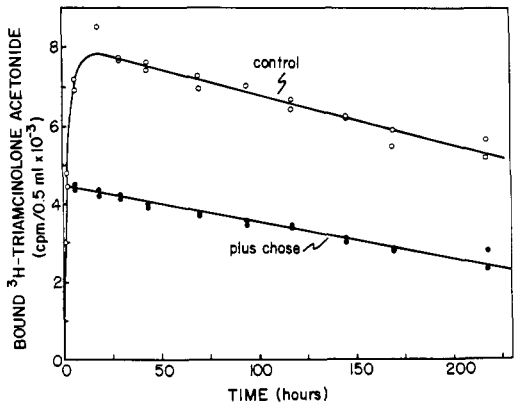

FIGURE 6: The effect of addition of a 1000-fold excess of nonradioactive triamcinolone acetonide on the time-dependent decrease of bound radioactive triamcinolone acetonide. A 105,000*g* supernatant fraction (385 μg of protein N/ml) was incubated at 0° with 10^{-8} M triamcinolone acetonide-*t*. At 2 hr, the incubation was divided into two portions and nonradioactive triamcinolone acetonide was added to one part at a final concentration of 10^{-5} M. The control incubation received an appropriate amount of vehicle. At the indicated times binding assays were performed on duplicate 0.5-ml aliquots of both the control and chase incubations. The lines determined by each set of values were calculated by the method of least squares.

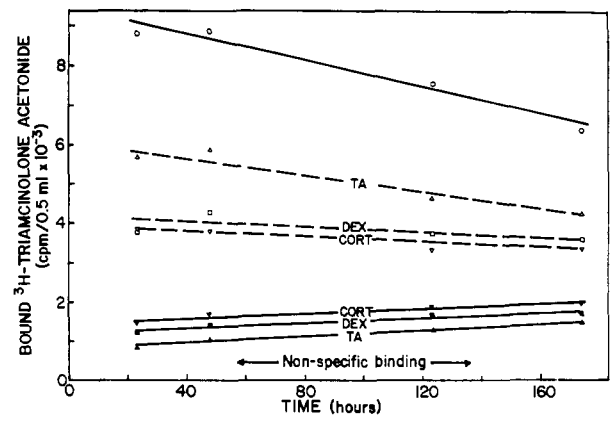

FIGURE 7: The effect of preincubation with nonradioactive dexamethasone and 11β-cortisol on the binding of triamcinolone acetonide-*t*.Nonradioactive steroid (2×10^{-8} M triamcinolone acetonide, 10^{-7} M dexamethasone, or 8×10^{-7} M 11β-cortisol) was added to each of three samples of a 105,000*g* supernatant and other aliquots received an appropriate amount of vehicle. All samples were then preincubated for 32 hr at 0°. At the end of the preincubation period, unlabeled steroid was added as above to three samples which had been preincubated with vehicle, and 2×10^{-8} M radioactive triamcinolone acetonide was then added to all samples including a control sample which had no nonradioactive steroid additions at all. Aliquots (0.5 ml) were taken from each incubation and assayed for bound radioactivity at the indicated times. (∇) 8×10^{-7} M 11β-cortisol, ($\square$) 10^{-7} M dexamethasone, and ($\triangle$) 2×10^{-8} M triamcinolone acetonide. The open symbols connected by a dashed line represent those samples preincubated with vehicle, the nonradioactive steroid being added after the preincubation simultaneously with the triamcinolone acetonide-*t*. The solid symbols connected by a solid line represent those samples preincubated with nonradioactive steroids. The open circles connected by a solid line (control) represent the binding in a sample to which no unlabeled steroid was added. The level of nonspecific binding is indicated by the arrows.

binding component is based on the assumption that one molecule of triamcinolone acetonide is bound per unit of specific binding component. The rate constant of association for triamcinolone acetonide calculated under these conditions is 7.9×10^5 M^{-1} min^{-1} for the experiment presented in part A and 8.2×10^5 M^{-1} min^{-1} for expt B. A third determination made in another experiment with varying concentration of the soluble fraction was 7.8×10^5 M^{-1} min^{-1}. The average for the three determinations is 8.0×10^5 M^{-1} min^{-1}.

Reversibility of Triamcinolone Acetonide Binding. In an attempt to show reversibility of binding a 1000-fold excess of nonradioactive triamcinolone acetonide was added to a sample of the 105,000*g* supernatant which had been preincubated with the radioactive steroid and the binding was assayed over a period of 10 days. The results of the experiment are presented in Figure 6. The control binding reached a maximum after approximately 10-hr incubation. The amount of binding then declined very slowly in a zero-order manner. The amount of binding in the incubation to which a 1000-fold excess of nonradioactive triamcinolone acetonide was added declined in a similar manner.

In another experiment, the soluble fraction was preincubated for 20 hr at 0° in the presence and absence of 1.5×10^{-8} M nonradioactive triamcinolone acetonide then 1.5×10^{-8} M triamcinolone acetonide-*t* and 2×10^{-5} M 11α- or 11β-cortisol was added to each portion. The specific binding was determined at various intervals for a period of 3 days. There was essentially no specific binding in the samples which were preincubated with unlabeled triamcinolone acetonide.

Further Attempts to Exchange the Bound Steroid. A similar experiment was carried out after preincubation of the soluble fraction in the presence or absence of near maximal concentrations of the less potent glucocorticoids, dexamethasone and 11β-cortisol. The results are presented in Figure 7. In those samples which were preincubated with unlabeled steroid there was a small amount of binding of 2×10^{-8} M triamcinolone acetonide-*t* which increased at a very slow rate for 170 hr. The amount of binding expected (preincubation without steroids) for 2×10^{-8} M triamcinolone acetonide-*t* in the presence of the appropriate amount of unlabeled steroid is presented in the curves determined by the dashed lines.

It is difficult to conduct chase experiments over very long time periods in whole cells at 0° because the cells will not remain intact. In the experiment presented in Table II intact L cells were incubated with triamcinolone acetonide-*t* (10^{-8} M), cooled in ice and a chase of a 1000-fold excess of nonradioactive triamcinolone acetonide was added to one half of the cell suspension. The cell suspensions were kept in ice for 45 hr and assayed for bound radioactive steroid. During the 45-hr period cell destruction was appreciable as demonstrated by a decrease in recovered protein. The specific activity of the bound radioactivity rose by about 25% indicating preferential loss of nonbinding component protein. There is however no difference in the specific activity of binding recovered in the control and the chase samples.

Stabilization of the Binding Component by Triamcinolone Acetonide. A 105,000*g* supernatant was incubated with 10^{-8} M triamcinolone acetonide-*t* and 11α- or 11β-cortisol after various periods of preincubation in the absence of any steroid. The results are presented in Figure 8. The longer the time of preincubation in the absence of steroid, the less the binding capacity of the supernatant. As presented in the insert of the figure, the rate of degradation at 0°. in the absence of steroid is approximately three times as rapid as in the presence of triamcinolone acetonide. In addition it has been observed in other experiments that the rate of degradation of the bound complex varies directly with the concentration of supernatant protein.

Binding of Triamcinolone Acetonide at a Constant Concen-

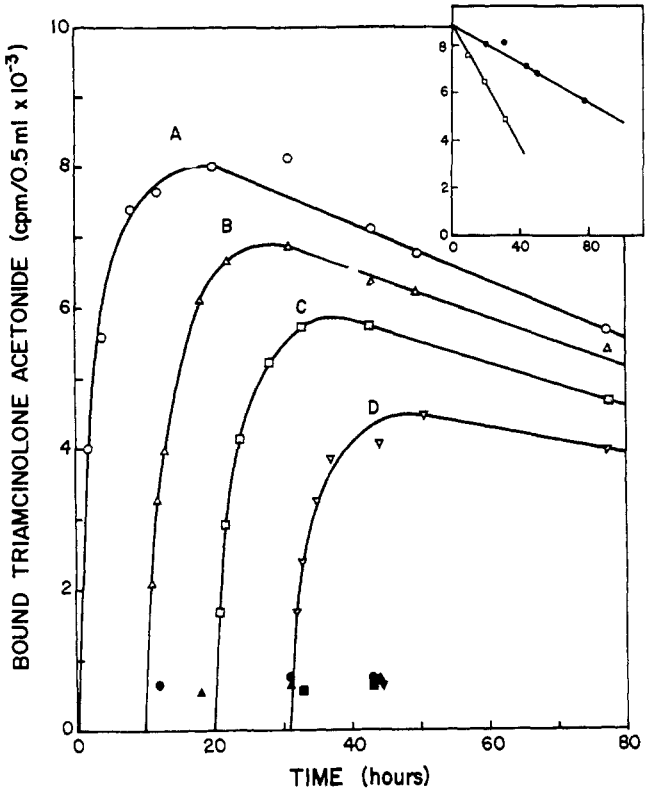

FIGURE 8: Stabilization of the soluble binding component at 0° by triamcinolone acetonide. A supernatant (860 μg of protein N/ml) was maintained at 0°, and at various times, a portion was incubated with 10^{-8} M triamcinolone acetonide-*t* and 2×10^{-5} M 11α- or 11β-cortisol. At the indicated times, 0.5-ml aliquots were removed and passed through a small Sephadex G-25 column. The values in the figure represent the total bound radioactivity in each 0.5-ml aliquot expressed as counts per minute. (○) Triamcinolone acetonide-*t* and 11α-cortisol added at zero time; (△) addition after 10 hr; (□) addition after 20 hr; (▽) addition after 31 hr. The solid symbols represent the bound radioactivity recovered from the incubations containing 11β-cortisol. The insert shows the decline of initial binding capacity plotted against time. The initial binding capacity was determined by extrapolating back to zero time the line defined by the points taken after complete saturation (20) in curve A. The binding capacity after preincubation in the absence of steroid (curves B, C, and D), was determined in a similar manner by extrapolating each back to the time at which triamcinolone acetonide-*t* was added to the incubation (10, 20 and, 31 hr, respectively). (●) The binding capacity remaining during incubation in the presence of triamcinolone acetonide (from curve A). (□) Binding capacity remaining after increasing time of incubation in the absence of triamcinolone acetonide.

tration of Free Steroid. If specific binding is not reversible, then the binding reaction at any concentration of steroid should proceed until there is either no free steroid or no unoccupied binding component. In order to test this prediction the experiment presented in Figure 9 was performed. A 105,000*g* supernatant was divided into three portions and incubated at 0° with three different concentrations of triamcinolone acetonide-*t*. The concentration of the free form of the steroid was maintained within a small range by monitoring the binding and continually adding small amounts of radioactive steroid to the incubations. At each concentration of free triamcinolone acetonide, binding continued until all of the receptor sites were occupied. This was demonstrated by the observation that the addition of a large amount of radioactive triamcinolone acetonide to all of the incubations after maximum binding had been achieved resulted in no increased

TABLE II: Attempt to Chase in the Intact Cell.[a]

Time (hr)	Additions	Amount of Supernatant/ Assay (μg of Protein N)	Bound Triamcinolone Acetonide-*t* Cpm	Bound Triamcinolone Acetonide-*t* Sp Act. (cpm/μg of Protein N)
0	None	659	10,526	16.0
		708	11,152	15.8
45	None	271	5,433	20.1
		225	5,267	23.4
45	10^{-5} M chase	197	4,087	20.8
		173	3,908	22.6

[a] Mouse fibroblasts (4.6×10^6 cells/ml) were incubated with 10^{-8} M triamcinolone acetonide-*t* at 37° in complete growth medium containing 25 mM Hepes buffer (pH 7.3). At the end of 1 hr, the incubation was cooled in ice, and replicate 7-ml aliquots were removed for assay of bound steroid (zero time). The remaining cold cell suspension was divided into two portions. Unlabeled triamiconolone acetonide (10^{-5} M) was added to one portion and the other received an equivalent amount of vehicle (0.5% ethanol). The incubations were continued for 45 hr at 0° and replicate 7-ml aliquots were removed as before. The cells were ruptured and the radioactivity and protein content of an aliquot of the supernatant were determined as described in Materials and Methods.

binding. As was presented in Figure 8, there is a degradation of the unbound binding component which is faster than that observed for the bound form. Thus, at the lower concentrations of free steroid the slower binding reaction permits more time for degradation of the unbound binding component and the maximum amount of binding achieved is correspondingly lower.

Binding of Triamcinolone Acetonide in the Soluble Fraction from Steroid-Sensitive and Resistant L Cells as a Function of Steroid Concentration. Soluble fractions prepared from glucocorticoid-sensitive and glucocorticoid-resistant L cells were incubated for 18 hr at 0° with various concentrations of triamcinolone acetonide-*t* at constant specific activity. Each incubation contained either 2×10^{-5} M nonradioactive fluocinolone acetonide (a potent growth-inhibitory steroid) or 11α-cortisol. The results of such an experiment are presented in Figure 10. From part A of the figure it can be seen that the binding of triamcinolone acetonide increases until a plateau is reached at a steroid concentration of approximately 3×10^{-8} M. At low concentrations of triamcinolone acetonide there is essentially no binding which is not competed for by the active compound, fluocinolone acetonide. However, at higher concentrations the nonspecific binding in the presence of fluocinolone acetonide increases. From part B of Figure 10 it is clear that there is considerably less specific binding of triamcinolone acetonide in the resistant cells. The time course of binding of the resistant cells was the same as for the sensitive cells. The amount of specific binding in sensitive and resistant cells is compared in part B of Figure 11. The resistant cell supernatant binds approximately 10% as much triamcinolone acetonide as that from the sensitive cells. Part A of

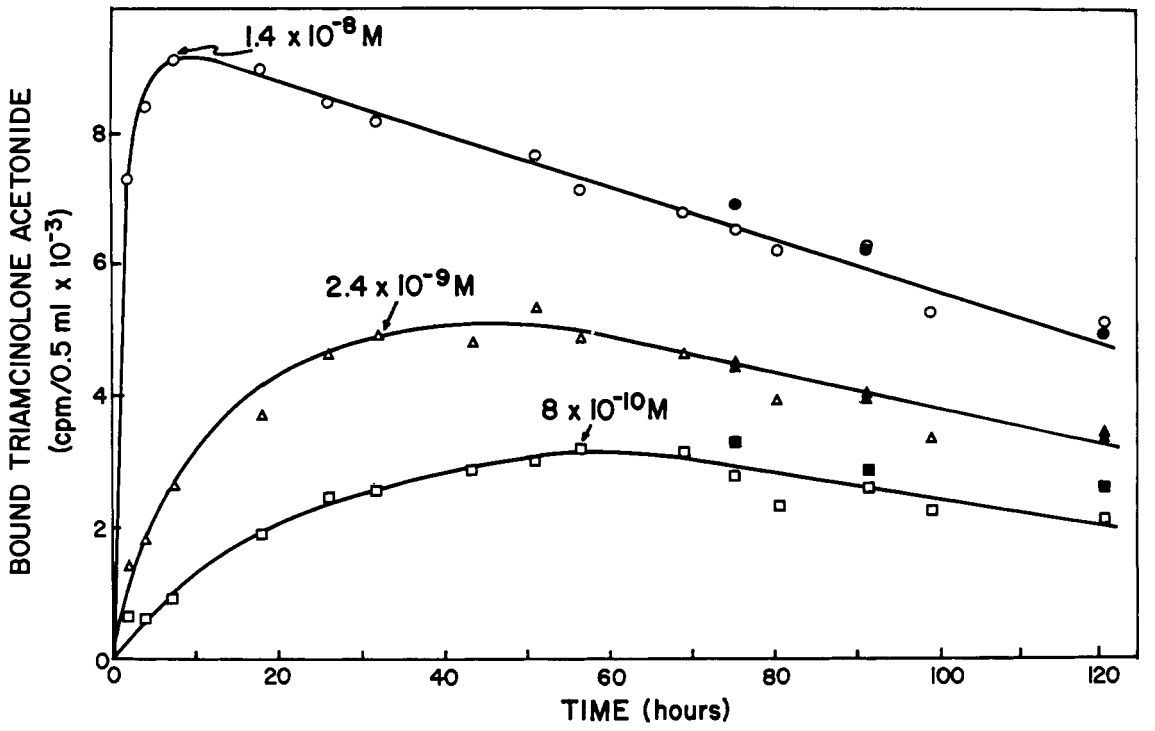

FIGURE 9: The binding of triamcinolone acetonide at a constant concentration of free steroid. A 105,000*g* supernatant (672 μg of protein N/ml) was divided into three 20-ml portions. Tritium-labeled triamcinolone acetonide was added to each portion at a final concentration of 2×10^{-8}, 2×10^{-9}, and 8×10^{-10} M. The preparations were incubated at 0° and 0.5-ml samples were assayed for bound radioactivity at the indicated times. The sample which was made 2×10^{-8} M in the beginning was allowed to proceed to maximum binding at which time the concentration of the free form of the steroid (calculated by subtracting bound radioactivity from total radioactivity) was 1.4×10^{-8} M. In the other two incubations the amount of binding was measured in 0.5-ml samples, and at each time of assay, an amount of triamcinolone acetonide-*t* equivalent to the amount which had become bound since the preceeding assay was added to the incubation. In this way the concentration of the free steroid was kept reasonably constant. At the lowest concentration of steroid the concentration of free drug varied from 3×10^{-10} to 8×10^{-10} M and in the middle curve from 1.6×10^{-9} to 2.4×10^{-9} M. The concentration of free steroid at the top of each binding curve is presented in the figure. At 70 hr, after the binding was maximum in all incubations, a 5-ml portion of each incubation was added to tubes containing 0.05 ml of additional triamcinolone acetonide-*t* at 10^{-6} M. The results of assays on these incubations are represented by the solid symbols. The results are expressed in all cases as the amount of radioactivity bound per 0.5 ml of incubation. The addition volumes were trivial with respect to the volume of the incubations.

Figure 11 presents the Scatchard plots of the binding recovered in the presence of 11α-cortisol for the experiments presented in Figure 10. The Scatchard lines of the high-affinity binding for both sensitive and resistant cells are linear with identical slopes, suggesting a single class of high-affinity-binding sites. It can be calculated from the intercept on the abscissa that there are 6.7 pmoles of triamcinolone acetonide bound per mg protein N in the sensitive cells. These binding determinations were made after 18-hr incubation, at which time there could not have been more than 10% degradation of the bound receptor.

Competition for Binding of Triamcinolone Acetonide with Active Growth-Inhibitory Steroids. Replicate aliquots of a 105,000*g* supernatant fraction from L cells were incubated with 2×10^{-8} M triamcinolone acetonide-*t* and various concentrations of nonradioactive fluocinolone acetonide, dexamethasone, 11α-cortisol, and 11β-cortisol. The apparent dose response curves of competition for binding are presented in Figure 12. The inset presents the effects of the competing steroids on the growth of mouse fibroblasts for purposes of comparing the potency of steroids in inhibition of triamcinolone acetonide binding and inhibition of cell growth. At 2×10^{-8} M triamcinolone acetonide, binding to the specific binding component is just reaching maximum (Figure 10). Therefore, a concentration of competing steroid which reduces the binding of 2×10^{-8} M triamcinolone acetonide at this time by 50% is a concentration which alone should occupy the maximum amount of binding component. Comparing the potencies of the three competing steroids in this manner, 2×10^{-8} M triamcinolone acetonide is equivalent to 2.4×10^{-8} M fluocinolone acetonide, 9.5×10^{-8} M dexamethasone, and 7.6×10^{-7} M 11β-cortisol.

Discussion

Most of the work which has been carried out on the binding of steroids in subcellular systems has utilized binding assay techniques designed to separate the bound form of the steroid from the free compound. In the event that the binding is readily reversible these techniques will not allow the accurate measurements afforded by equilibrium dialysis (Chader and Westphal, 1968). We have found however, that it is very difficult to obtain consistent results by equilibrium dialysis of the crude L cell supernatant fraction. The Sephadex assay which we have employed is very much like charcoal absorption techniques (*e.g.*, Baxter and Tomkins, 1971), or density gradient assays (*e.g.*, Toft *et al.*, 1967) in that equilibrium conditions cannot be maintained during the period required for assay. The advantage of the Sephadex binding assay is that it is rapid (6–8 min) and complete separation of the bound from the free form is obtained (Figure 1) even when the amount bound is only a small fraction of the total radioactivity.

The best results with the Sephadex G-25 assay are obtained

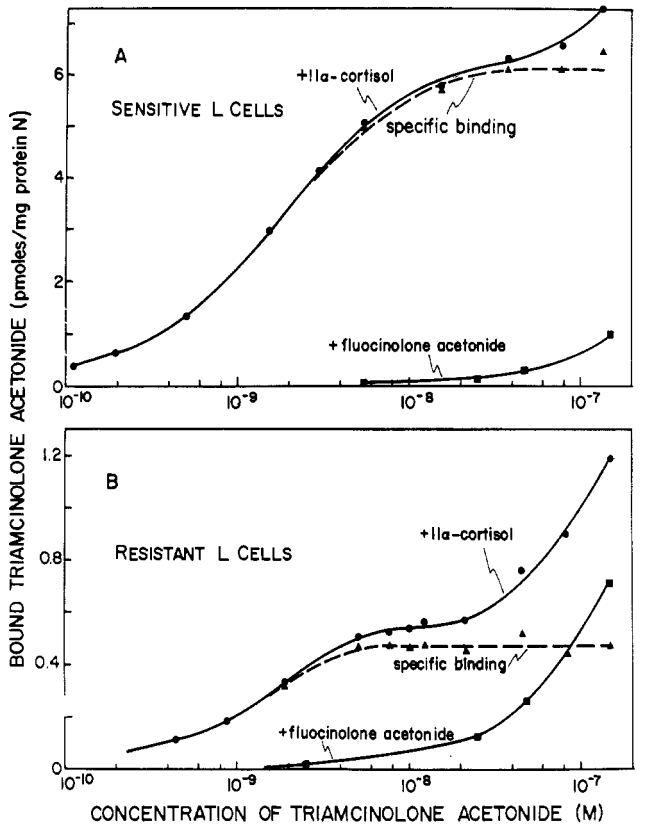

FIGURE 10: The binding of tiramcinolone acetonide in the soluble fraction of sensitive and resistant L cells as a function of steroid concentration. The 105,000g supernatant fractions were prepared from glucocorticoid-sensitive and resistant L cells, and 0.5-ml aliquots were incubated for 18 hr at 0° with various concentrations of triamcinolone acetonide-t (20 Ci/mmole) and 2×10^{-5} M nonradioactive 11α-cortisol or fluocinolone acetonide. A portion of each incubation was assayed for total radioactivity, and the remainder was passed through small columns of Sephadex G-25 for determination of bound radioactivity. The results are expressed as the number of picomoles of triamcinolone acetonide bound per mg of protein N. The concentration of free triamcinolone acetonide was determined by subtracting the total bound steroid from the total steroid in each sample. Protein concentration in mg of protein N/ml: incubations from sensitive cells, 1.92; incubations from resistant cells, 2.04. (●) Binding in the presence of 11α-cortisol, (■) binding in the presence of fluocinolone acetonide, and (▲) specific binding. Specific binding represents the total binding (binding in the presence of 11α-cortisol) minus the curve for nonspecific binding (residual binding still observed in the presence of the active glucocorticoid, fluocinolone acetonide).

when low concentrations (under 10^{-7} M) of the most potent glucocorticoids are employed. When 10^{-6} M radioactive cortisol is incubated with the soluble fraction from L cells and an aliquot is filtered through Sephadex G-25, over 80% of the observed binding is not competed for by active steroids. This large background of nonspecific binding at high steroid concentrations has not permitted us to obtain accurate binding assays when using weak glucocorticoids such as 11β-cortisol. Wira and Munck (1970) have, however, successfully employed the technique in assaying bound cortisol in the thymus cell system.

The specific binding complex in the soluble fraction from mouse fibroblasts is rapidly degraded at 37° (Figure 4). This very rapid degradation at higher temperatures necessitates that experiments concerned with the kinetics of binding be carried out at 0°. Thus, at the moment, degradation prevents us from exploring the nature of the steroid binding at physiological temperatures. This may be a considerable drawback, as it has been well demonstrated that the association constant of corticosteroid-binding globulins varies with temperature; in that system it is much higher at 4° than at 37° (Westphal, 1967).

It is clear that the degradation which proceeds in the L cell supernatant at 37° results in a complete, apparently irreversible loss of binding capacity. If supernatant which has been incubated to saturation at 0° with triamcinolone acetonide-t is heated to 37°, 80–90% of the specific binding is lost within 1 hr. The rate of degradation of the binding complex in the soluble fraction from L cells at 0° is very slow. Schaumburg (1970) has demonstrated that the binding complex in rat thymus is rapidly degraded at 4°. Wira and Munck (1970) found that there was a complete loss of radioactive cortisol from the bound form after 15-min incubation of rat thymus extracts at 37°. The slow 0° rate of degradation in our system permits us to make an accurate determination of the rate constant of association at that temperature. This value of 8.0×10^{5} M^{-1} min^{-1} for triamcinolone acetonide is much slower than that which would be predicted by experiments with other glucocorticoid-binding systems. Baxter and Tomkins (1971) have published an association-rate constant of 4.1×10^{6} M^{-1} min^{-1} at 0–4° for dexamethasone in a cytoplasmic extract from hepatoma cells grown in tissue culture. Dexamethasone is a less potent glucocorticoid than triamcinolone acetonide in most assay systems.

Previously bound radioactive triamcinolone acetonide in L cell soluble fraction is not displaced by the addition of a 1000-fold excess of the unlabeled steroid (Figure 6). Therefore at 0° there is negligible or no exchange of bound for free steroid. This is also the case in the supernatant when the temperature is raised. When the supernatant fraction containing triamcinolone acetonide-t bound at 0° is switched to 37°, there is no difference in the rate of loss of bound radioactivity in the presence or absence of a chase with a 1000-fold excess of unlabeled steroid (W. B. Pratt and D. N. Ishii, work in preparation). If intact cells containing bound triamcinolone acetonide-t are incubated for 45 hr at 0° with a 1000-fold excess of the unlabeled compound there is no effect on the amount of binding in relation to nonchase controls (Table II). Thus, the complex of triamcinolone acetonide with the binding component behaves in an essentially irreversible manner. There is no covalent bound formed between the binding component and the drug, however, as it has been shown that the bound steroid can be readily extracted with organic solvents and demonstrated to be the unaltered compound (Hackney *et al.*, 1970). From the experiment presented in Figure 8, it is clear that the physiologically less potent steroids, dexamethasone and 11β-cortisol, also interact with the binding component such that there is negligible exchange between bound and free steroid.

The observation that the binding is not readily reversible in the L cell preparation is not an expected result given the experience with other glucocorticoid-binding systems. Baxter and Tomkins (1971) have demonstrated that the binding of radioactive dexamethasone in the soluble fraction of mouse hepatoma cells is rapidly decreased by the addition of excess unlabeled steroid and they have calculated a dissociation-rate constant of 3×10^{-3} min^{-1} at 0–4°. The steroid which is associated with the binding component in rat thymus readily dissociates from the bound to the free form. Indeed the elegant binding assay developed by Munck and Brinck-Johnsen (1968) relies on measuring the differential dissociation of steroids from high-affinity, specific binding components as opposed to

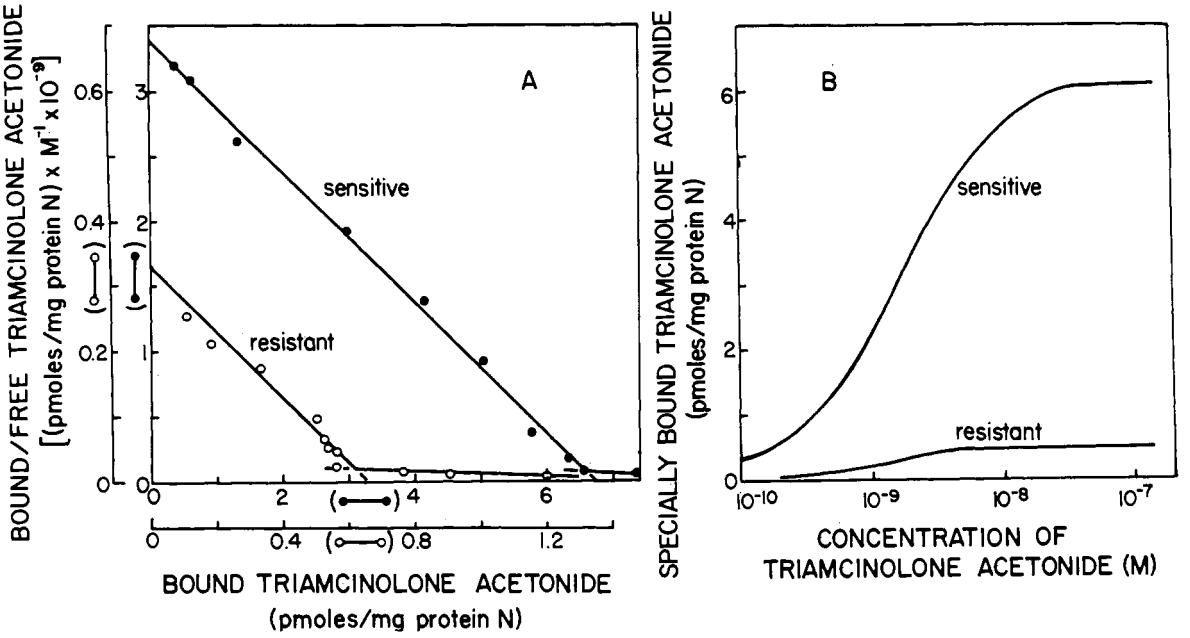

FIGURE 11: Scatchard plot and specific binding. (A) Scatchard plot of the binding in the presence of 11α-cortisol for sensitive and resistant cell supernatant fractions. Data from Figure 10. (B) Specific binding in the 105,000g supernatant fraction from sensitive and resistant cells. Data from Figure 10.

dissociation from nonspecifically bound, low-affinity forms. Schaumburg and Bojessen (1968) have calculated a dissociation-rate constant of 1.77×10^{-3} min^{-1} for the corticosterone receptor complex in rat thymus. From $T_{1/2} = \ln e^2/k_{diss}$ one would predict a dissociation half-time for the complex of steroid and binding component of 392 min. In experiments utilizing a chase with nonradioactive steroid, the $T_{1/2}$ for dissociation of corticosterone from the bound complex was found to be 168 min at 4°. The binding of cortisol by corticosteroid-binding globulin is reversible and the dissociation increases with increasing temperature (Seal and Doe, 1962; Westphal, 1967). There is, therefore, good, consistent precedent in all of the glucocorticoid systems studied so far for predicting that the steroid binding component complex in the supernatant fraction of mouse fibroblasts should readily dissociate. The fact that it does not stand out as a major difference between the specific glucocorticoid-binding component in the L cell system and those existing elsewhere.

The resistant cell line has a maximum specific-binding capacity which is about 10% of that of the sensitive cell line. This clearly cannot be the result of a decreased affinity of the binding component in the resistant cell supernatant for the steroid. It probably represents, therefore, a decreased number of binding molecules. We have not as yet determined the rates of degradation of the resistant cell-binding component at any temperature. Therefore, it is not known whether the decreased amount of binding component is due to a decreased rate of synthesis, an increased rate of degradation, or an alteration in both processes. A similar depression of binding capacity in a resistant cell line has been demonstrated *in vitro* in supernatant fractions prepared from P 1798 mouse lymphosarcoma (Hollander and Chiu, 1966; Kirkpatrick *et al.*, 1972).

The concept of dose response is classically considered in terms of the reversible interaction of the steroid with its receptor

$$[R] + [S] \underset{k_2}{\overset{k_1}{\rightleftharpoons}} RS$$

However, we are observing one of two phenomena. (1) The steroid is interacting with the binding component in a reversible manner but a second process is rendering the binding irreversible (RS′).

$$[R] + [S] \underset{k_2}{\overset{k_1}{\rightleftharpoons}} [RS] \xrightarrow{k_3} RS'$$

If this is the case then the difference in potency between steroids could be explained in the usual manner. In addition the observations that the binding can be readily competed for but cannot be chased are consistent with this model. (2) The second alternative is that the interaction of the steroid with the binding component may cause a change in the conformation of the binding component such that a specific hydrophobic receptor site becomes buried and dissociation cannot take place.

$$[R] + [S] \xrightarrow{k_1} RS'$$

In this case the difference in potencies is determined by large differences in k_1, the binding can be competed for and exchange cannot take place.

In the intact cell under growth conditions there are two other factors which must be considered, the rate of synthesis and the rate of degradation of the receptor. Thus the amount of bound steroid which is seen at any one time will be determined by the rate constant of synthesis of the receptor (k_4), the rate constant of degradation of the unbound receptor at 37° (k_5), and the rate constant of degradation of the bound receptor at 37° (k_6). We are now examining the steady-state

$$\xrightarrow{k_4} [R] + [S] \underset{k_2}{\overset{k_1}{\rightleftharpoons}} [RS] \xrightarrow{k_3} RS'$$
$$\downarrow k_5 \qquad\qquad\qquad\qquad \downarrow k_6$$

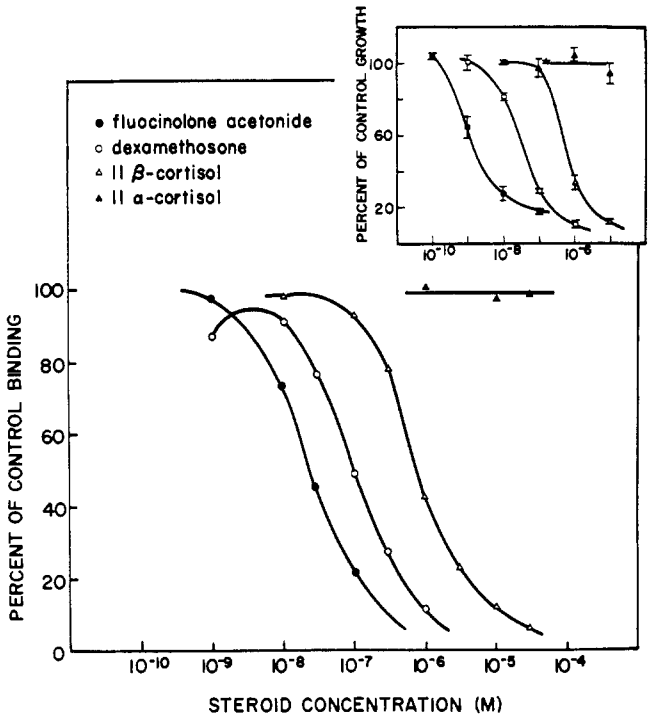

FIGURE 12: The relative potencies of three glucocorticoids in competing for the binding of triamcinolone acetonide to the soluble fraction of L cells. Aliquots (0.5 ml) of a 105,000*g* supernatant (containing 980 μg of protein N/ml) were incubated at 0° for 17 hr with 2×10^{-8} M triamcinolone acetonide-*t* and various concentrations of nonradioactive competing steroid. At the end of the incubation, the amount of bound radioactivity per μg of protein N was determined. The values in the figure are presented as a percentage of the binding assayed in a control incubation containing triamcinolone acetonide-*t* and vehicle. The inset presents the rate of growth of L cells. Each value represents the mean and standard deviation of three replicate cultures, expressed as a percentage of growth attained in control cultures which received vehicle alone.

level of receptor in intact steroid-sensitive and resistant cells in order to try to define the significance of these processes in the maintenance of the steady-state levels of specific binding which we observe in intact L cells at 37°.

This discussion has been restricted to the literature on the binding of the glucocorticoid series of steroids. The extensive literature on the binding of other groups of steroids, in particular the estrogens, has been recently reviewed in detail (Jensen *et al.*, 1971).

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