Subcellular Distribution of Glucocorticoid Receptors in Mouse Fibroblasts†

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ABSTRACT: Mouse fibroblasts contain a macromolecular binding component (receptor) which binds glucocorticoids specifically and with high affinity. This study shows that there are three different cellular forms of bound receptor and that it is experimentally possible to markedly alter the subcellular distribution of these three forms. Cells incubated with [^{3}H]triamcinolone acetonide were broken after hypotonic shock and a 7000*g* hypotonic supernatant was obtained; the pellet was extracted with 0.3 M KCl, yielding a nuclear extract; the remaining pellet was resuspended in water, sonicated, and assayed for "nuclear residual" (*i.e.*, nonextractable) radioactivity. If whole cells are incubated at 0° in a growth medium, almost all of the bound steroid is located in the hypotonic supernatant fraction. Incubation at 37° produces a shift of the steroid-bound macromolecule into the nuclear extractable form, while omission of glucose and addition of KCN at 37° markedly increase the nuclear residual form at the expense of both the nuclear-extractable and supernatant forms. Since DNase treatment of chromatin liberates a soluble steroid–receptor complex, we believe that the nuclear residual form may be steroid–receptor complex tightly bound to chromatin. We propose a model suggesting that an energy-requiring process is required to generate free receptor from the chromatin complex to complete the normal cellular recycling system.

The mouse fibroblast (L929) is one of many types of cells which respond to glucocorticoids. The pharmacological response elicited in fibroblasts is growth inhibition, in contrast to the glucocorticoid response observed in hepatoma (HTC) cells which is induction of the enzyme tyrosine aminotransferase (Baxter *et al.*, 1972). Lymphosarcoma P1798 (Baxter *et al.*, 1971) and thymocytes (Hallahan *et al.*, 1973) both exhibit cytolysis in the presence of glucocorticoids. Although there are differences in physiological response, glucocorticoid-sensitive cells have in common macromolecular binding components (receptors) which specifically and with high affinity bind glucocorticoids.

Extensive investigations have led to the notion that glucocorticoids may operate by receptor mechanisms similar to the well-studied estrogens (*cf.* reviews by Munck and Wira, 1971; Jensen and DeSombre, 1973). Current theory holds that upon entering the cell, the steroid binds to a cytoplasmic receptor, causing (or associated with) a transformation which permits a translocation of the steroid–receptor complex into the nucleus. After gaining entrance to the nucleus the steroid-bound receptor is thought to initiate events (presumably gene repression or derepression) which eventually result in the physiological response.

In mouse fibroblasts the concept that the macromolecular binding component is the receptor mediating the growth-inhibitory effect is supported by the following three observations. (1) Active glucocorticoids compete for the binding of triamcinolone acetonide,[1] 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 but chemically similar steroids do not compete for this binding. (2) The specific binding of triamcinolone acetonide is saturable at the concentration of drug which produces a maximal growth inhibitory effect. (3) A drug resistant cloned subline of L929 cells contains only 10–15% of the amount of binding component found in the sensitive cells.

Some of the factors which maintain the steady-state level of glucocorticoid receptor binding in mouse fibroblasts have been examined (Ishii *et al.*, 1972). Triamcinolone acetonide binding occurs initially in the cytosol to form what appears to be an essentially irreversibly bound complex. There is an active turnover or cycling of the complex between the cytosol and nuclear fractions in the intact cell whereby the steady-state level of cytoplasmic complex is maintained. The cyclical release of receptor from the nucleus to the cytoplasm in an unbound form is temperature dependent, requires glucose metabolism, and is inhibited by cyanide or dinitrophenol. The addition of excess unlabeled glucocorticoid will result in a rapid decrease in bound radioactive steroid in the cytosol, but this chase effect is apparently not due to exchange. Rather, it is due to competition for rebinding as the binding component cycles through the nucleus. This cycling hypothesis has subsequently been confirmed in HTC cells (Rousseau *et al.*, 1973).

Since it seems reasonable that the specific glucocorticoid receptor mediates the cellular response, a knowledge of factors affecting its subcellular location should provide insights into its mechanism of action. In this paper we examine the subcellular distribution of the steroid-bound receptor under various conditions. In addition to the well known steroid-

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[1] Abbreviations used are: triamcinolone acetonide, 9α-fluoro-11β,16α,17α,21-tetrahydroxypregna-1,4-diene-3,20-dione-16,17-acetonide. 11α-cortisol, 11α,17α,21-trihydroxypregn-4-ene-3,20-dione; 11β-cortisol, 11β,17α,21-trihydroxypregn-4-ene-3,20-dione; Hepes buffer, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

bound cytosol and nuclear salt extractable forms of the receptor, we report the finding of a new type of specifically bound glucocorticoid–receptor complex, which preliminary evidence indicates is very tightly bound to chromatin.

Materials and Methods

Materials. [1,2,4-^{3}H]Triamcinolone acetonide[1] (9.2, 10.7, or 4.0 Ci/mmol) was purchased from Schwarz Bio-Research, Inc., Orangeburg, N.Y., or from New England Nuclear (18.9 Ci/mmol), Boston, Mass. 11α-Cortisol[1] was donated by the Squibb Institute for Medical Research while 11β-cortisol[1] was purchased from Mann Research Laboratories, Inc., New York, N.Y. Electrophoretically pure DNase I was obtained from Schwarz/Mann, Orangeburg, N.Y., protease-free RNase from Sigma Chemical Co., St. Louis, Mo., and B Grade Pronase from Calbiochem, Los Angeles, Calif. Norit A charcoal and Dextran T70 were purchased from Pfanstiehl Laboratories, Inc., Waukegan, Ill., and Pharmacia Fine Chemicals, Uppsala, Sweden, respectively. All other chemicals were reagent grade and were used without further purification.

Cells and Media. Suspension cultures of L929 fibroblast cells were maintained in basal medium (Eagle, 1955) modified as previously described (Gray *et al.,* 1971). Cultures were maintained with constant stirring in an atmosphere of humidified air.

Incubation of Intact Cells. Cells were harvested by centrifugation at 600 *g* for 10 min at 0–4°. Depending upon the experiment, the cells were resuspended in cold (0°) or warm (37°) growth medium without serum containing 25 m*M* Hepes[1] buffer (pH 7.35). In most cases the cell suspension was then divided and incubated with 10^{-8} M [^{3}H]triamcinolone acetonide in the presence of either 10^{-5} M 11α-cortisol or 10^{-5} M 11β-cortisol. Cells were resuspended at a concentration no greater than 3–4 × 10^6 cells/ml since higher cell concentrations will not reliably maintain a steady-state level of steroid-bound receptor (Ishii *et al.,* 1972).

Glucose and KCN in water were added at appropriate concentrations in $^1/_{100}$ the incubation volume. All steroids were prepared in 10% ethanol at 100 times the final concentration employed and added as above. Competing steroids, *i.e.* 11α- and 11β-cortisol, were always added to the cell suspension immediately before the [^{3}H]triamcinolone acetonide.

Cell Homogenization and Fractionation. Aliquots (10.0 ml) of a cell suspension were harvested by centrifugation at 1500*g* for 1 min and the supernatant medium was rapidly removed by aspiration. All subsequent procedures were performed at 0–4°. The cell pellets (~0.10 ml) were immediately suspended in 2.0 ml of chilled hypotonic buffer (0.01 M Tris (pH 7.35)–0.1 mM EDTA) for 5 min and homogenized with 20 strokes of a tight fitting pestle in a Dounce-type all-glass homogenizer. If only a hypotonic preparation was desired the homogenate was immediately spun at 7000*g* for 10 min. If a comparison between isotonic and hypotonic preparations was desired, exactly 0.1 vol of hypertonic (1.43 M NaCl, 0.11 M KCl, and 0.11 M Tris (pH 7.35)) buffer was added to a portion of the homogenate to bring it to isotonicity before centrifugation. The other portion of the homogenate received 0.1 vol of hypotonic buffer for control purposes.

An aliquot of the 7000*g* supernatant fraction[2] was assayed for bound radioactivity, and the remainder was immediately removed and retained for protein determination. The pellet, composed of nuclei and cytoplasmic debris, was extracted for 10 min by resuspension in 2.0 ml of extraction buffer (0.3 M KCl–0.01 M Tris (pH 7.35)) followed by centrifugation at 7000*g* for 10 min. As before, the supernatant was removed and an aliquot (nuclear extractable fraction) was assayed for bound radioactivity. The residual pellet was then sonicated in 2.0 ml of hypotonic buffer using a Bronwill Biosonik III at a setting of 30 until no visible particles remained. An aliquot of this suspension was directly assayed for total radioactivity.

Assay for Bound Radioactivity. We employed the dextran coated charcoal adsorption technique for all our binding assays (Baxter *et al.,* 1971; Beato and Feigelson, 1972). An aliquot of the sample to be assayed was added to a 0.5 vol of charcoal dextran suspension (3.75% Norit A and 0.375% Dextran T70 in isotonic buffer). The mixture was incubated 5 min and spun at 7000*g* for 10 min. A volume equal to the original aliquot was removed and counted in a scintillation counter. Controls were run to demonstrate that the charcoal method removed at least 99% of free radioactive steroid under all conditions employed in these experiments.

Assays for Radioactivity and Protein. The aliquot to be assayed was added to 10 ml of a scintillation solution prepared according to Bray (1960) and counted in a Packard Tri-Carb liquid scintillation spectrophotometer, Model 3310. Quenching was determined by the use of internal standards. Protein determinations were performed according to the method of Oyama and Eagle (1956).

Results

Optimum Conditions for Extraction of Nuclear Bound Radioactivity. The following variables were investigated to determine optimal nuclear extraction conditions: salt concentration and pH of the extraction buffer, temperature of the extraction procedure, volume required for total extraction, contamination of extract by cytosol receptor, and time course of the extraction.

A suspension of L cells was divided in half and incubated 60 min at 37° with 10^{-8} M [^{3}H]triamcinolone acetonide in the presence of either 10^{-5} M 11α-cortisol or 10^{-5} M 11β-cortisol. The steady-state level of cellular binding is achieved in 30 min and can be maintained at least 6 hr under these conditions (Ishii *et al.,* 1972). The cells were centrifuged at 600*g* and homogenized in hypotonic buffer. Figure 1 shows the results of an experiment examining the effect of various concentrations of KCl in extracting bound radioactivity from the particulate fraction.

In the case of cells incubated with noncompeting 11α-cortisol, the amount of bound radioactivity extracted from the pellet increases markedly with salt concentration up to about 0.3 M KCl where a plateau is attained (part A). Correspondingly, the residual radioactivity remaining in the extracted pellet decreases with increasing salt concentration, leveling off at about 0.3 M KCl. The residual pellet and bound extracted radioactivity do not give a constant total over the salt concentration range studied because the extracted free radioactivity (*i.e.* total extracted − bound extracted radioactivity) also exhibits a salt dependence in its yield. However, the sum of total extracted and residual pellet radioactivity is constant. With those cells incubated in the presence of 11β-cortisol similar patterns are seem but they are very much reduced reflecting the ability of 11β-

[2] No significant differences were noted between 7000-, 10,000-, or 105,000*g* supernatants.

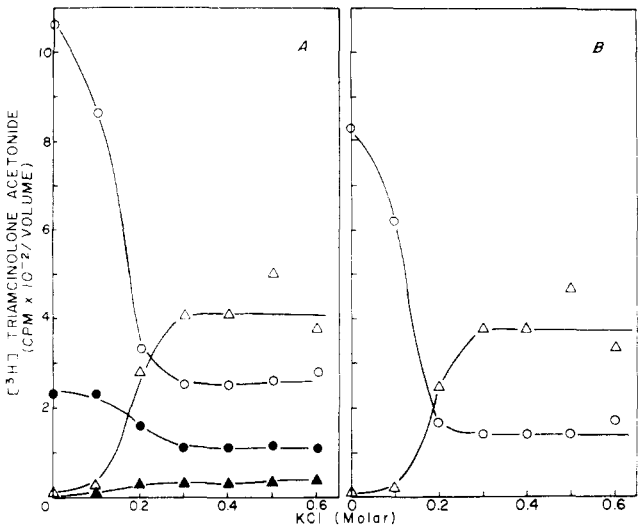

FIGURE 1: The effect of salt concentration on extraction of bound radioactivity from the nuclear pellet. Replicate cell suspensions (1×10^7 cells/ml) were incubated with 10^{-8} M tritium-labeled triamcinolone acetonide and either 11α- or 11β-cortisol (10^{-5} M) for 40 min at 37°. The cells were centrifuged at 600g and homogenized in hypotonic buffer. Aliquots were removed and centrifuged at 7000g, and the supernatants discarded. The remaining pellets were resuspended and extracted for 10 min with Tris buffer containing various concentrations of KCl (in duplicate) and subsequently resedimented. The supernatants were removed and assayed for bound radioactivity. The pellets were resuspended in a volume of buffer similar to that used for extraction, sonicated, and assayed for radioactivity: (A) bound radioactivity in the extraction supernatant using cells bound with 11α- (△) or 11β-cortisol (▲); sonicates with 11α- (○) or 11β-cortisol (●); (B) the difference between samples bound in the presence of 11α- or 11β-cortisol which we define as specifically bound nuclear extractable radioactivity (△) or specific nuclear residual radioactivity (○).

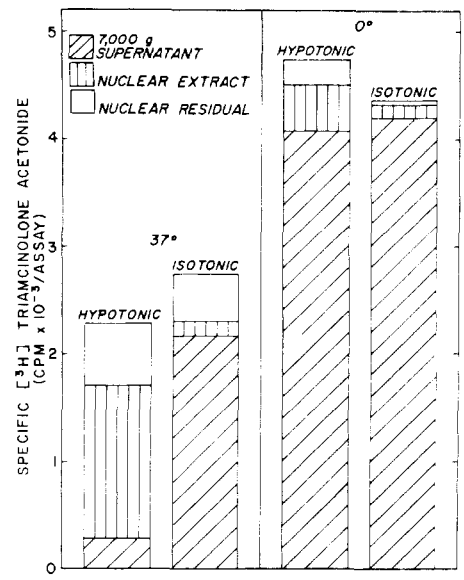

FIGURE 2: The effect of temperature and salt concentration on the subcellular distribution of bound [^{3}H]triamcinolone acetonide. An ice chilled suspension of L cells (3.6×10^6 cells/ml) in growth media without serum containing 25 mM Hepes buffer was divided in half. Each half received 10^{-8} M [^{3}H]triamcinolone acetonide and either 10^{-5} M 11α-cortisol or 10^{-5} M 11β-cortisol. The two portions were again split in half, one of which was incubated at 0° for 21 hr and the other at 37° for 1 hr. After incubation was complete 10-ml aliquots were taken and worked up as explained under Materials and Methods to obtain the 7000g supernatant, nuclear extract, and nuclear residual radioactivity fractions. At both temperatures a comparison between isotonic and hypotonic sample preparation was made by adding a 0.1 vol of either hypertonic or hypotonic buffer to the homogenate prior to the 7000g centrifugation. The data shown here are the specific radioactivites, *i.e.* counts per minute in 11α-cortisol sample − counts per minute in 11β-cortisol sample.

cortisol to compete for virtually all of the specific glucocorticoid receptor binding to radioactive triamcinolone acetonide.

Since 11α-cortisol is an inactive steroid and 11β-cortisol is an active glucocorticoid, the difference between incubation in the presence of 11α- and 11β-cortisol represents the specific radioactivity (Figure 1B). The amount of bound specific radioactivity extracted from the pellet is henceforth referred to as "nuclear extractable radioactivity" or "nuclear extractable receptor" while the nonextractable radioactivity is referred to as "nuclear residual radioactivity." Based upon this experiment we have chosen a 0.3 M KCl salt buffer to use in all further nuclear extractions.

At 0.5 M and higher salt concentrations the nuclear pellet becomes quite viscous and resists pelleting by centrifugation, indicating lysis of the nuclei and subsequent release of chromatin into solution; phase contrast microscopy confirmed this notion. This finding gave us concern that our 0.3 M KCl extraction might also be lysing a fraction of the nuclei and leading to unclear results. However, microscopic examination of nuclei before and after our extraction procedure showed no significant nuclear lysis.

Using an experimental design similar to that above, i.e. obtaining replicate nuclear pellets from steroid-bound cells, we investigated the effect of buffer pH and temperature on the 0.3 M KCl nuclear extraction. Within the range of pH 6.5–8.5, we found that the yield of extractable bound radioactivity showed no pH dependence. Also, the yield was not significantly improved by temperatures above 0°. Thus we chose to use a 0.01 M Tris buffer (pH 7.35 and 0°) for our 0.3 M KCl extractions. The time course of extraction was also examined, and maximal extraction of bound radioactivity was achieved in less than 10 min.

To determine the optimum volume of extraction buffer needed to quantitatively obtain all soluble nuclear counts the following experiments were performed. Extraction buffer containing ^3H_2O was prepared and added in various volume ratios to nuclear pellets obtained from a measured volume of (unbound) whole cells. The pellets were resuspended in the buffer and centrifuged, and an aliquot of the supernatant was counted. We observed that the nuclear pellet was much smaller after extraction and we anticipated this shrinking should lead to a dilution of our 0.3 M KCl buffer. Our data (not shown) confirmed this and indicated a volume of 7:1 or 8:1 buffer–pellet was needed to attain the final salt concentration desired.

Next, cells were incubated with [^{3}H]triamcinolone acetonide and aliquot portions were harvested yielding replicate cell pellets of 1 ml. The cells were broken hypotonically and nuclear pellets obtained which were extracted three times in succession with 10 ml of extraction buffer. The extractions yielded bound counts of 2128, 128, and 26 cpm/ml demonstrating that ~95% of the total extractable bound radioactivity could be obtained with one tenfold extraction volume. This finding, in conjunction with the results of the experiment immediately above, led us to use a single tenfold or greater volume of extraction buffer for determination of nuclear extractable radioactivity.

The experiment in Figure 1 also permits an estimate of cytosol receptor contamination due to nonspecific adsorption or trapping in the nuclear pellet. Extraction with hypo-

tonic buffer is shown on the ordinal axis; this extraction produced specifically bound counts of 5 cpm/ml. The cytosol supernatant contained 128. Since our hypotonic buffer contains no salt, this extraction is actually a "wash" of the pellet. Thus, one may readily calculate that approximately 4% ($^5/_{128}$) of the cytosol receptor is trapped in or associated with the nuclear pellet. Similarly, knowing the 0.3 M KCl nuclear extract contained 372 cpm/ml, one may calculate that contaminating cytosol appears to represent at most about 1% ($^5/_{372}$) of those counts obtained by our 0.3 M KCl extraction. These results demonstrate that a wash of the nuclear pellet after cell homogenization would not significantly alter the results obtained for cytoplasmic or nuclear extracted bound radioactivity.

Intracellular Distribution of Bound Receptor as a Function of Binding Temperature. The intracellular distribution of specifically bound radioactive triamcinolone acetonide was determined in cells incubated at 37° for 1 hr and in cells incubated with steroid at 0° for 21 hr. After binding, samples were taken from each suspension to measure cytosol, nuclear extractable, and nuclear residual radioactivity as outlined under Materials and Methods, using a "hypotonic" and an "isotonic" procedure. The only difference between the hypotonic and isotonic preparations was that the broken cell suspension of one remained at hypotonicity throughout, while the other suspension was exposed to isotonic salt concentration prior to separation of the nuclear and cytoplasmic fractions. Figure 2 shows the results obtained in one such experiment expressed as total specific counts per minute/cell compartment (*i.e.*, bound 7000*g* supernatant, bound nuclear extract, or nuclear residual). Note that one obtains more total binding at 0° than at 37° primarily located in the soluble, 7000*g* supernatant fraction. This finding is in agreement with previously published results (Ishii *et al.*, 1972). Note also that one may obtain a considerably different distribution pattern in hypotonic compared to isotonic conditions at each temperature of binding. This temperature and salt dependent behavior is better illustrated in Table I.

In Table I the specific counts found in each subcellular fraction or compartment are given as a percentage of the total (data from three experiments). It is important to recognize that, due to experimental design (see Materials and Methods), the cytoplasmic and nuclear extract determinations are glucocorticoid-specific, macromolecular-bound counts while the residuals are total counts (but glucocorticoid specific), not all of which may have been originally bound to a receptor moiety. The most striking feature of Table I is the different receptor distribution found at 0° as compared to 37° in the hypotonic preparations. At 0° ~90% of the radioactivity is in the 7000*g* supernatant as opposed to ~20% at 37°. A similar situation does not obtain for the isotonic preparations; here the distribution is not substantially different at the two temperatures, although there appears to be a slightly higher percentage of cytoplasmic radioactivity at 0°.

Another useful comparison to be made is between the two types of preparations at the same temperature. At 0° there is very little difference between the isotonic and hypotonic preparations while at 37° most (~80%) of the radioactivity is in the nuclear fractions under hypotonic conditions, but in the 7000*g* supernatant fraction (~80%) under isotonic conditions.

Distribution of Bound Receptor after Energy Deprivation. In a previous publication (Ishii *et al.*, 1972) it was shown that depriving L cells of glucose and/or addition of dinitrophenol or cyanide resulted in a major change in the subcellular distribution of bound receptor shifting it from the 7000*g* supernatant fraction into the pellet fraction. This study did not distinguish between the residual and extractable bound nuclear specific radioactivity as described here, but gave the total specific radioactivity associated with the nuclear fraction. Thus, we reinvestigated the effect of energy deprivation with particular regard to the change (if any) in the type of nuclear radioactivity.

TABLE I: Effect of Temperature and Salt Concentration on the Subcellular Distribution of Bound [^{3}H]Triamcinolone Acetonide.[a]

	% of Total Specific Radioact. in Cell			
	37°		0°	
	Hypotonic	Isotonic	Hypotonic	Isotonic
7000*g* supernatant	18 ± 1	80 ± 8	87 ± 4	94 ± 2
Nuclear extract	63 ± 3	11 ± 7	9 ± 4	3 ± 1
Nuclear residual	19 ± 3	9 ± 4	3 ± 1	3 ± 1

[a] The data from three experiments like that shown in Figure 2 were normalized on the basis of the cell concentration and then the average radioactivity (with standard error) found in each subcellular compartment was calculated as a percentage of the total in the cell.

A suspension of cells was split in half and bound for 40 min at 37° with 10^{-8} M [^{3}H]triamcinolone acetonide and either 10^{-5} M 11α- or 11β-cortisol. The cells were spun down and resuspended in growth medium without glucose, and each suspension was again divided into two portions; one α and one β suspension received glucose (final concentration 5 mM) while the other two suspensions received KCN (final concentration 5×10^{-4} M). Incubation was continued at 37° for 90 min with triplicate 10-ml samples taken at various time intervals. Each sample was treated as explained under Materials and Methods to obtain the bound 7000*g* supernatant, bound nuclear extractable, and nuclear residual counts. Results of one such experiment are shown in Figure 3. Both the supernatant and nuclear extractable bound counts undergo a substantial reduction over the course of 90-min incubation in glucose-free medium containing cyanide. Concomitantly, the specific nuclear residual counts exhibit a quantitatively equal rise as is shown by summation to give total counts in the system. The drop in nuclear extractable counts occurs immediately while the drop in cytosol counts appears to have an initial lag. The results of this experiment are in agreement with those previously published with the important new finding that it is only the nuclear residual and not both types of nuclear radioactivity that increases after energy deprivation.

Nature of Nuclear Residual Counts. A suspension of cells was prebound with radioactive steroid for 45 min and subjected to glucose deprivation and cyanide addition for 90 min as above. All the glucose deprived cells were harvested and homogenized in hypotonic buffer and aliquots were taken. After centrifugation, the replicate nuclear pellets were extracted twice with 0.3 M KCl. Further extractions do not release measurable amounts of additional bound steroid. A buffer of 0.5 M NaCl, 0.05 M $MgCl_2$, and 0.01 M Tris (pH 7.35) containing 10^{-5} M nonradioactive triamci-

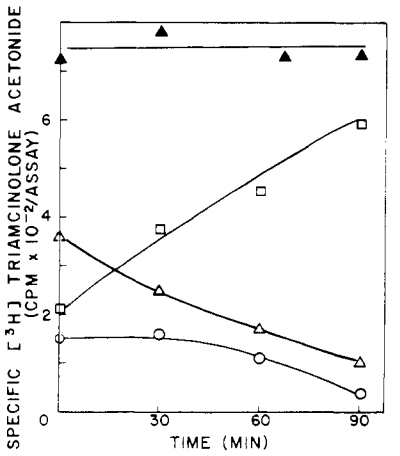

FIGURE 3: The effect of KCN and glucose deprivation on the subcellular distribution of radioactive triamcinolone acetonide in L cells. Replicate suspensions of cells were preincubated for 1 hr in growth medium containing 5 mM glucose and 25 mM Hepes (pH 7.35), with 10^{-8} M [^{3}H]triamcinolone acetonide and either 10^{-5} M 11α- or 11β-cortisol. The cells were centrifuged and resuspended in glucose-free medium containing Hepes buffer and steroids as above. Both suspensions were made 5×10^{-4} M in KCN and incubation at 37° was continued. Triplicate samples at the times indicated were worked up as in Figure 2; similarly, the data presented are specific radioactivities. The symbols are as follows: 7000g supernatant (○), nuclear extract (△), nuclear residual (□), and total radioactivity (▲). Control experiments (not shown) of cells incubated in the continuous presence of glucose without cyanide show a distribution of radioactivity after 90 minutes which is identical with the distribution at zero time.

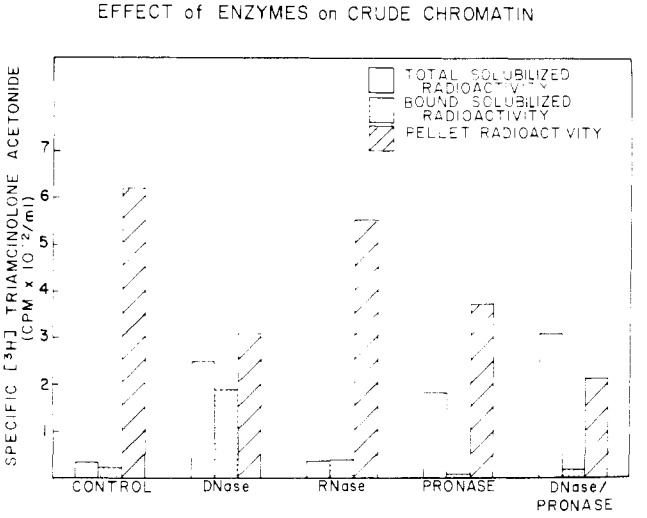

FIGURE 4: The nature of the specific nuclear residual radioactivity. Suspensions of L cells were incubated with steroids in normal media and further incubated 90 min in glucose-deficient media as described in the legend to Figure 3. The cells were homogenized in hypotonic buffer and centrifuged and the nuclear pellets extracted twice with extraction buffer. The extracted pellets were lysed in a 0.5 M NaCl–0.05 M $MgCl_2$–0.01 M Tris (pH 7.35) buffer. Enzymes were added as indicated to the lysed nuclei (duplicates) and incubation was continued for 1 hr at 0°. The digested suspensions were centrifuged at 10,000g and both bound and total solubilized radioactivities were assayed in the supernatants. The pellets were sonicated in a volume of buffer equal to the supernatants, and an aliquot assayed for radioactivity. The values presented represent specific radioactivity as defined previously.

nolone acetonide was added to the extracted pellets to lyse the nuclei (Pratt *et al.*, 1968) and the crude chromatin suspension was incubated at 0° for 60 min with the following enzymes: DNase, RNase, Pronase, or DNase + Pronase. Following digestion each incubation was centrifuged at 10,000g and the supernatant assayed for both bound and total radioactivity; the remaining pellets were resuspended in buffer, sonicated, and assayed for radioactivity. The results are shown in Figure 4. Digestion with DNase, Pronase, or DNase + Pronase leads to an increase in total supernatant radioactivity and a concomitant decrease in pellet radioactivity; RNase has no significant effect relative to the control. However, it is only with DNase treatment that we observe an increase in *bound* supernatant radioactivity. Similar results could be obtained using crude chromatin preparations from cells incubated with steroid under normal media conditions at 37° although, as expected, the level of 10,000g supernatant bound radioactivity following DNase treatment is much lower. Controls were run with bound 7000g cytosol receptor to demonstrate that DNase and RNase had no effect while Pronase lowered the bound radioactivity to nonspecific levels. These experiments demonstrate that a significant fraction of the nuclear residual radioactivity is bound to protein and that it is in some manner associated with DNA. This finding strongly suggests that the nuclear residual radioactivity represents steroid–receptor complex tightly bound to chromatin.

Our inability to solubilize all radioactivity from the pellet using DNase treatment (Figure 4) reflects insufficient enzyme digestion and possibly some nonspecific absorption of released bound radioactivity in the pellet. In later experiments (not shown) where longer DNase treatment was used, approximately 70% of the nuclear residual radioactivity could be solubilized in the bound form. Using numbers from these experiments, we calculate that cells bound with steroid at 37° have at least 6% of their total receptor in the nuclear residual form and correspondingly, under conditions of glucose deprivation, the percentage of bound receptor in the nuclear residual form can increase to 53%. Cells bound to equilibrium at 0° had less than 0.5% bound residual radioactivity.

Effect of Temperature and Chase on the Subcellular Distribution of Triamcinolone Acetonide in L Cells. We have demonstrated that there is a marked temperature dependence in the subcellular distribution of steroid-bound receptor (Figure 2, Table I). At 0° there is no detectable nuclear residual and very little nuclear extractable receptor present. At 37° there is a high level of nuclear extractable receptor and a small but measurable amount of nuclear residual. By binding cells initially at 0° for 4 hr and then jumping the temperature to 37° we hoped it might be possible to measure the kinetics of translocation and gain insights into its mechanism. This experiment is shown in part A of Figure 5. Raising the temperature to 37° causes a very rapid loss of bound radioactivity from the cytosol fraction and equally rapid gains in both nuclear extractable and nuclear residual levels. The translocation process is essentially complete in about 5 min. We did not detect any significant difference in the kinetics of the appearance of the two forms of nuclear receptors, indicating that the equilibrium between the two must take place faster than the translocation of cytosol receptor to the nucleus.

The addition of a 1000-fold unlabeled triamcinolone acetonide chase immediately prior to the temperature jump demonstrates that the change in subcellular receptor distribution results from a redistribution of prebound receptor and not from additional steroid receptor binding (Figure 5B). However, rather than attaining a steady state (Figure 5A) the specific radioactivity eventually drops as it is com-

peted for by the nonradioactive chase in the cellular cycling mechanism (Ishii *et al.*, 1972). This result indicates that both types of nuclear receptors are involved in the cycling process and presents further evidence for the physiological importance of the nuclear residual form of the receptor.

Discussion

For some time now many laboratories studying steroid receptors have discussed their systems in relation to two forms of the steroid-bound receptor, a cytoplasmic form and a nuclear salt-extractable form (Jensen and DeSombre, 1973; Chamness and McGuire, 1972; Beato *et al.*, 1969; Abraham and Sekeris, 1973; Kaiser *et al.*, 1973; Munck *et al.*, 1972; Melnykovych and Bishop, 1971). Our laboratory has studied the cytoplasmic form of the fibroblast glucocorticoid receptor in some detail (Hackney *et al.*, 1970; Ishii and Aronow, 1973; Pratt and Ishii, 1972; Ishii *et al.*, 1972); however, the nature of the nuclear form was largely unexplored. This study examines the conditions necessary to obtain the nuclear glucocorticoid receptor form from the fibroblasts in order to study it in similar detail. We found that extraction of the particular fraction with 0.3 M KCl resulted in a maximal yield of solubilized bound radioactivity (other results indicate nearly any salt at an equivalent ionic strength works equally well). Using unlabeled competing steroids to determine the nonspecific binding levels, we could show that a large fraction of this extractable bound radioactivity (~90%) was specific. Furthermore, in a preliminary communication (Aronow and Middlebook, 1973) we demonstrated that the particulate salt-extractable bound radioactivity exhibited physical properties differing significantly from those exhibited by the cytoplasmic form of the receptor. Based on these findings, we believe the specifically bound radioactivity extracted from the particulate fraction by 0.3 M KCl qualifies for the term nuclear extractable glucocorticoid receptor.

During the course of experiments designed to obtain the nuclear extractable receptor we noted that there was always residual radioactivity associated with the pellet after extraction was complete. We found that 11β-cortisol could only compete for about 30–50% of the radioactive triamcinolone acetonide found in the presence of 11α-cortisol, indicating a high level of nonspecific binding (not unexpected considering the lipophilic nature of the drug).

Evidence that the residual radioactivity was of potential importance came when we incubated cells prebound with steroid under energy deprivation conditions (Figure 3). A dramatic rise in specific residual radioactivity was accompanied by an equal drop in cytosol and nuclear extractable receptor levels. Previous studies have shown that L cells in normal media will maintain a constant subcellular distribution of bound radioactivity for up to 5 hr, that the change in subcellular distribution of steroid-bound receptor brought about by energy deprivation is readily reversible on return to normal media conditions, and that neither this reversibility nor the maintenance of the control distribution is dependent on simultaneous protein synthesis (Ishii *et al.*, 1972). These results make it highly improbable that the changes shown in Figure 3 are due to either new receptor synthesis or cell death but rather represents an alteration in the subcellular distribution of the steady-state level of prebound receptor.

The results of the experiment depicted in Figure 4 demonstrate that a significant fraction of the residual radioactivity observed after energy deprivation is protein bound.

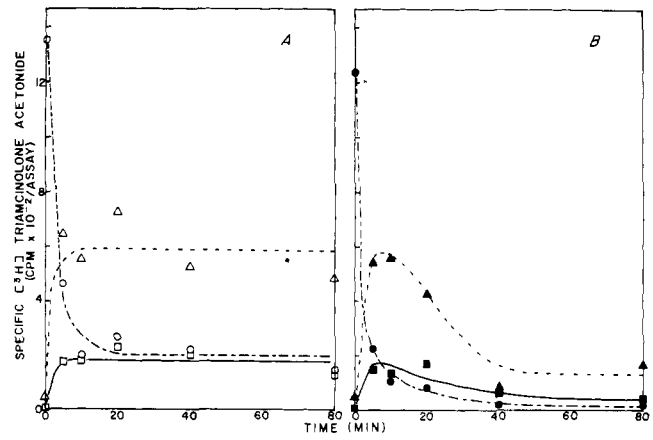

FIGURE 5: The effect of unlabeled chase on the temperature-dependent subcellular distribution of [^{3}H]triamcinolone acetonide in L cells. Replicate suspensions of L cells (4 × 10^6 cells/ml) were preincubated at 0° for 4 hr with 10^{-8} M [^{3}H]triamcinolone acetonide and either 10^{-5} M 11α- or 11β-cortisol. The incubations were divided and continued at 37° in the presence or absence of 10^{-5} M nonradioactive triamcinolone acetonide. Part A presents the effect of the incubation temperature change (no chase) on the specific radioactivity found in the 7000*g* supernatant (○), nuclear extract (△), and nuclear residual (□) fractions. Part B shows the same effect in the presence of 10^{-5} M unlabeled triamcinolone acetonide: 7000*g* supernatant (●), nuclear extract (▲), and nuclear residual (■). Variation between replicate incubations was less than 10%.

Cells incubated in the presence of glucose have a smaller proportion of nuclear residual radioactivity, and, as above, most of it is protein bound. Moreover, through the use of 11α- and 11β-cortisol it was determined that the radioactive triamcinolone acetonide was specifically bound. Equally important was the demonstration (Figure 5) that the residual radioactivity level rose significantly under conditions where the cytoplasmic receptor should translocate to the nucleus and that this increase was chasable. The effect of the chase indicates that the nuclear residual receptor is very probably involved in receptor recycling mechanism. Although previous reference has been made to glucocorticoid nuclear residual radioactivity (Abraham and Sekeris, 1973), we believe this is the first report showing not only that a significant portion of the residual radioactivity is bound, but also that it is specifically bound and, under certain conditions, can represent the majority of specifically bound steroid found in the cell. This finding demonstrates that, at least in fibroblasts, there are three forms of the steroid-bound glucocorticoid receptor: a cytoplasmic, a nuclear extractable, and a nuclear residual form. There have been reports (Lebeau *et al.*, 1973, 1974) of estradiol-bound nuclear receptors which are not salt extractable; however, the relationship of that work and the studies reported here is uncertain at the present time.

The experiment in Figure 4 is also of interest from the standpoint of the enzyme specificity required for solubilization of bound radioactivity from the extracted particulate fraction. We found that DNase but not RNase released specifically bound radioactivity from chromatin as compared to the control. Pronase alone or following DNase treatment released largely unbound radioactivity into the supernatant. Since the fibroblast glucocorticoid receptor has already been shown to be sensitive to proteolytic enzymes (Hackney *et al.*, 1970), the effect of Pronase was hardly an unexpected result. Apparently the steroid-bound receptor is susceptible to Pronase action whether it is attached to or has been released from the chromatin.

The release of bound steroid by DNase indicates that the structural integrity of DNA is required for the nuclear residual receptor to remain associated with the crude chromatin pellet. Since the hydrolysis of DNA would also result in the disruption of chromatin this experiment does not necessarily indicate that the nuclear residual receptor is bound directly to DNA, but merely to chromatin. The differential extractability of the nuclear receptors reflects different types of nuclear association, *i.e.* the extractable receptor is less tightly or not bound to chromatin.

The observations delineated above permit a refinement of the model for glucocorticoid binding and cycling previously proposed by this laboratory (Ishii *et al.*, 1972). In that model it was suggested that steroid entered the cell and bound in a reversible fashion to a receptor located in the cytoplasm. The steroid-bound cytoplasmic receptor rapidly undergoes an irreversible alteration that converts it to an activated complex which subsequently migrates to the nucleus in a temperature-dependent manner. There, in a temperature and glucose-dependent, dinitrophenol or KCN sensitive step, the receptor is regenerated or recycled back into the cytoplasm as unbound receptor. Evidence presented in this paper demonstrates that there are at least two forms of bound nuclear receptors and that one of these forms is much more tightly associated with chromatin than is the other.

It seems reasonable to propose that after activation, the steroid-bound receptor first migrates to the nucleus (*cf.* Figure 5) where an equilibrium is established between an extractable and nonextractable form. We have no evidence whether or not the nuclear migration, the chromatin binding, or the regeneration of unbound cytoplasmic receptor is a reversible process. However, due to the marked accumulation of nuclear residual receptor after glucose deprivation and/or KCN treatment it seems likely that the energy-dependent step in this recycling system involves the release of the nuclear residual receptor from chromatin. Possibly the regeneration of unbound receptor and its reappearance in the cytosol are associated with this step.

The subcellular distribution of the three forms of steroid-bound receptor is temperature and energy dependent. The observed distribution also appears to be dependent on the manner in which the cells are homogenized and subcellular fractions obtained (Figure 2 and Table I). Specifically, at 0° there is very little difference between the isotonic and hypotonic preparations while at 37° most (~80%) of the radioactivity is in the cytoplasmic fraction under isotonic conditions as compared to 18% under conditions of hypotonic cell breakage. We believe the explanation for this difference is simply that by raising the broken cell suspension to isotonicity before separating the cytoplasmic and nuclear fractions, one extracts a portion of those receptors which, at 37°, had translocated and were nuclear in location. This leads to a much different *apparent* receptor distribution between 37° hypotonic and isotonic preparations. At 0°, only a small fraction (if any) of the steroid–receptor complex has migrated to the nucleus and thus raising to isotonicity cannot extract a significant amount of radioactivity. Therefore, at 0° the hypotonic and isotonic preparations are similar in receptor distribution. However, it should be pointed out that the L cell interior is isotonic and our hypotonic lysis method may introduce distribution artifacts. The question of which subcellular fractionation method best represents the true subcellular receptor distribution would probably best be answered by autoradiographic experiments.

An examination of Figures 2 and 3 shows that, depending on experimental conditions, a wide range exists in the relative abundance of each of the three forms of the steroid-bound receptor. Thus, when studying the physicochemical properties of the three forms it is possible to obtain a high yield of any one form desired. Binding with steroid at 0° will yield more than 80% cytosol receptor. Binding at 37° produces a distribution wherein ~60% of the receptor is in the nuclear extractable form. Finally, initial binding at 37°, followed by glucose deprivation, will yield more than 50% residual receptor.

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