Steady-State Level of the Specific Glucocorticoid Binding Component in Mouse Fibroblasts†

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ABSTRACT: When L cells are incubated at 37° in the presence of radioactive triamcinolone acetonide, the amount of steroid bound in a specific manner remains constant for several hours. Chase experiments reveal that the specific binding component (receptor) in the soluble fraction of the intact cell is being continually inactivated or recycled (half-time 30–40 min). New, unoccupied binding component is continually entering the system by a process that is temperature dependent, energy dependent, and does not require simultaneous protein synthesis. The complex of steroid and binding component in the 7000*g* supernatant is in equilibrium with steroid bound to the 7000*g* particulate fraction at 37°. Energy deprivation reduces the bound steroid in the supernatant fraction but increases it in the particulate fraction. This effect is reversible upon addition of glucose to the cell suspension. At 0°, the rate of binding is much slower, there is more total binding in the cell, and there is no detectable inactivation of the binding component in the soluble fraction. At the same time, there is markedly diminished binding in the particulate fraction at 0° compared to 37°. Preincubation with radioactive steroid at 0°, followed by a chase of nonradioactive steroid and a temperature shift to 37°, clearly demonstrates the movement of bound label from the cytosol fraction into the particulate fraction, and its subsequent loss as the chase effect proceeds. A model is presented to demonstrate how the rapid recycling of the binding component may play an important role in determining the degree of target cell response to changing concentrations of free glucocorticoid.

Mouse fibroblasts (L929) growing *in vitro* contain a binding component for glucocorticoid steroids which is recovered largely in the soluble fraction of the cell after hypotonic rupture (Hackney *et al.*, 1970). This molecule or molecular aggregate has an apparent molecular weight of 620,000 as determined by filtration through Sephadex G-200, and has been purified approximately 2000-fold (Hackney and Pratt, 1971).

The following observations support the concept that the binding component is the receptor for the glucocorticoid effect. Active glucocorticoids compete for the binding of triamcinolone acetonide, both in the intact cell (Hackney *et al.*, 1970) and in the 105,000*g* supernatant (Pratt and Ishii, 1972) in a manner which reflects their potency as inhibitors of growth and hexose transport (Gray *et al.*, 1971). Inactive steroids do not compete for binding with the exception of cortexolone, an analog of 11β-cortisol which is capable of blocking the effect of active glucocorticoids but does not produce any effect itself (Mosher *et al.*, 1971; Samuels and Tomkins, 1970; D. N. Ishii and W. B. Pratt, unpublished data). The specific binding of triamcinolone acetonide is saturable at the concentration of drug which produces a maximal growth inhibitory effect. A resistant subline of L929 cells contains only 10–15% of the amount of binding component recovered from the sensitive cells.

One characteristic of steroid binding in mouse fibroblasts that is quite different from the binding observed in other glucocorticoid-sensitive systems like thymocytes (Munck and Brink-Johnsen, 1968; Schaumburg, 1970) and hepatoma cells (Baxter and Tomkins, 1971) is the nearly irreversible nature of the steroid-binding component complex at 0° (Pratt and Ishii, 1972). In this paper we will examine the steady-state level of the steroid–receptor complex at 37° in the soluble fraction of intact cells. The steady-state level of bound receptor in the intact cell can best be explained by a continuous rapid loss of the binding component accompanied by new input of the receptor into the soluble system. The ability of nonradioactive steroid to decrease the amount of radioactive triamcinolone acetonide bound to the receptor in the intact cell at 37° does not appear to represent an exchange of bound steroid for unbound steroid; rather the apparent chase effect is primarily the result of competition for binding sites arising from the continuous input into the steady state. The results are summarized in a model which demonstrates how the L cell is able to bind steroids very tightly and yet respond rapidly to variations in the concentration of free glucocorticoid.

Materials and Methods

Materials. [1,2,4-^{3}H]Triamcinolone acetonide[1] was purchased from Schwarz BioResearch Inc., Orangeburg, N. Y. Two separate lots were employed in these experiments, one with a specific activity of 20 Ci/mmole and the other at 9.2 Ci/mmole. Fluocinolone acetonide was a gift from Dr. Ralph Dorfman of Syntex Corp. and 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.

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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. 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.

Cell Culture. Suspension cultures of L929 cells were maintained in basal medium (Eagle, 1955) modified as described previously (Gray *et al.*, 1971). Cultures were maintained with constant stirring in an atmosphere of humidified air. The experiment on cells in monolayer culture was carried out as described previously (Pratt and Aronow, 1966). The selection and cloning of steroid-resistant subline of L929 cells have also been described (Hackney *et al.*, 1970; Hackney and Pratt, 1971).

Incubation of Intact Cells. Cells were harvested from suspension culture by centrifugation at 600*g* for 10 min in a refrigerated centrifuge. The cells were suspended in growth medium without serum, with 25 mM Hepes[2] buffer at pH 7.35 and with 10^{-8} M [^{3}H]triamcinolone acetonide. Replicate portions of the cell suspension were incubated under the conditions described in the individual experiments. Cells were suspended at a density of 1.5×10^6 to 3.5×10^6 cells per ml. The stock cells in suspension culture with serum are normally kept in logarithmic growth between 0.5×10^6 and 2.5×10^6 cells per ml. It is important that the cell density in the experiments not be higher than 5×10^6 cells/ml since at higher cell densities the level of specifically bound radioactive steroid will decrease with time. In most of the experiments, a cell suspension was preincubated in the presence of radioactive triamcinolone acetonide and 5 mM glucose. After the preincubation, the cells were centrifuged at 600*g*, resuspended in the same medium with radioactive steroid but without glucose and divided into replicate portions for further incubation under the conditions described in the legends to the figures.

2,4-Dinitrophenol (Dnp) was added in warm 50% ethanol, and KCN in water, in $^1/_{100}$th the incubation volume to yield a final concentration of 5×10^{-4} M. Nonradioactive fluocinolone acetonide or triamcinolone acetonide was dissolved in ethanol at 10^{-2} M, diluted ten times with warm water, and immediately added to the incubation volume to give a final concentration of 10^{-5} M. 11β-Cortisol was added in a similar manner from a 10^{-3} M stock solution at room temperature. All cultures in the same experiment received appropriate amounts of vehicle solutions.

Assay for Bound Radioactivity. Aliquots (7.0 ml) of a cell suspension were harvested by centrifugation at 1500*g* for 30 sec and the medium was rapidly removed. All subsequent procedures were carried out at 0–4°. The cell pellets were immediately suspended in 1.4 ml of a cold hypotonic solution of 0.01 M Tris buffer at pH 7.35 and 0.1 mM EDTA for 2.5 min and homogenized with 15 strokes of a tight-fitting pestle in a Dounce-type glass homogenizer. The hypotonic buffer contained 10^{-5} M nonradioactive triamcinolone acetonide in order to stop any possible additional binding during the assay procedure (this is, however, probably not necessary as the binding values are identical in the presence or absence of the cold steroid in the hypotonic buffer). 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 7000*g* for 3 min.

The bound radioactivity in the 7000*g* supernatant was separated from the free compound by passing a 1.0-ml aliquot of the supernatant through a 1×25 cm column of Sephadex G-25 with an elution buffer of 0.01 M Tris–0.04 M KCl (pH 7.35). The fractions containing the material excluded from the gel were collected, combined, and assayed for radioactivity and protein (Pratt and Ishii, 1972). In those experiments where only the specifically bound radioactivity is presented, the amount of binding recovered in identical cell suspensions incubated with 10^{-8} M [^{3}H]triamcinolone acetonide and 10^{-5} M nonradioactive fluocinolone acetonide (a potent growth inhibitory steroid) has been subtracted from that found in samples incubated with the radioactive compound alone. This nonspecific binding value for the 7000*g* supernatant is always less than 10% of the total bound radioactivity in control samples.

The radioactivity in the 7000*g* pellet is measured by suspending the unwashed pellet in 1.0 ml of water, sonicating for 5 sec with a Bronwill Biosonik III at a setting of 30, and assaying the amount of radioactivity in a 0.5-ml aliquot of the sonicate. Nonspecifically bound radioactive steroid associated with the 7000*g* pellet is a larger fraction of the total bound material than in the supernatant solution, and constitutes about half of the total pellet radioactivity. Accordingly, a correction is made for nonspecific binding by assaying the radioactivity in a 7000*g* pellet from cells incubated with 10^{-8} M [^{3}H]triamcinolone acetonide and 10^{-5} M nonradioactive fluocinolone acetonide, which competes for the binding specific for growth inhibition. Each value for specifically bound triamcinolone acetonide in the pellet represents an average of three assays from cells incubated with radioactive steroid alone minus the average of three determinations on an identical suspension incubated with 10^{-5} M nonradioactive fluocinolone acetonide.

Assay for Binding in the 105,000g Supernatant. A 105,000*g* supernatant was prepared from L cells, incubated with radioactive triamcinolone acetonide (10^{-8} M), and assayed for binding as previously described.

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. In those experiments where the bound radioactivity in both the pellet and supernatant were determined, appropriate quench corrections were carried out using internal standards. In all other experiments the samples being counted were replicates and no quench correction was necessary for comparison of values within the experiment. Protein determinations were carried out according to the method of Oyama and Eagle (1956) and the amount of binding in all 7000*g* supernatants is expressed as counts per minute bound per microgram of protein N.

Results

Specific Binding of [^{3}H]Triamcinolone Acetonide in the Soluble Fraction (7000g Supernatant) of Intact L Cells. Tritium-labeled triamcinolone acetonide (10^{-8} M) was added to a cold suspension of mouse fibroblasts, and the suspension was divided into three portions and incubated in ice, at room temperature, and at 37°. The maximum binding at 37° (Figure 1) was achieved by 20 min and was considerably faster if the radioactive steroid was added to cells preequilibrated at 37°. The amount of binding in the soluble fraction of the cells at 37° remained constant for 6 hr. This will be referred to as the steady-state level of the bound complex. The rate of binding was slower at 22 and 0°, and the amount of binding did not plateau during the 6-hr period. In addition, larger amounts of bound steroid were recovered in the soluble fraction from the cells incubated at the lower temperatures.

[2] Abbreviations used are: Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; Dnp, 2,4-dinitrophenol.

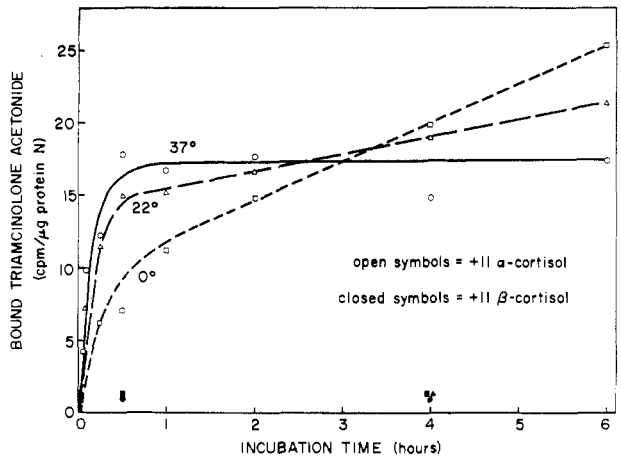


FIGURE 1: Time and temperature dependence of triamcinolone acetonide binding in the soluble fraction of intact mouse fibroblasts. Replicate suspensions (5×10^6 cells/ml) were incubated with 10^{-8} M tritium-labeled triamcinolone acetonide and either 11α- or 11β-cortisol (2×10^{-5} M) at the indicated temperatures. Aliquots were removed at various times and assayed for bound radioactivity.

Effects of Addition of Nonradioactive Steroid and Puromycin on the Steady-State Binding. When 10^{-5} M nonradioactive triamcinolone acetonide was added to a cell suspension that had been preincubated with radiolabeled steroid at 37° for 90 min, the level of bound radioactive steroid remained constant if the cells were maintained at 0°, but declined rapidly if the incubation was continued at 37° (Figure 2). This chase effect at 37° could represent an exchange of unlabeled steroid for prebound radioactive steroid which dissociates

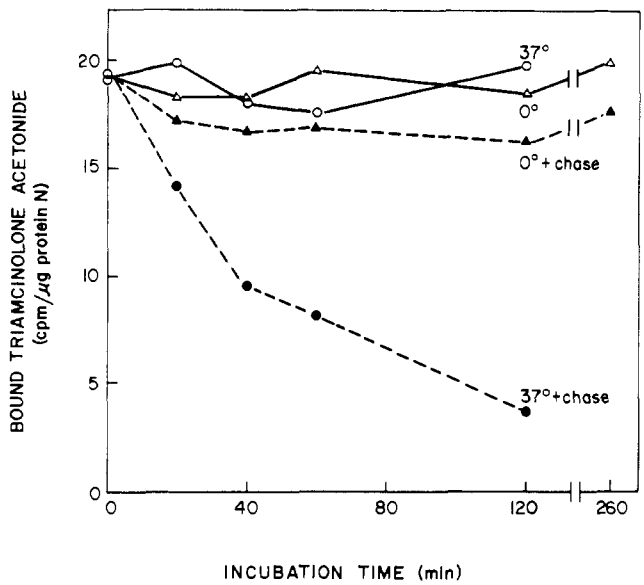


FIGURE 2: The rate of loss of bound radioactive triamcinolone acetonide from the soluble fraction of mouse fibroblasts after the addition of a chase of 10^{-5} M nonradioactive triamcinolone acetonide. Fibroblasts were incubated at 37° for 90 min with 10^{-8} M [^{3}H]triamcinolone acetonide and then divided into four portions. Unlabeled triamcinolone acetonide (10^{-5} M) was added to two of the portions and the incubations were continued at either 37° or switched to 0°. At the intervals shown in the figure, aliquots were removed and assayed as in Figure 1. Incubations in the absence of unlabeled steroid: (△) 0°; (○) 37° Incubations in the presence of 10^{-5} M unlabeled triamcinolone acetonide are indicated by the corresponding solid symbols.

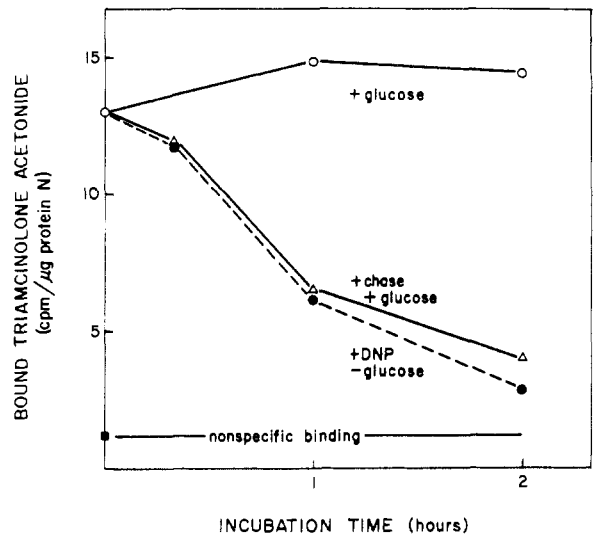


FIGURE 3: The effect of Dnp or unlabeled chase on the rate of loss of bound radioactive triamcinolone acetonide from the soluble fraction of L cells. A suspension of cells was preincubated for 1 hr in growth medium containing 5 mM glucose and 10^{-8} M [^{3}H]triamcinolone acetonide. The cells were centrifuged and resuspended in glucose-free medium containing 10^{-8} M [^{3}H]triamcinolone acetonide. The culture was then divided, the appropriate additions were made, and the incubation was continued for 2 hr. Aliquots were removed at various times and the 7000g supernatant was assayed for bound radioactivity. The additions were as follows: (○) plus 5 mM glucose (control); (△) plus 5 mM glucose and 10^{-5} M unlabeled triamcinolone acetonide (chase); (●) plus 5×10^{-4} M Dnp. Binding in a replicate sample preincubated with 10^{-5} M 11β-cortisol and [^{3}H]triamcinolone acetonide (nonspecific binding) (■).

from a static number of binding components at 37° but not at 0°. Alternatively, the steady-state level of receptor maintained at 37° may be the result of a continuous inactivation or degradation of the binding component and rapid activation or synthesis. In this case, the effect of the addition of unlabeled steroid at 37° would represent competition for binding to newly activated or synthesized receptor, and the amount of prebound radioactive triamcinolone acetonide would fall at a rate which represented the rate of inactivation or degradation of the binding component.

In order to determine if the maintenance of the steady-state level of binding component depended upon the maintenance of a rapid rate of protein synthesis the effect of puromycin was investigated. The addition of puromycin, 2×10^{-4} M, a concentration which halts the incorporation of radioactive leucine into protein within 5 min, did not affect either the steady-state level of binding or the rate of loss of bound radioactivity in incubations to which nonradioactive chase steroid was added. Similarly, puromycin added to cells at 37° for 1 hr in the absence of steroid had no effect on the amount of binding component which could be detected by an *in vitro* binding assay of the 7000g supernatant (data not shown).

Effect of Energy Deprivation on the Amount of Bound Steroid in the Soluble Fraction of the Cell. Cells were preincubated with radioactive triamcinolone acetonide, centrifuged, and suspended in medium containing the radioactive drug but no glucose. The culture was divided into three identical portions one of which was supplemented with dinitrophenol, another received glucose, and the third glucose plus a steroid chase (Figure 3). The amount of bound triamcinolone acetonide decreased at the same rate in th Dnp-treated culture as the

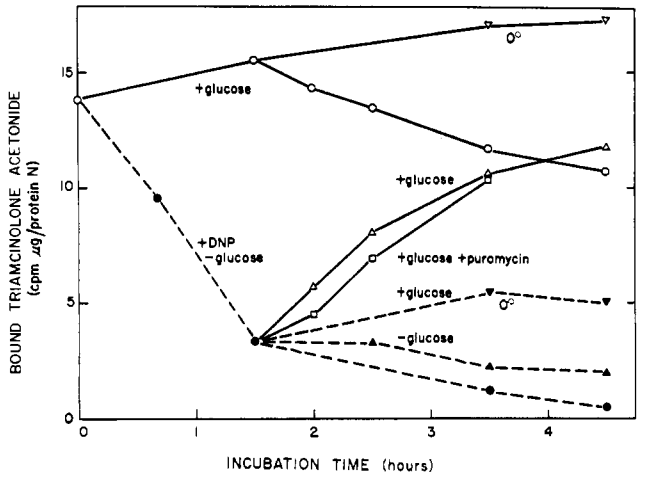


FIGURE 4: The effect of glucose, puromycin and temperature on the rate of return of binding in the soluble fraction of fibroblasts treated with Dnp. The fibroblast suspension was preincubated with [^{3}H]-triamcinolone acetonide, then incubated with Dnp in the absence of glucose as in Figure 3. After 90 min of incubation with Dnp, the cells were again centrifuged, resuspended, and incubated at 37° (except where noted) under the following conditions: (△) plus glucose; (□) plus glucose and puromycin (2×10^{-4} M); (▼) plus glucose, 0°; (▲) minus glucose; (●) minus glucose plus Dnp (5×10^{-4} M). The control curves, from incubations which were not exposed to Dnp, are: (○) plus glucose, 37°; and (▽) plus glucose, 0°. The binding was assayed as in Figure 3. Each point, except the 4.5-hr values, represents the average of duplicate samples.

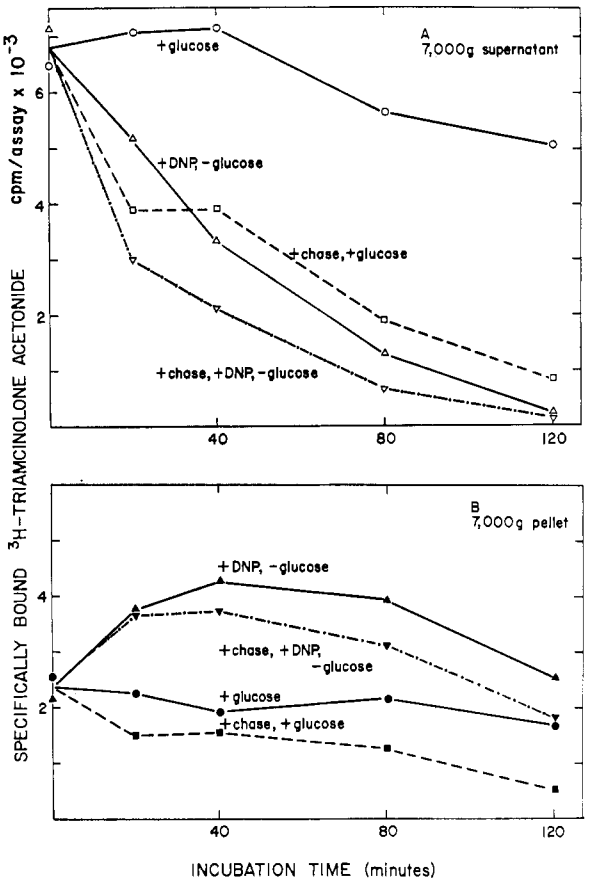


FIGURE 5: The effect of Dnp and/or fluocinolone acetonide chase on the distribution of bound radioactive triamcinolone acetonide in the soluble and particulate fractions. A suspension of L cells was preincubated and treated as explained in the legend to Figure 3 except that the 7000*g* pellet was also assayed for bound radioactive steroid as described under Materials and Methods. Part A of the figure presents the total specifically bound [^{3}H]triamcinolone acetonide in the supernatant fraction and part B presents the corresponding pellet values. The additions were as follows: (○) plus glucose; (△) plus Dnp minus glucose; (□) plus 10^{-5} M fluocinolone acetonide plus glucose; (▽) plus Dnp plus 10^{-5} M fluocinolone acetonide minus glucose. The solid symbols indicate the corresponding pellet values. The nonspecific binding determined in parallel incubations containing 10^{-5} M fluocinolone acetonide has been subtracted from each time point. The supernatant values represent averages of two determinations and the pellet values are averages of three determinations at each time point.

one exposed to a chase of nonradioactive steroid. The loss of binding observed in the Dnp-treated cells could be due to a nonspecific effect such as cell death and lysis or an inhibition in the rate of steroid binding, or it could be the result of the inhibition of an energy-dependent activation or redistribution of the receptor. However, Dnp-treated cells remain visually intact and Dnp has no effect on the rate of binding at 0° in the 105,000*g* supernatant prepared from L cells or in intact cells at 0°.

The reversibility of the Dnp effect on binding was examined by preincubating a suspension of L cells at 37° for 1 hr with [^{3}H]triamcinolone acetonide, centrifuging, and resuspending the cells in medium with glucose or with Dnp in the absence of glucose. The incubations were continued at 37° for 90 min, and the cells were centrifuged again and suspended under the conditions defined in Figure 4. As with all of these experiments, 10^{-8} M [^{3}H]triamcinolone acetonide was present in all incubation media throughout the experiment. Dnp treatment resulted in a marked loss of the bound radioactivity. When Dnp-treated cells were resuspended and incubated at 37° in the presence of glucose, the amount of bound radioactivity rose toward normal, and this return was not affected by puromycin. If Dnp-treated cells were resuspended in medium without glucose or Dnp, the binding did not rise on further incubation at 37°. Dnp-treated cells resuspended in glucose-containing medium demonstrated only a small increase in bound radioactivity if the incubation was continued at 0°. There was some decline of the binding in control samples at 37° during this extended experiment, but no decline in that portion of the control cells which were incubated at 0° after resuspension at 90 min.

The experiment demonstrates that the decrease in binding in the 7000*g* supernatant fraction of the cells which occurs as a result of Dnp treatment can be reversed and hence cannot be attributed to cell lysis. The input of steroid-binding capacity into the system after Dnp treatment requires the presence of glucose, is not affected by puromycin, and is temperature dependent, and time dependent.

Effect of Energy Deprivation and Chase on the Amount of Bound Steroid in the Particulate Fraction. The amount of specific binding recovered in the 7000*g* pellet of cells incubated with [^{3}H]triamcinolone acetonide at 37° is between 20 and 35% of the total specific binding of the cell. In order to determine if depression of binding observed in the supernatant cell fraction with exposure to Dnp or after chase with nonradioactive triamcinolone acetonide results from a redistribution of specifically bound radioactivity from the soluble to the particulate fraction, the experiment presented in Figure 5 was performed. A suspension of cells was preincubated for 1 hr with [^{3}H]triamcinolone acetonide, centrifuged, and resuspended in the presence of glucose, glucose plus chase, Dnp, or Dnp plus chase, and the incubations were continued at 37°. The necessary correction for nonspecific binding was

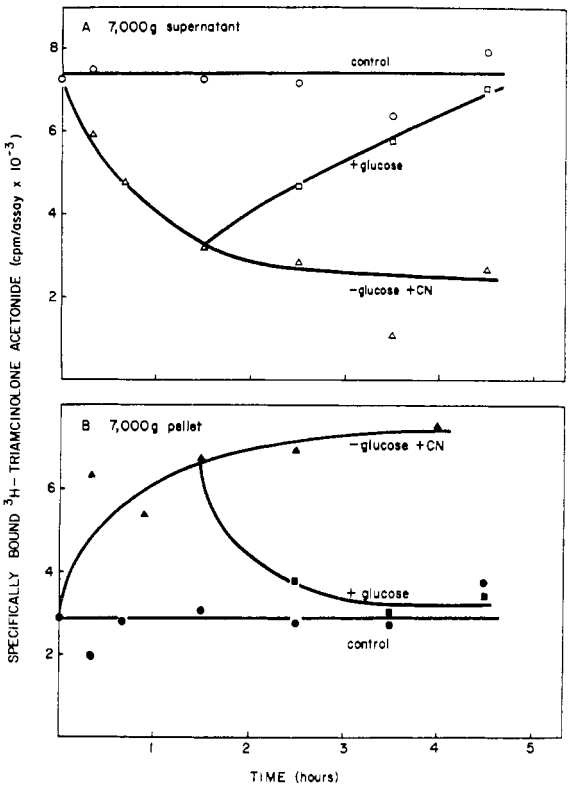


FIGURE 6: The effect of KCN on the distribution of bound radioactive triamcinolone acetonide in soluble and particulate cellular fractions. A suspension of L cells was preincubated with steroid and treated as described in the legend to Figure 5, except that 5×10^{-4} M KCN was added instead of Dnp. Part A: total specifically bound [^{3}H]triamcinolone acetonide in the supernatant fraction (open symbols); part B: corresponding pellet values (solid symbols). The additions were as follows: (○) plus glucose (control); (△) plus KCN minus glucose; (□) glucose added after removal of cyanide by washing.

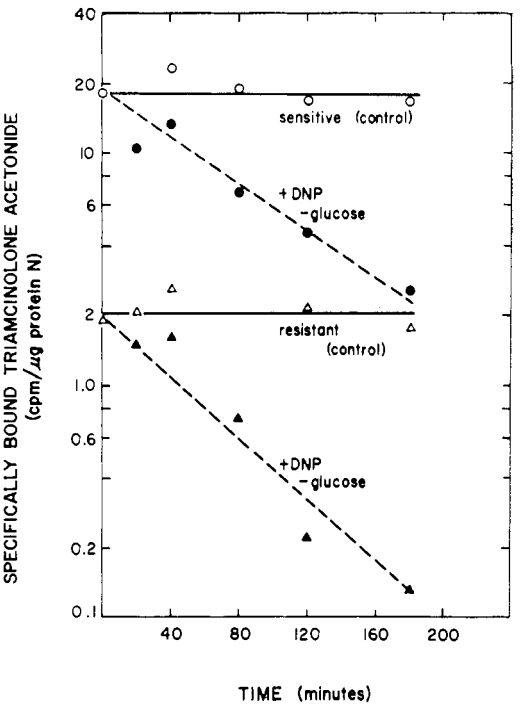


FIGURE 7: The rate of loss of bound radioactive triamcinolone acetonide in resistant as compared to sensitive L cells in the presence of Dnp. Equivalent suspensions of resistant (Res) and sensitive (Sen) fibroblasts were preincubated for 1 hr at 37° with 10^{-8} M [^{3}H]triamcinolone acetonide in the presence or absence of 10^{-5} M 11β-cortisol. After resuspension in glucose-free medium containing radioactive steroid, the cultures were divided, appropriate additions were made, and the incubations were continued for 180 min at 37°. The additions were as follows: (○) Sen cells plus 5 mM glucose; (●) Sen cells plus Dnp minus glucose; (△) Res cells plus 5 mM glucose; (▲) Res cells plus Dnp minus glucose.

obtained by treating replicate suspensions identically, except that both the preincubation and subsequent incubations were carried out in the presence of 10^{-5} M fluocinolone acetonide as well as the radioactive steroid. The amount of binding not blocked by the cold fluocinolone acetonide at each time point was subtracted from all of the corresponding soluble and particulate fractions to give the specific binding in each fraction at each assay point.

As can be seen from part A of Figure 5, approximately 6500 cpm of radioactive steroid was specifically bound in the supernatant fraction and the amount of binding decreased in the presence of chase or with Dnp treatment. About 2500 cpm was specifically bound in the particulate fraction (part B of the figure) and the addition of chase resulted in a loss of binding, but the rate of loss was somewhat slower than in the soluble fraction. Dnp treatment resulted in a rapid increase in the amount of radioactivity which was bound to the particulate fraction. The specific binding in the particulate fraction of Dnp-treated cells always rises to a maximum of about twice the control value, an observation that has been repeated several times. The accumulation of radioactivity in the particulate fraction also rises in cells treated with both Dnp and chase.

The results seen in the presence of Dnp could be due to some sort of direct effect of Dnp on steroid binding to the particulate fraction rather than secondary to energy deprivation. Consequently, cyanide was used instead of dinitrophenol as an inhibitor of aerobic energy production, with similar results (Figure 6). Specific binding in the supernatant fraction decreased sharply with cyanide treatment in the absence of glucose, while glucose addition after 90 min restored the binding to control levels. Specific binding in the particulate fraction mirrored these changes, rising with cyanide treatment in the absence of glucose, and dropping back to control levels with the addition of glucose. Quantitatively, in this experiment almost all of the specifically bound steroid lost from the supernatant solution after glucose deprivation and cyanide treatment can be accounted for in the pellet.

The results of these experiments support the hypothesis that the steroid-bound complex in the soluble and particulate fraction is in some form of energy-dependent equilibrium at 37°. In the presence of Dnp or cyanide the complex can still enter the particulate fraction but it is not readily released again into the soluble fraction unless an energy-yielding substrate such as glucose is present. This recycling hypothesis was confirmed in a temperature-dependent chase experiment discussed below.

Effect of Dnp on the Binding Component in the Soluble Fraction of Resistant Cells. A resistant subline of mouse fibroblasts contains only 10–15% of the specific steroid binding component as the sensitive parent strain (Pratt and Ishii, 1972). This cell line is not inhibited by concentrations of triamcinolone acetonide as high as 10^{-5} M (Hackney *et al.*, 1970). Equivalent suspensions of resistant and sensitive cells were preincubated for 1 hr in the presence of glucose and 10^{-8} M [^{3}H]triamcinolone acetonide. The cell suspensions were centri-

fuged and resuspended in the presence of glucose or in the presence of Dnp minus glucose. The rate of loss of binding from the soluble fraction of both sensitive and resistant cells was the same (Figure 7).

Degradation of the Binding Component in the 105,000g Supernatant. If, instead of incubating radioactive steroid with whole cells, a 105,000*g* supernatant is prepared from L cells, it is found to contain a component which binds [^{3}H]-triamcinolone acetonide at 0° in a manner that is specific for the growth inhibitory effect.

In the experiment presented in Figure 8, a portion of 105,000*g* supernatant was preincubated for 20 hr at 0° with 10^{-8} M [^{3}H]triamcinolone acetonide, divided into four portions, and incubated at 0 and 37° in the presence and absence of a high concentration of nonradioactive steroid. There was no detectable decrease in binding with continued incubation at 0° over this relatively short time of 60 min, although over a much longer time (see insert) there was a slow first-order degradation, not zero order as we thought previously (Pratt and Ishii, 1972). The degradation and/or inactivation which takes place at 37° is a first-order phenomenon. There was no detectable effect of the chase at either temperature.

In a separate experiment (not shown) the 105,000*g* supernatant was preincubated with 10^{-8} M [^{3}H]triamcinolone acetonide at 0°, then incubated at 37°. Rapid loss of binding function was observed (half-time of 20–30 min). If at any time the sample was returned to 0°, degradation stopped. But the addition of fresh radioactive steroid did not produce any increase in binding after a further 90 min of 0° incubation indicating that the decrease in binding observed at 37° was not due to a temperature-dependent metabolism of the steroid. Also, a replicate portion of the 105,000*g* supernatant incubated in the absence of steroid for 1 hr at 37° lost its capacity to bind steroid when returned to 0°. Therefore, in the 105,000*g* supernatant both the unoccupied and steroid-occupied form of the binding component are inactivated or degraded in a temperature-sensitive manner to a form which cannot bind steroid. This process occurs in the absence of a particulate fraction.

The rate of decrease in binding at 37° in the 105,000*g* supernatant is somewhat faster than either the chase or Dnp effects in the intact cell (half-times of 20–30 min *vs.* 30–40 min), and there are no data suggesting that the decrease observed in the subcellular preparation represents the same phenomenon that is taking place in the intact cell.

Effect of Temperature on the Total Specific Binding of Triamcinolone Acetonide in Intact Cells. As was seen in Figure 1, the amount of binding in the soluble fraction of intact cells at 0° is higher than at 37°. A complete time course of binding in the supernatant and particulate fractions of replicate suspensions of intact cells incubated at 37 and 0° is presented in Figure 9A. As before, there is more binding in the soluble fraction of 0° cells than those maintained at 37°, but there is more triamcinolone acetonide bound in the particulate fraction from the 37° incubation. Hence, binding of the steroid complex to the particulate fraction is a temperature-dependent process. The total amount of specific binding is greater in cells incubated at 0° than at 37°. In Figure 9B cells were incubated with steroid for 2 hr at 37° and then switched to 0°. The amount of binding in the particulate fraction did not change but the amount of soluble binding increased to a new plateau. The greater binding capacity of the 0° cells is not well understood; it may be due to a negative temperature coefficient for the affinity constant, well known to occur with steroid hormones (Schaumburg and Bojesen, 1968; Westphal, 1967), but other explanations such as a temperature-dependent redistribution of unbound receptor within the cell may be involved.

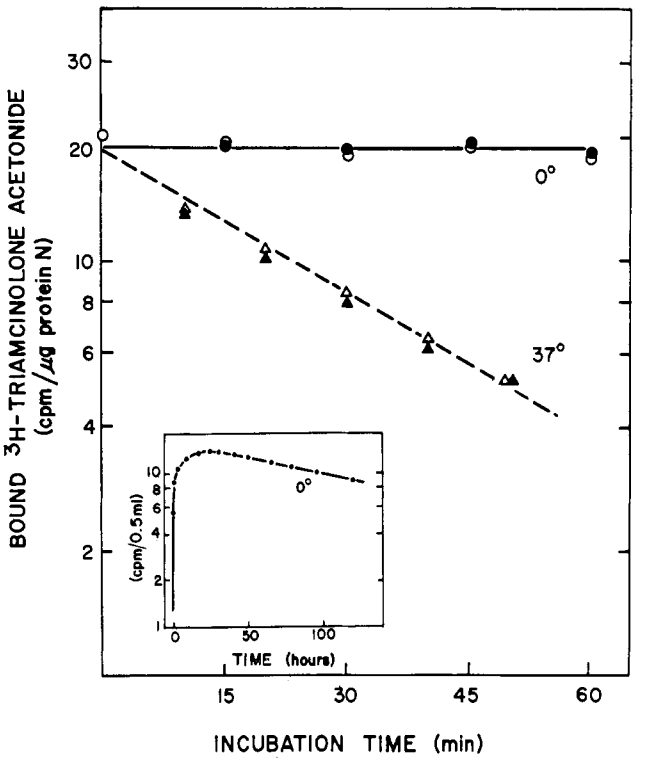


FIGURE 8: Loss of the binding component from the 105,000*g* supernatant at 37 and 0°. A 105,000*g* supernatant (788 μg of protein N/ml) was preincubated with 10^{-8} M [^{3}H]triamcinolone acetonide for 20 hr at 0°. The incubation was divided and continued at 0° or at 37° in the presence (open symbols) or absence (closed symbols) of 10^{-6} M unlabeled triamcinolone acetonide: (●) 0°; (▲) 37°. The insert presents the binding curve of a 105,000*g* supernatant incubated at 0° with 10^{-8} M radioactive triamcinolone acetonide for 120 hr.

Since binding to the particulate fraction is temperature dependent, it is possible to examine the hypothesis of a transfer of cytosol steroid-bound receptor in a chase experiment (Figure 10). Incubation at low temperature resulted in an accumulation of bound radioactivity in the cytosol, with little in the particulate fraction. Addition of 1000-fold excess of nonradioactive steroid, followed by a temperature jump to 37°, clearly demonstrated the transfer of prebound, labeled steroid to the particulate fraction and its subsequent loss.

Discussion

In this work we have chosen to examine the steady-state level of the bound complex of triamcinolone acetonide and the specific glucocorticoid binding component in mouse fibroblasts as it is maintained over short intervals of 2–6 hr in intact cells. In cell cultures containing serum growing under normal conditions, the glucocorticoid binding component is presumably synthesized and degraded in a manner that requires protein synthesis, ultimately controlling the long-term steady-state level of the unbound component in the cell. The maintenance of the steady-state level of the bound complex for the short time interval examined in this work apparently does not require simultaneous protein synthesis. This is inferred from the fact that the steady state, the decrease of binding observed with chase, and the energy-dependent re-

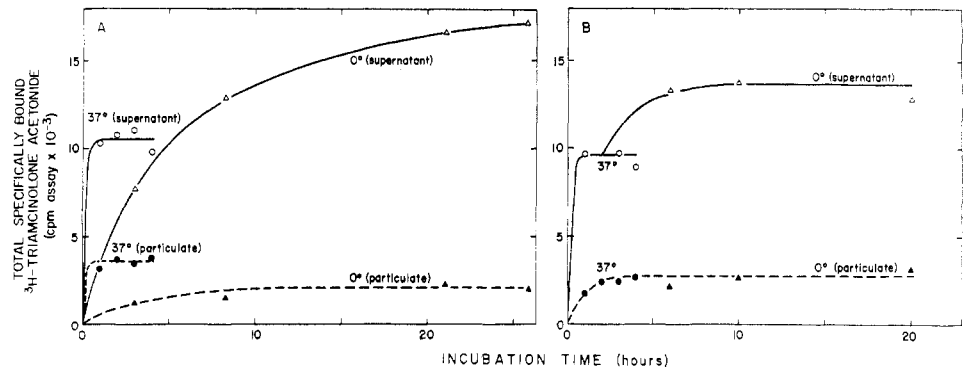


FIGURE 9: The effect of temperature on the distribution of bound radioactive triamcinolone acetonide between particulate and supernatant fractions. In part A of the figure, a suspension of L cells (2.6×10^6 cells/ml) was incubated at 0 and 37° in the presence of 10^{-8} M [^{3}H]triamcinolone acetonide. Parallel incubations were prepared in the presence of 10^{-5} M fluocinolone acetonide. Both the supernatant and the 7000*g* pellet were assayed for bound radioactive steroid as explained in the Methods section, and the specifically bound triamcinolone acetonide is presented in the figure. In part B of the figure, a suspension of L cells (2.3×10^6 cells/ml) incubated under the same steroid conditions as above at 37° was divided at 2 hr. One portion was continued at 37° and the other portion was continued in ice. The distribution of the bound radioactive steroid was determined as for part A. The symbols are as follows: (○) 37°; (△) incubation continued at 0° after temperature switch. The solid symbols indicate the corresponding 7000*g* pellet values.

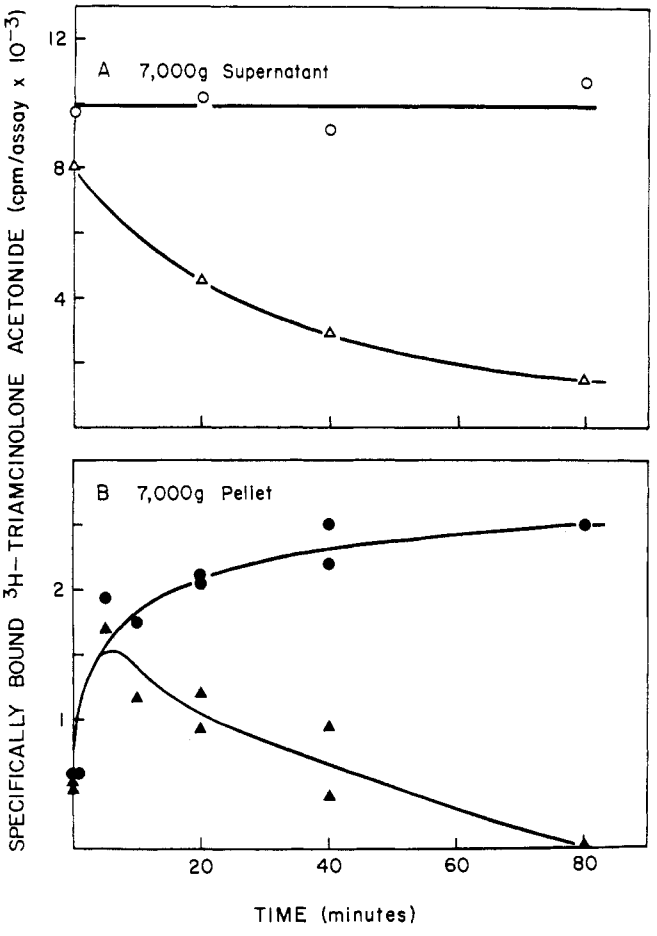


FIGURE 10: The effect of unlabeled chase on the temperature-dependent distribution of bound radioactive triamcinolone acetonide in soluble and particulate cellular fractions. A suspension of L cells (4×10^6 cells/ml) was preincubated for 4 hr with 10^{-8} M [^{3}H]triamcinolone acetonide at 0°. The incubation was divided and continued at 37° for 80 min in the presence or absence of 10^{-5} M nonradioactive triamcinolone acetonide. Part A of the figure presents the total specifically bound [^{3}H]triamcinolone acetonide in the supernatant fraction (open symbols) and part B presents the corresponding pellet values (closed symbols). Part B contains additional data from a second experiment performed only on the particulate fraction. The incubations were as follows: (○) control; (△) plus 10^{-5} M unlabeled triamcinolone acetonide.

appearance of binding after Dnp treatment are all puromycin insensitive. Preincubation of whole cells with puromycin during the absence of steroid does not alter the amount of receptor detectable, at least over a 1-hr period; hence, the steady state could not be explained by steroid stabilization against degradation.

When cells are deprived of a glucose energy source and incubated in the presence of Dnp or cyanide, the amount of bound complex decreases in the supernatant of whole cells in spite of the continued presence of steroid in the incubation medium. At the same time there is an accumulation in the 7000*g* particulate fraction (Figures 5 and 6). The return of binding component from the particulate to the soluble fraction is apparently energy dependent as the particulate-associated portion of the bound complex doubles on treatment with Dnp or cyanide, and is reversed on addition of glucose (Figures 5 and 6). The movement into the particulate fraction is temperature dependent (Figures 9 and 10).

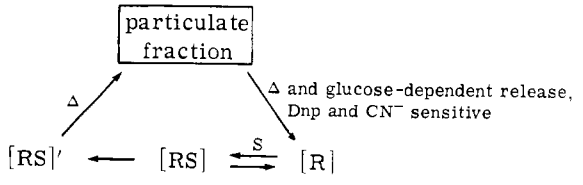
The depression of total bound radioactivity in the supernatant observed with Dnp is reversible. As the reversal is energy dependent, temperature dependent, and unaffected by puromycin, we propose that the binding component has been inactivated in the presence of Dnp and is reactivated or recycled by an energy-requiring process to a form which can bind the steroid. An alternative possibility is that the glucose molecule itself or perhaps a metabolite of it may associate with the binding component and be required for the binding of steroid (*i.e.*, that the glucose has a function other than providing energy).

The ability of a cytoplasmic steroid–receptor complex to form at 0°, but to be transferred to the particulate fraction of the cell only at 37°, is in accord with similar studies of Munck and Wira (1971), in the rat thymus cell. In this system, studied extensively in Munck's laboratory, it is known that the decreased rate of glucose uptake produced by glucocorticoids can be blocked if the cells are kept under anaerobic conditions (Munck, 1968). Young (1969) showed that glucose is required in order to detect cortisol effects on protein and nucleic acid synthesis. Total specific binding of cortisol to thymocytes is decreased in the absence of oxygen and glucose (Munck and Brink-Johnsen, 1968). Some of these effects may well be due to loss of receptor capacity under conditions of glucose deprivation or anaerobiosis.

A detailed study of the thymocyte glucocorticoid receptor by Munck *et al.* (1972) reveals many similarities in the properties of receptor macromolecules from these two sources. As in the present study, the initial glucocorticoid–receptor interaction occurs in the cytoplasm and is temperature independent. Transfer to the nucleus occurs only at 37°, however. Also in accord with the present studies is the observation that ATP or some form of energy is required to regenerate receptor in an active form, and Munck *et al.* (1972) postulate a recycling system much as we do in the present study. The two cell systems do not appear to be identical in all respects—the unbound cytosol receptor in thymocytes appears to be less stable at 3° than the fibroblast receptor, and also appears to bind steroid reversibly. A greater fraction of the bound glucocorticoid is found in the nuclear fraction of thymocytes than fibroblasts, but this may be a consequence of the larger nuclear to cytoplasmic ratio in thymocytes.

The fact that binding can be decreased in an intact cell at 37° by the presence of a chase of excess nonradioactive glucocorticoid does not necessarily mean that the association between a steroid and a specific binding component is reversible. As we have demonstrated, the bound complex is in a dynamic steady state and the chase effect could represent a competition of the unlabeled drug for binding to newly introduced binding component. The evidence for nonreversibility of the steroid–receptor complex at 37° is not completely compelling, but includes the fact that none of the specifically bound steroid in the 105,000*g* supernatant will exchange for unlabeled chase over a 10-day period of incubation at 0° and there is no exchange in the intact cell over a period of 2 days at 0° (Pratt and Ishii, 1972). One would have to presume that if dissociation takes place it must be very temperature sensitive; however, there is no observable dissociation of steroid from the bound complex in the 105,000*g* supernatant with 50 min of incubation at 37° (Figure 8). If the chase effect in the intact cell represented solely dissociation of bound radioactive steroid from a binding component which became occupied by the unlabeled compound, then from Figures 2, 3, 5, and 10 one could expect approximately 50% exchange in the 105,000*g* supernatant within 30–40 min of incubation. In the presence of Dnp or cyanide there is apparently little or no release of the bound complex from the particulate to the soluble fraction and no reappearance of binding component for which unlabeled chase could compete. All of these observations support the proposal that the steroid is associated with the binding component in a manner which does not readily permit dissociation even though the binding is noncovalent (Hackney *et al.*, 1970).

We have earlier suggested a model for steroid binding to receptor macromolecules in an *in vitro*, 105,000*g* supernatant fraction of mouse fibroblasts (Pratt and Ishii, 1972). We can now expand the model to encompass events in the whole cell as follows:



The initial binding of the steroid (S) to receptor (R) is first detected in the cytosol fraction of the cell, although this does not rule out a possible membrane site for the native receptor—it could well be that the hypotonic rupture we employ frees the receptor from an original membrane component. The fact that both R and RS′ degrade rapidly at 37° in an isolated 105,000*g* supernatant suggests that R exists in a protected form in the intact cell.

In any event, the initial binding reaction occurs readily at low temperatures, and the complex is converted to an essentially irreversible form, RS′, which then binds to the particulate fraction (probably the nucleus) in a temperature-dependent manner (Figure 10). Release of the receptor from the particulate fraction, and its regeneration to naked receptor available for new binding, is temperature sensitive, requires glucose, and is inhibited by cyanide or Dnp. The half-time for this recycling process is in the order of 30–40 min for the mouse fibroblast at 37° (Figures 2, 3, 5, and 10).

The cell which has evolved so that it responds to glucocorticoids must be able to respond to low concentrations of these hormones and be able to deliver an effect that is proportional to the concentration of circulating steroid. This is true whether one is thinking of diurnal variations in the level of these hormones or rapid responses to increased levels of hormone released under stress conditions. In addition to producing an initial effect which represents the degree of response desired (this is controlled by those processes that determine the circulating level of hormone), the target cell must be able to de-respond as the level of free steroid declines. We propose the following model in order to explain how the mouse fibroblast has developed the ability to react to low concentrations of hormone and produce a response which continually reflects the changing free hormone concentration to which it is exposed.

As the concentration of circulating glucocorticoid is low, the receptor in the target cell has a high affinity for the hormone in order that it can bind the hormone in the appropriate concentration range. The high affinity of the receptor for the hormone, although favorable perhaps for a rapid response to low hormone concentrations, is not favorable for a rapid recovery from the hormone effect. If dissociation of the drug from the receptor requires many hours or days, the target cell's response might be determined by concentrations of glucocorticoid which have long since dropped to lower levels. The regulation system which has evolved in the L cell appears to involve a continuous turnover or recycling of the specific binding component. This involves a temperature-dependent relocation of the bound complex and an energy- and temperature-dependent regeneration of the receptor to a form which can once again bind the steroid. Thus, in spite of the very tight binding of the receptor by the steroid, the cell is constantly producing free receptor capable of interacting with the hormone. As the circulating level of hormone falls, the target cell response diminishes while new receptor is produced. This leaves the cell in a state to again respond rapidly to a change in the hormone level and permits a titration of the target cell response by variations in the circulating level of free hormone. In this model, both the tight binding and the rapid turnover which determines the steady state are necessary for the appropriate physiological response of the target cell.

Acknowledgments

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Purification and Properties of a Microsomal Cyclic Adenosine Monophosphate Binding Protein Required for the Release of Tyrosine Aminotransferase from Polysomes†

G. Donovan and I. T. Oliver*

ABSTRACT: The isolation and partial purification of a microsomal protein which binds adenosine 3′,5′-cyclic monophosphate are described. The protein, together with cAMP, is required for the release of tyrosine aminotransferase from neonatal rat liver polysomes. Experiments on heat stability and degradation by pronase, ribonuclease and deoxyribonuclease confirm the protein nature of the binding factor. The protein has a molecular weight of 40,000 by zone sedimentation and binds cAMP with an intrinsic association constant of 3.6×10^{-9} M. cAMP remains unchanged as a result of binding. The most powerful competitors of the binding of [^{3}H]cAMP to the protein are: unlabeled cAMP, monobutyryl-cAMP, dibutyryl-cAMP, cGMP, and cIMP. None of the cyclic pyrimidine nucleotides which were tested are competitors and deoxy-cAMP is only a very weak competitor. 2′,3′-cAMP and a range of other nucleotides are without effect on binding of cAMP.

In previous work from this laboratory a cytoplasmic translational control mechanism for enzyme synthesis has been characterized (Chuah and Oliver, 1971, 1972). The system was found during investigations on the mechanism of induction of tyrosine aminotransferase in neonatal rat liver, and by experiments *in vitro* it was demonstrated to operate at the release step in the synthesis of subunits of the enzyme on microsomal-bound polysomes (Chuah and Oliver, 1971, 1972). Adenosine 3′,5′-cyclic monophosphate[1] was shown to be a specific effector involved in the mechanism, and by fractionation of the system *in vitro* a further component was demonstrated. This component, a release factor, is normally found in microsomes of fetal, neonatal, and adult rat liver (Chuah and Oliver, 1971) and it binds cAMP with high specificity and high affinity. The factor has no activity in the release function in the absence of cAMP and is not active with other cyclic nucleotides. In the present paper, the purification and characterization of the release factor are described.

Methods

Animals. Adult male or female rats of the Wistar strain of *Rattus norwegicus* were used without special preparation. Animals were killed by a blow to the head and cervical fracture. Livers were excised immediately and chilled in ice-cold 0.25 M sucrose in TKM buffer.

Preparation of the Crude Microsomal Extract. All steps in the preparation of the crude microsomal extract were at 0–4°. Approximately 40 g of rat liver was homogenized in a coaxial homogenizer in two volumes of 0.25 M sucrose in TKM buffer. The homogenate was centrifuged at 600*g* for 10 min in the PR-2 International centrifuge and the resultant supernatant was further centrifuged at 6000*g* for 15 min. The 6000*g* supernatant was centrifuged at 105,000*g* (1 hr) in the type 50

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[1] Abbreviations used for nucleoside 3′,5′-cyclic monophosphates have the word cyclic (or the symbol c) preceding the conventional abbreviation for 5′-nucleoside monophosphates: adenosine 2′,3′-cyclic monophosphate (2′,3′-cAMP); N^6,2′-*O*-dibutyryladenosine 3′,5′-cyclic monophosphate (dibutyryl-cAMP); N^6-monobutyryladenosine 3′,5′-cyclic monophosphate (monobutyryl-cAMP); deoxyadenosine 3′,5′-cyclic monophosphate (deoxy-cAMP). Other nucleotides are abbreviated conventionally. The Tris–EDTA–boric acid buffer of Aronsson and Grönwall (1957) is abbreviated as TEB. Double strength TEB is written as 2 × TEB. Other abbreviations used are: TTM buffer, 50 mM Tris-HCl–8 mM theophylline–6 mM mercaptoethanol (pH 7.4); TM buffer, 50 mM Tris-HCl–6 mM mercaptoethanol (pH 7.4); TKM buffer, 50 mM Tris-HCl–25 mM KCl–5 mM $MgCl_2$ (pH7.4).