Properties of the Partially Purified Activated Glucocorticoid Receptor of Rat Liver. Binding to Chromatin Subunits†

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ABSTRACT: The activated glucocorticoid receptor from rat liver has been purified over 3000-fold by repeated adsorption to phosphocellulose. The partially purified receptor–triamcinolone complex is stable for several weeks at −75 °C in the presence of 0.1% bovine serum albumin, and sediments at 2.9 (±0.1) S in sucrose gradients containing 0.15 M NaCl. The Stokes' radius of the partially purified receptor is 26.5 Å, and the frictional ratio is 1.14, indicating that at near physiological salt concentration the activated receptor is slightly elongated and has a molecular weight of 33 500. These preparations of receptor are free of exo- and endonucleases and bind to DNA and chromatin, as well as to the native chromatin subunits, the nucleosomes. Removal of histone H1 and further digestion of the nucleosomal DNA to the core size of about 140 base pairs indicate that the integrity of the nucleosome structure determines the extent of interaction with the glucocorticoid receptor. Although the binding of receptor to unfractionated rat liver chromatin is more efficient than to chicken erythrocyte chromatin, the nucleosomes of both tissues bind equal amounts of receptor. Therefore, the factors responsible for this tissue difference do not reside in the nucleosomes, but rather in a higher order structure of the chromatin or in that part of the genome which is not organized as nucleosomes.

The study of the interaction of the steroid hormone receptors with the genome has been hindered by the use of crude receptor preparations. As initially pointed out by Chamness et al. (1974), and later confirmed by several investigators (Rousseau et al., 1974; Milgrom and Atger, 1975; Simons et al., 1976; Bugany and Beato, 1977), the use of crude receptor preparations can give rise to an apparent saturation of chromatin, suggesting the existence of a limited number of receptor binding sites in the genome of the target cell (Kalimi et al., 1973; Beato et al., 1973). These artifacts are probably due to factors in the crude cytosol which interfere with the binding of the receptor to the genome, making a quantitation of the acceptor sites in the target cell chromatin virtually impossible (for a review see Yamamoto and Alberts, 1976).

Partially purified steroid receptors bind very efficiently to the target-tissue chromatin, but with the exception of the progesterone receptor of chick oviduct (Buller et al., 1975) no indication of a limited number of acceptor sites has been obtained (Simons et al., 1976; Bugany and Beato, 1977). In addition, the partially purified glucocorticoid receptor of rat liver and hepatoma tissue culture cells binds to a large number of sites in purified DNA of various sources, including bacteria and phages, and this complicates the demonstration of hypothetical specific acceptor sites in the target-cell genome (Simons et al., 1976; Climent et al., 1976; Bugany and Beato, 1977). A better characterization of the partially purified receptor preparations, including a study of their interactions with different fractions of chromatin and DNA, will be required in order to answer the fundamental questions concerning the existence and nature of specific receptor recognition sites, acceptors, in the genome.

In the last few years, eukaryotic chromatin has been shown to exhibit universally an ordered structure composed of repeated spherical subunits, the ν bodies (Olins and Olins, 1974) or nucleosomes (Oudet et al., 1975), built up by two histone tetramers and a defined length of DNA varying around 200 base pairs (see Allfrey et al., 1976). Although the function of this structural organization is still not clear, it is interesting to compare the chromatin subunits and unfractionated chromatin in respect to different structural and functional properties, including the binding of regulatory proteins.

In this paper, we present several physical properties of the partially purified activated glucocorticoid receptor of rat liver, and describe its interaction with chromatin subunits derived from target and nontarget tissues.

Materials and Methods

[^{3}H]Triamcinolone acetonide, specific radioactivity 33.7 Ci/mmol, [^{3}H]dexamethasone, specific radioactivity 22 Ci/mmol, and [*methyl*-^{3}H]thymine, specific radioactivity 50 Ci/mmol, were obtained from New England Nuclear Corp. (Boston, Mass.). The nonradioactive steroids were purchased from Sigma Chemical Co. (St. Louis, Mo.). Activated charcoal was obtained from Mallinckrodt Inc. Dextran-500, Dextran blue, and the Sephadex gels were from Pharmacia, Sweden. Micrococcal nuclease was obtained from either Boehringer (Mannheim) or Worthington (Freehold, N.J.). Globin cDNA (24×10^5 cpm/μg) was prepared with AMV reverse transcriptase and purified rabbit globin mRNA in the absence of actinomycin D and using [^{3}H]thymidine (specific radioactivity 80 Ci/mmol) as label. *Escherichia coli* [^{3}H]DNA, 27 500 cpm/μg, was prepared by incubating *E. coli* (strain K12T71) in L broth (Müller, 1972) containing [^{3}H]thymine, and extracting the DNA by a modified Marmur procedure including treatment with ribonuclease and Pronase (Beato et al., 1970). The standard proteins (bovine liver catalase, rabbit muscle aldolase, ovalbumin, and horse myoglobin) were obtained from Serva (Heidelberg). Bovine serum albumin was from the Behringwerke (Marburg).

Male Sprague-Dawley rats weighing about 260 g were used throughout. The animals were adrenalectomized between 5 and 7 days before the beginning of the experiments and received 0.9% NaCl ad libitum. The animals were killed by cervical dislocation and the liver perfused in situ through the

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portal vein with 10 mL of cold TSS buffer (50 mM Tris-HCl,[1] pH 7.55, containing 25 mM KCl, 0.25 M sucrose, 5 mM $MgCl_2$, 1 mM Na_2EDTA, and 1 mM β-mercaptoethanol). The livers were excised and either immediately used or frozen in liquid nitrogen and stored at −75 °C until use.

Purification of the Activated Receptor–Triamcinolone Complex. The isolation of the activated receptor was carried out as previously described (Climent et al., 1976), with minor modifications. The livers were weighed (50 g), minced, and homogenized in 2 volumes of TSS buffer. The cytosol was prepared by centrifugation of the postmitochondrial supernatant at 50 000 rpm for 60 min in the Beckman rotor 60 Ti. The cytosol was incubated with 5×10^{-8} M [^{3}H]triamcinolone acetonide, at 0 °C for 20 min, and applied to a phosphocellulose column (2.5 × 10 cm) equilibrated with TSS buffer without sucrose. The flow through of this column was applied to a second phosphocellulose column (2 × 7 cm) equilibrated and eluted with the same buffer. The flow through of this second column was collected and incubated at 20 °C for 30 min, cooled to 0 °C in an ice bath, and applied to a third phosphocellulose column (2 × 5 cm), equilibrated with TSS buffer without sucrose. The column was washed with TGA buffer (10 mM Tris-HCl, pH 7.5, containing 10% glycerol, 0.1% bovine serum albumin, 1 mM Na_2EDTA, 1 mM 2-mercaptoethanol, and 0.1 M NaCl) until no radioactivity was detected in the eluate, and the activated receptor was eluted from the column by raising the NaCl concentration to 0.5 M. The radioactive fractions were pooled and precipitated overnight with 0.5 volume of saturated ammonium sulfate, titrated to pH 7.0 with ammonium hydroxide. The partially purified receptor, contained in the precipitate after centrifugation at 50 000*g* for 1 h, was resuspended in a small volume of TGA buffer without NaCl. The recovery of the receptor in the different fractions was checked by a microcharcoal procedure. Five microliters of the different fractions were incubated with 5 μL of activated-charcoal-coated dextran (Climent et al., 1976) for 10 min at 0 °C, and, after centrifugation at 3000*g* for 5 min, 5 μL of the supernatant was used for radioactivity determination in 5 mL of Bray's solution (Bray, 1960). On the average, the yield of this procedure was around 15% and the purification 3000- to 5000-fold.

Sucrose Gradient Centrifugation. Unless otherwise mentioned, the samples used for centrifugation in sucrose gradients were treated with dextran-coated charcoal and adjusted to a final concentration of NaCl of 0.15 M. Linear sucrose gradients of 5–20% were made in 10 mM Tris-HCl, pH 7.5, containing 0.15 M NaCl, 1 mM Na_2EDTA, and 1 mM 2-mercaptoethanol. The gradients were centrifuged for 19 h at 60 000 rpm and 0 °C in the Beckman rotor SW 60 Ti, and collected into three-drop fractions using an ISCO Collector equipped with an UV recorder (280 nm). The radioactivity in each fraction was determined in 5 mL of Bray's solution (Bray, 1960). The following standard proteins were run in parallel gradients: horse myoglobin ($s_{20,w}$ = 2.0), ovalbumin ($s_{20,w}$ = 3.5), and bovine serum albumin ($s_{20,w}$ = 4.4). The sedimentation coefficients were taken from Tanford (1963). In some experiments, the partially purified receptor was run together with the standard proteins in order to obtain a more reliable estimation of the sedimentation coefficient.

Chromatography on Sephadex G-100. For the gel-filtration experiments a column (1.5 × 143 cm) of Sephadex G-100 superfine was equilibrated in the cold with TGA buffer containing 0.15 M NaCl and no albumin. The samples were applied in a final volume of 1.5 mL, and contained various concentrations of NaCl. The column was eluted at a flow rate of 4 mL/h, and 2-mL fractions were collected. The absorbance at 280 nm was recorded, and 0.5 mL was used for the determination of radioactivity in 7 mL of Bray's solution (Bray, 1960). The column was calibrated with Dextran blue 2000, bovine serum albumin, ovalbumin, horse myoglobin, and [^{3}H]triamcinolone acetonide. The apparent partition coefficient, K_D, was calculated according to the following formula:

$$K_D = \frac{V_e - V_0}{V_t - V_0}$$

where V_e is the elution volume of the corresponding radioactive peak, and V_t and V_0 are the elution volumes of Dextran blue and the radioactive steroid, respectively. A Porath plot (1963) of the $K_D^{1/3}$ vs. the Stokes' radius of the standard proteins was used for the determination of the Stokes' radius of the receptor–steroid complex. The Stokes' radii of the standards are taken from Siegel and Monti (1966), and Tanford (1963).

Test of Nuclease Activity. For the determination of deoxyribonuclease activity, 10 μg of *E. coli* DNA was incubated for 1 h at 25 °C in 100 μL of TSS buffer without sucrose in the presence of bovine serum albumin (1 mg/mL) and various amounts of the partially purified receptor. When staphylococcal nuclease was used as control, $CaCl_2$ was added to a final concentration of 4 mM. At the end of the incubation, 5-μL aliquots were spotted on Whatman 3MM filter-paper disks for the determination of acid-insoluble radioactivity (Bollum, 1968). The other part of the assay mixture was divided into two equal parts and used for centrifugation in neutral and alkaline sucrose gradients. The samples were made 0.8 M in NaCl, and 0.1 volume of 2 M NaOH was added to the samples to be centrifuged in alkaline gradients. The sucrose gradients (5–30% in 0.8 M NaCl, 1 mM Na_2EDTA, and for the alkaline samples 0.2 M NaOH) were run in the Beckman rotor SW 41 at 40 000 rpm and 4 °C for 11 h, and 200-μL fractions were collected. Aliquots (150 μL) were spotted into filter-paper disks and used for the determination of acid-insoluble radioactivity (Bollum, 1968). The samples from the alkaline gradients were neutralized with 2.0 M HCl before applying to the filters. Bovine liver catalase, rabbit muscle aldolase, and calf liver tRNA were used as markers.

Preparation and Fractionation of Chromatin. Chromatin subunits were prepared from rat liver nuclei, which had been isolated by a modification (Beato et al., 1970) of the procedure of Chauveau et al. (1956). Nuclei were suspended in buffer A (Hewish and Burgoyne, 1973) and 100 units of micrococcal nuclease (Worthington, Freehold, N.J.) was added per mL. Nuclease digestion was started by addition of $CaCl_2$ (final concentration 1 mM) and the samples were incubated at 37 °C. The optimal incubation time had to be newly established for each enzyme batch to assure an optimal yield of nucleosome monomers and its multiples, respectively. Reactions were terminated by chilling and addition of Na_2EDTA (final concentration 2 mM). After centrifugation at 4000*g* for 5 min, the nuclear pellets were lysed gently in buffer B (5 mM sodium phosphate buffer, pH 6.8, 0.2 mM Na_2EDTA) using a Pasteur

[1] Abbreviations used are: Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; EDTA, (ethylenedinitrilo)tetraacetic acid. The following trivial names are used for steroids: triamcinolone acetonide, 9α-fluoro-11β,16α,17α,21-tetrahydroxypregnane-1,4-diene-3,20-dione cyclic 16,17-acetal with acetone; dexamethasone, 9α-fluoro-11β,21-dihydroxypregnane-1,4-diene-3,70-dione; cortisone, Δ^4-pregnene-21-hydroxy-3,11,20-trione; corticosterone, Δ^4-pregnene-11β,21-dihydroxy-3,20-dione; 17β estradiol, 1,3,5(10)-estratriene-3,17β-diol. Enzymes used are: micrococcal nuclease, from *Staphylococcus aureus* (EC 3.1.4.7); AMV reverse transcriptase:RNA-dependent DNA nucleotidyltransferase from avian myeloblastosis virus.

pipet. The lysed material was then centrifuged at 4000*g* for 2 min and the supernatant was taken and fractionated on isokinetic sucrose gradients as described by Finch et al. (1975). The peak fraction of nucleosome monomers and multiples thereof were dialyzed against buffer B, concentrated in Minicon B15 cells (Amicon, Oosterhout, The Netherlands), and recentrifuged until homogeneous fractions were obtained.

For the preparation of nucleosomes from chicken erythrocytes, the procedure had to be modified. Nuclei were prepared by hypotonic lysis of erythrocytes and washed several times in RSB (Weintraub and Groudine, 1976). Then, they were suspended in a solution of 0.5% Triton X-100 (Serva, Heidelberg), applying ten strokes with a loose-fitting Dounce homogenizer. After centrifugation at 4000*g* for 5 min, this step was repeated twice, followed by two washings with Triton-free RSB. Then, the nuclei were suspended in buffer A (see above) and digested with micrococcal nuclease as described above. Compared to rat liver nuclei, the digestion time had to be increased fourfold. Lysis of nuclei and fractionation of chromatin were then done as described for rat liver nucleosomes.

In an alternative procedure, chromatin from detergent nuclei was prepared by repeated washings in 0.075 M NaCl, 0.02 M Na_2EDTA, 0.01 M Tris, pH 7.4 (Weintraub and Groudine, 1976), followed by hypotonic lysis of the nuclei with 0.01 M Tris, pH 7.4. The gelatinous pellet was suspended in buffer B and either sheared by passing it through a french press at 1000 psi or digested with micrococcal nuclease (50 units/mL for 20 min at 37 °C).

As in the case of rat liver, nucleosomes from chicken erythrocytes were then separated on isokinetic sucrose gradients from the integral multiples of the monomer nucleosome.

Removal of Histone H1. Two procedures were used alternatively to remove histone H1 from chromatin or from isolated nucleosomes (dimers). In the first procedure, chromatin was exposed to 0.6 M NaCl (Ohlenbusch et al., 1967; Oudet et al., 1975) in buffer B (see above) for 12 h at 4 °C, and then the chromatin was centrifuged on isokinetic sucrose gradients as described above, but the gradients were supplemented with 0.6 M NaCl. After centrifugation for 20 h in a Beckman SW 27 rotor at 26 000 rpm, the fractions were pooled and dialyzed against buffer B.

In an alternative procedure (Ilyin et al., 1971), chromatin was exposed to tRNA (yeast tRNA, 1 mg/mL) at 0 °C for 12 h at 1 mM $MgCl_2$. Subsequently, the chromatin was separated from tRNA and histone H1 by centrifugation on isokinetic sucrose gradients, supplemented with 1 mM $MgCl_2$. Removal of histone H1 was monitored by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Laemmli, 1970).

Preparation of Nucleosome Core Particles. H1-depleted nucleosome dimers were digested with micrococcal nuclease (100 units/mL) for 5 min at 37 °C, the reaction was stopped by addition of Na_2EDTA (final concentration 2 mM), and the core nucleosome particles were isolated by centrifugation on isokinetic sucrose gradients.

Polyacrylamide Gel Electrophoresis of DNA Fragments. The DNA was extracted from the chromatin fragments as described by Noll et al. (1975) and dissolved in half-strength electrophoresis buffer supplemented with 10% sucrose (Serva, Heidelberg, nuclease-free). Ten micrograms of DNA was applied per slot and electrophoresed on 5% polyacrylamide slab gels (1.5 × 100 × 140 mm) prepared as described by Loening (1969). The running buffer was 36 mM Tris, 30 mM NaH_2PO_4, 1 mM Na_2EDTA, pH 7.7. After preelectrophoresis at 8 mA for 1 h, samples were applied on the gel and the electrophoresis was performed at 40 mA (constant) for 2.5 h. The gel was then stained with *stains all* (Serva, Heidelberg) overnight, destained in water, and photographed using an orange filter.

Binding of Receptor to Isolated Nucleosomes. The binding of partially purified receptor to chromatin subunits was studied by two different methods: sucrose gradient centrifugation and precipitation with streptomycin sulfate (Webster et al., 1976).

For the sucrose gradient experiments, nucleosomes (2 A_{260} units in 0.1 mL of 1 mM phosphate buffer, pH 7.0) were incubated with partially purified receptor (2 pmol in TGA buffer without NaCl) in a final volume of 300 μL, adjusted with TGA buffer and 2 M NaCl to give the desired salt concentration. Incubation was carried out at 22 °C for 1 h. The samples were then applied to linear sucrose gradients (5–20% in 10 mM Tris-HCl, pH 7.5, containing 0.2 mM Na_2EDTA and, in some cases, 90 mM NaCl). Centrifugation was performed in the Beckman SW 60 rotor for 3 to 5 h at 60 000 rpm and 0 °C, using bovine catalase as marker. Fractions (three drops) were collected by puncturing the bottom of the centrifuge tube and pushing the gradient through an ISCO monitor cell where the absorbance at 260 nm was registered. The radioactivity in each fraction was measured in 5 mL of Bray's solution (Bray, 1960).

For the precipitation experiments, incubation was carried out as above, but using 3 A_{260} units of nucleosomes and 1.5 pmol of partially purified receptor. The final NaCl concentration was 0.15 M. At the end of the incubation, the assay mixtures were diluted with 700 μL of cold TGA buffer containing 0.15 M NaCl, and 3 mg of streptomycin sulfate dissolved in 100 μL of buffer was added. After 30 min at 0 °C, the samples were centrifuged at 5000*g* for 5 min, and the pellets washed with 1 mL of incubation buffer containing 0.02% streptomycin sulfate. The final pellet was dissolved overnight in 0.2 mL of 1 M NaCl, 1 M NaOH, and aliquots were taken for determination of DNA (Burton, 1956) and radioactivity. Controls were carried out with free [^{3}H]triamcinolone acetonide in the absence of the receptor in order to correct for adsorption of free steroid to the nucleosomes (less than 5% of the value observed with partially purified receptor). Aggregation of the receptor was quantitated by including a sample without added nucleosomes.

The binding of the receptor to unfractionated chromatin from rat liver and chicken erythrocyte was determined as previously described (Bugany and Beato, 1977), as well as by the streptomycin sulfate method described above. The DNA content of chromatin was determined by the procedure of Burton (1956) using calf thymus DNA as standard.

Results

Stability of the Partially Purified Glucocorticoid Receptor. Knowledge of the conditions which improve the stability of the receptor is an essential requisite for the study of its binding to chromatin or DNA. The influence of various conditions of storage on the stability of the partially purified receptor–triamcinolone complex is depicted in Figure 1a. In the absence of bovine serum albumin the receptor is rapidly inactivated when stored at 0 °C, but exhibits a half-life of about 2 days at −75 °C. Addition of bovine serum albumin to a concentration of 1 mg/mL stabilizes the receptor, leading to a half-life of 10 days in the refrigerator and several weeks at −75 °C. Lyophilization of the partially purified receptor, both in the absence and in the presence of bovine serum albumin, leads to complete inactivation (data not shown). For the rest of our studies the receptor was either used immediately after preparation or stored at −75 °C in TGA buffer without NaCl.

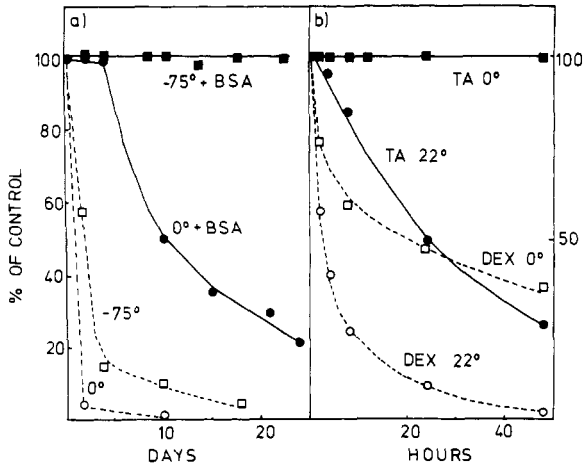

FIGURE 1: Stability of the partially purified glucocorticoid receptor. Aliquots (5 μL) of freshly prepared glucocorticoid receptor were stored at different temperatures in 10 mM Tris-HCl, pH 7.5, containing 10% glycerol, 1 mM Na_2EDTA, 1 mM 2-mercaptoethanol, and, in some cases, bovine serum albumin (1 mg/mL). At the indicated time intervals, duplicate aliquots were taken for the determination of protein-bound radioactivity by a microcharcoal technique (see Materials and Methods). (a) Influence of temperature and bovine serum albumin on the stability of the receptor-triamcinolone complex: at 0 °C in the absence (○) and in the presence (●) of bovine serum albumin, and at −75 °C in the absence (□) and in the presence (■) of bovine serum albumin. (b) Influence of the steroid ligand on receptor stability in the presence of bovine serum albumin: receptor-triamcinolone complex at 0 °C (■) and 22 °C (●); receptor-dexamethasone complex at 0 °C (□) and 22 °C (○).

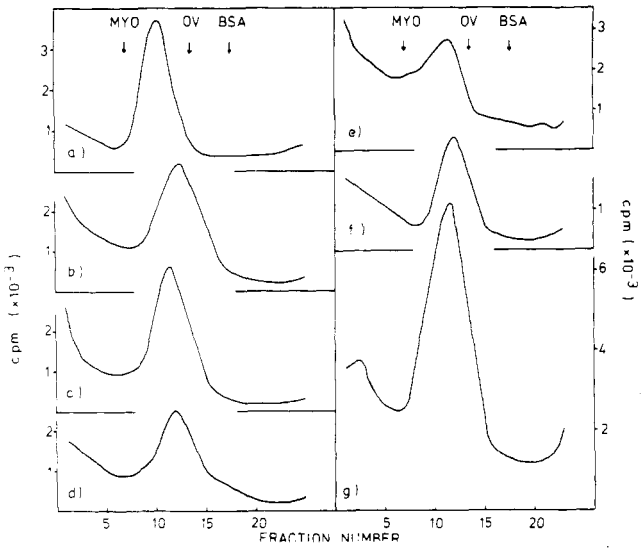

FIGURE 2: Centrifugation of the different glucocorticoid receptor preparations in sucrose gradients containing 0.15 M NaCl. For these experiments, equivalent amounts of protein-bound radioactivity were applied to linear sucrose gradients (5–20% in buffer containing 0.15 M NaCl, see Methods). The gradients were centrifuged for 19 h at 60 000 rpm and 0 °C in a Beckman rotor SW 60 and three-drop fractions were collected. The radioactivity in each fraction is plotted against the fraction number, starting at the top of the gradient. The position of the protein markers, myoglobin, ovalbumin, and bovine serum albumin, is indicated by arrows. (a) Partially purified receptor-triamcinolone complex. (b) Rat liver cytosol labeled for 1 h at 0 °C with 50 nM [^{3}H]triamcinolone acetonide and treated with charcoal before application to the gradient. (c) Rat liver cytosol treated as in b but made 0.5 M in NaCl 15 min before application to the gradient. (d) Rat liver cytosol treated as in b but incubated at 22 °C for 30 min before charcoal treatment. (e) Rat liver cytosol treated as in b but using [^{3}H]dexamethasone instead of [^{3}H]triamcinolone acetonide. (f) Rat liver cytosol treated as in d was passed through a phosphocellulose column (see Methods) and the material which did not bind to the column was treated with charcoal and applied to the gradient. (g) Partially purified activated glucocorticoid receptor was mixed with an equal amount of nonactivated receptor-triamcinolone (prepared as in f) and applied to a sucrose gradient after treatment with charcoal.

A more detailed analysis of the influence of the steroid ligand on the stability of the receptor during the first two days of storage is shown in Figure 1b. For this experiment the complex of receptor and dexamethasone was partially purified following the procedure described under Materials and Methods but using [^{3}H]dexamethasone instead of [^{3}H]triamcinolone acetonide. All samples were stored in the presence of 1 mg/mL bovine serum albumin. At 0 °C the complex of receptor and triamcinolone acetonide is stable during the first 2 days, whereas the complex of receptor and dexamethasone has a half-life of around 20 h. At 22 °C both complexes are unstable and the half-life of the receptor-triamcinolone complex is 24 h, whereas the dexamethasone-receptor complex is inactivated with a half-life of 2–3 h. The inactivation rate is not affected by nonradioactive steroids (triamcinolone acetonide, dexamethasone, corticosterone, cortisone, or 17β-estradiol) present in a 1000-fold excess over the radioactive hormone (data not shown).

Centrifugation in Sucrose Gradients. The partially purified receptor-triamcinolone complex was analyzed by centrifugation through linear 5–20% sucrose gradient in buffer containing 0.15 M NaCl (Figure 2). The purified receptor sediments as a single radioactive peak between 2.8 and 3.0 S as calculated from the sedimentation coefficients of bovine serum albumin, ovalbumin, and myoglobin which were included as internal markers or centrifuged in a parallel gradient (Figure 2a). This value is slightly lower than that observed when cytosol is incubated with triamcinolone acetonide at 0 °C and applied to a similar gradient (Figure 2b). Under these conditions a broader radioactivity peak is observed sedimenting between 3.1 and 3.4 S. Treatment of this crude cytosol preparation with 0.5 M NaCl or incubation at 22 °C for 30 min lead to a sedimentation pattern very similar to that of the partially purified receptor, with a radioactive peak centered around 3.1 S (Figure 2c,d).

A similar sedimentation behavior but exhibiting much more free radioactivity is observed when cytosol is incubated with radioactive dexamethasone and submitted to similar sucrose gradient analysis (Figure 2e). The partially purified complex of receptor and dexamethasone sediments in the same position as the complex of the activated receptor and triamcinolone acetonide (data not shown).

Also shown in Figure 2 is the radioactive profile obtained after centrifugation of the receptor-triamcinolone complexes which do not bind to phosphocellulose (see Methods) and, therefore, represent nonactivated complexes (Figure 2f). They sediment as a homogeneous radioactive peak with a maximum at 3.3 S. An equimolar mixture of this nonactivated receptor-triamcinolone complex with partially purified activated complex sediments as a single wide peak with the maximum around 3.1 S (Figure 2g). This result indicates that the differences in the sedimentation behavior of the activated and nonactivated receptor-triamcinolone complexes are small and not sufficient to allow a separation into two different peaks in this type of sucrose gradient.

In sucrose gradients prepared in buffers of low ionic strength (below 0.1 M NaCl) a considerable fraction of the receptor molecules aggregate and sediment to the bottom of the gradient into the pellet (data not shown). The use of buffer containing high salt concentrations (above 0.3 M NaCl) for the preparation of the sucrose gradients apparently does not change the sedimentation behavior of the receptor steroid complexes, which continue to sediment at 2.9 (±0.1) S. Sim-

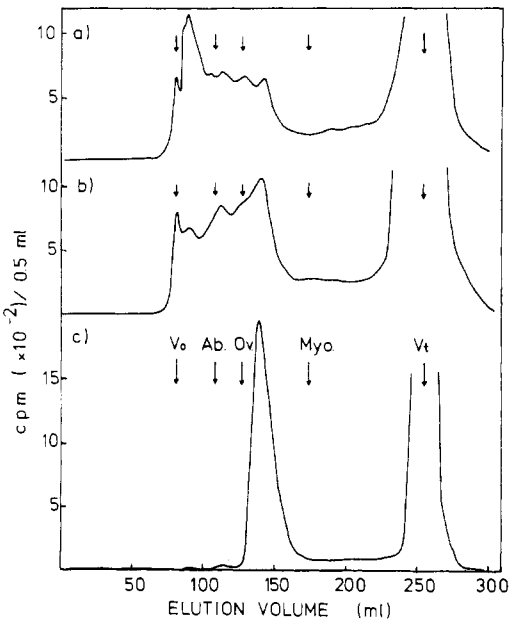

FIGURE 3: Chromatography of the rat liver glucocorticoid receptor on columns of Sephadex G-100. (a) Liver cytosol (1 mL), prepared as described under Materials and Methods, was incubated for 1 h at 0 °C with 50 nM [^{3}H]triamcinolone acetonide in TGA buffer without albumin, and applied to a Sephadex G-100 column. (b) Liver cytosol (1 mL), treated as under a but made 0.5 M in NaCl 15 min before application to the column. (c) Partially purified activated receptor–triamcinolone complex, applied to the same column in TGA buffer, together with the protein markers (bovine serum albumin, ovalbumin, and myoglobin). The columns were equilibrated and eluted as described under Materials and Methods. Two-milliliter fractions were collected and the figure represents the radioactivity determined in 0.5-mL aliquots. The void volume, V_0, and the total volume, V_t, were determined in a previous chromatography with Dextran blue 2000 and [^{3}H]triamcinolone acetonide, respectively. The positions where the marker proteins elute are indicated by arrows.

ilarly, no change in the sedimentation of the receptor is observed after incubation of the partially purified receptor with crude cytosol at near physiological ionic strength.

Column Chromatography on Sephadex G-100. At near physiological ionic strength (0.15 M NaCl), the partially purified receptor–triamcinolone complex elutes from a Sephadex G-100 column as a single peak located between the ovalbumin and the myoglobin markers. Very little radioactivity is detected in the position of the excluded fraction, indicating that the receptor does not aggregate under these conditions (Figure 3c). A Porath plot of the elution volume vs. the Stokes' radius of the internal marker proteins (bovine serum albumin, ovalbumin, and myoglobin) yields a Stokes' radius of 26.5 Å for the partially purified receptor. The crude cytosol labeled with triamcinolone acetonide and chromatographed through the same Sephadex G-100 column in 0.15 M NaCl shows a complex radioactivity elution profile, including a small peak in the position of the partially purified receptor (Figure 3a). Most of the radioactivity elutes from this column close to the excluded volume, but intermediate peaks are detected in the elution regions of bovine serum albumin and ovalbumin. Treatment of the cytosol with 0.5 M NaCl before application to the column results in a marked change of the elution profile with a more pronounced peak in the region of partially purified receptor and a corresponding diminution of the peak close to the excluded volume (Figure 3b). Under these conditions, the peak of the radioactivity eluting just after bovine serum albumin becomes also more evident.

The value of the Stokes' radius calculated from Sephadex gel chromatography and the sedimentation coefficient obtained from sucrose gradient centrifugation can be used to calculate the molecular weight, M, according to Einstein's modification of the Svedberg equation (Siegel and Monty, 1966):

$$M = (6\pi\eta N/1 - \bar{\nu}\rho)sa$$

where η is the solvent viscosity, ρ the solvent density, s the sedimentation coefficient, and a the Stokes' radius in cm. Assuming a partial specific volume ($\bar{\nu}$) of 0.74 cm^3 × g^{-1}, a molecular weight of 33 540 ± 950 is obtained for the partially purified receptor. The Stokes' radius in conjunction with the molecular weight permits an estimate of the frictional ratio, according to the formula (Siegel and Monty, 1966):

$$f/f_0 = a(4\pi N/3M(\bar{\nu} + \delta/\rho))^{1/3}$$

Assuming that the degree of solvation (δ) equals 0.2 g/g of protein, a frictional ratio of 1.14 is obtained, indicating that the partially purified receptor has a slightly elongated form with an axial ratio of 3–4 (Schachman, 1959).

Nuclease Activity. Receptor preparations to be used for DNA-binding studies should be free of nucleases. We have tested the ability of the partially purified receptor preparations to degrade radioactively labeled DNA to acid-soluble products. As already reported (Climent et al., 1976), there is considerable nuclease activity in crude cytosol and also in the flow through of the phosphocellulose column. However, the partially purified receptor preparations are free of exonuclease activity (Climent et al., 1976). To examine the possibility that they still contain endonuclease activity, double-stranded *E. coli* DNA, highly labeled with tritium, was incubated with partially purified receptor and submitted to analysis in neutral and alkaline sucrose gradients (Figure 4). The partially purified receptor did not change the sedimentation behavior of double- or single-stranded [^{3}H]DNA, indicating that it is free of endonuclease activity. Similar results were obtained with globin [^{3}H]cDNA prepared with reverse transcriptase (Climent et al., 1976, data not shown). In order to make sure that small amounts of nuclease activity are detected by this procedure, the same amounts of [^{3}H]DNA were incubated with 5 and 25 units of micrococcal nuclease, and the radioactivity insoluble in 10% trichloroacetic acid was determined. The high concentration of nuclease transformed almost all the radioactive DNA in acid-soluble products, whereas the lower concentration degraded about half the [^{3}H]DNA. The DNA treated with 5 units of nuclease was submitted to centrifugation in neutral sucrose gradients (Figure 4A). It is clear that this small amount of nuclease results in a marked reduction of the amount of radioactive DNA sedimenting in the heavy region of the sucrose gradient.

Binding of the Partially Purified Receptor to Chromatin Subunits. In previous reports we have shown that the partially purified glucocorticoid–receptor is able to bind to liver chromatin and DNA–cellulose (Climent et al., 1976; Bugany and Beato, 1977). Although the binding to DNA–cellulose was unspecific in respect to the source of DNA, Bugany and Beato (1977) have detected a clear quantitative difference in the binding of partially purified receptor to rat liver and chicken erythrocyte chromatin (see also Table I). Since this finding could reflect differences in the structure of rat liver and avian erythrocyte chromatin, it was interesting to study the interaction of the partially purified receptor with the chromatin subunits, prepared from both tissues. The result of an experiment in which the partially purified receptor was incubated with a mixture of mono-, di-, tri-, and multimers prepared from rat liver chromatin and centrifuged through a sucrose gradient is depicted in Figure 5. This experiment shows that the partially purified receptor can interact with the isolated chromatin

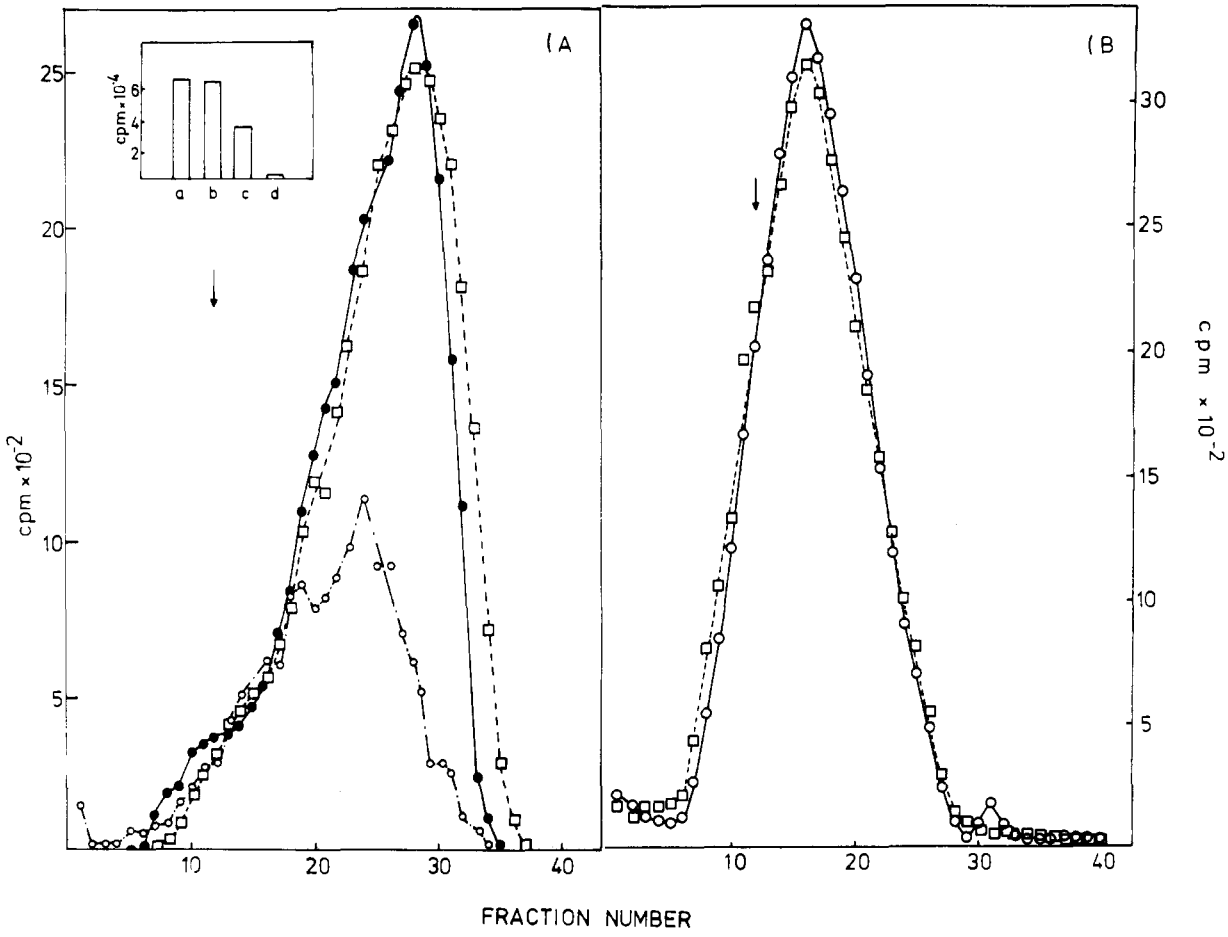

FIGURE 4: Test for endonuclease activity of the partially purified receptor preparations. Aliquots of the partially purified receptor–triamcinolone complex (3 pmol) were incubated for 1 h at 25 °C with 10 μg of [^{3}H]DNA$_{E.coli}$ in 100 μL of TSS buffer without sucrose containing 1 mg/mL bovine serum albumin. As controls, similar amounts of [^{3}H]DNA were incubated under similar conditions in the absence of the receptor or with micrococcal nuclease (5 and 25 units) in the presence of 4 mM $CaCl_2$. At the end of the incubation, 5-μL aliquots were taken for the determination of acid-insoluble radioactivity, as described under Materials and Methods. The *inset* shows the result of this experiment. (a) Control incubation without receptor. (b) Incubation with partially purified receptor. (c and d) Incubations with 5 and 25 units of micrococcal nuclease, respectively. The remaining material was analyzed in sucrose gradients prepared in neutral or alkaline buffers (see Materials and Methods). (A) Neutral sucrose gradient of [^{3}H]DNA$_{E.coli}$ incubated in the absence (●) and in the presence (□) of partially purified receptor, or with 5 units of micrococcal nuclease (○). (B) Alkaline sucrose gradients of [^{3}H]DNA$_{E.coli}$ incubated in the absence (○) and in the presence (□) of partially purified receptor. The arrows indicate the position of bovine liver catalase ($s_{20,w}$ = 11.3).

subunits. There was a linear relationship between the concentration of free receptor–steroid complex (range 1–10 nM) and the amount of radioactivity bound to the chromatin subunits (data not shown). The monomer peak at 11 S shows a higher radioactivity than the dimers and trimers, and a small amount of radioactivity migrates with nucleosome multimers. These differences are probably due to the longer distance that the heavier particles have to sediment through the sucrose gradient (see below). Free steroid does not bind to the nucleosomes (data not shown), as the chromatin used was obtained from adrenalectomized rats which have lost their nuclear receptors (Beato et al., 1974).

Most of the receptor molecules bound to the chromatin subunits in sucrose gradients are in the form of weak ionic complexes, as demonstrated by the fact that raising the salt concentration in the gradient buffer to 90 mM NaCl markedly decreases the radioactivity sedimenting with the nucleosomes (Figure 5b). However, even at 0.15 M NaCl a certain amount of the radioactive receptor still sediments with the chromatin subunits (data not shown), indicating that part of the binding is stable at near physiological ionic strength. This is confirmed by experiments where receptor binding was measured by precipitation with streptomycin sulfate, a method which allows a more quantitative estimate of receptor-binding capacity (Table I). At physiological ionic strength the amount of receptor bound to subunit oligomers is even higher than that sedimenting with the monomers. Similar results were obtained with chromatin subunits from chicken erythrocyte (Table I). Using unfractionated chromatin, the results obtained with the classical precipitation by 0.15 M NaCl (Bugany and Beato, 1977) were indistinguishable from those observed with streptomycin sulfate, indicating that it is a suitable method for studying receptor binding to chromatin subunits.

A comparison in sucrose gradients of the ability of liver and erythrocyte nucleosome monomers to bind the partially purified receptor also shows that at both low and high ionic strength there are no major quantitative differences between the two tissues (Figure 6). This is in contrast with the higher ability of unfractionated liver chromatin to bind the receptor as compared to erythrocyte chromatin found by Bugany and Beato (1977) and also in the present work (Table I). In addition, it is clear that unfractionated chromatin of both sources binds more activated receptor than the chromatin subunits (Table I). Therefore, we conclude that the higher and differential receptor binding capacity of the unfractionated chromatin does not reside in the structure of its nucleosomes but

TABLE I: Binding of Partially Purified Receptor to Chromatin Subunits Derived from Rat Liver and Chicken Erythrocytes.[a]

Fraction	Receptor-triamcinolone bound (pmol/mg of DNA)	
	Liver	Erythrocyte
Monomers	0.34 ± 0.14	0.31 ± 0.01
Dimers	0.40 ± 0.14	0.36 ± 0.04
Trimers	0.39 ± 0.23	0.34 ± 0.02
Multimers	0.56 ± 0.19	0.54 ± 0.09
Chromatin	2.94 ± 0.24	1.45 ± 0.21

[a] Chromatin and chromatin subunits were prepared from rat liver or chicken erythrocytes, and incubated with partially purified receptor-triamcinolone complex in TGA buffer containing 0.15 M NaCl. Receptor binding was measured by precipitation with streptomycin sulfate (see Methods). The numbers represent the mean values and standard deviations obtained with three different chromatins and three separate receptor preparations. The numbers are corrected for receptor aggregation by subtraction of the values obtained in the absence of nucleosomes or chromatin. This value represents less than 2% of the total amount of receptor, and amounts to 38% of the receptor bound to erythrocyte monomers, and 6% of the receptor bound to liver chromatin.

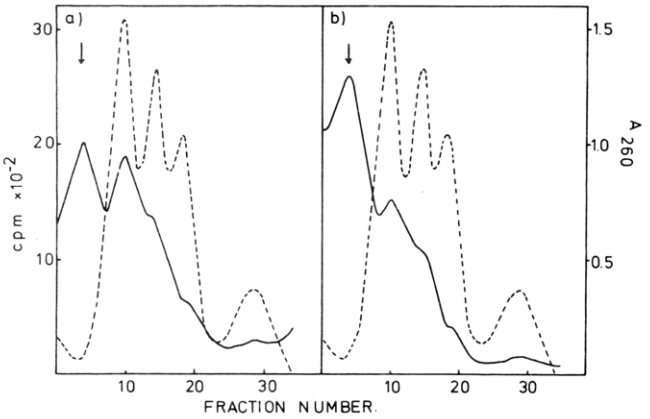

FIGURE 5: Binding of partially purified receptor to chromatin subunits of rat liver. To a mixture containing 2 A_{260} units of each of the different chromatin subunits, mono-, di-, tri-, and multimers, 8 pmol of partially purified receptor-triamcinolone complex was added and the salt concentration adjusted with 2 M NaCl (see Methods). The samples were incubated for 1 h at 22 °C and then applied to a linear sucrose gradient (5–20%) containing 10 mM Tris-HCl, pH 7.5, 0.2 mM Na_2EDTA, and, when indicated, 90 mM NaCl. After centrifugation for 3 h at 60 000 rpm and 0 °C in the SW 60 Beckman rotor, three-drop fractions were collected and the radioactivity was measured (full line). The ultraviolet absorbance at 260 nm is indicated by the dashed line (see Methods). Centrifugation was from left to right and the arrows indicate the position where free receptor-triamcinolone complex sediments. (a) Gradients prepared in buffer of 0.01 ionic strength. (b) Gradients prepared in buffer of 0.10 ionic strength.

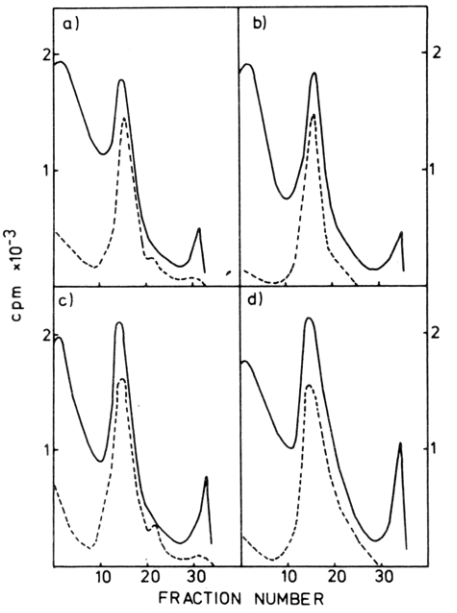

FIGURE 6: Binding of partially purified receptor to chromatin monomers from rat liver and chicken erythrocytes. Aliquots (2 A_{260} units) of chromatin monomers prepared from either rat liver or chicken erythrocytes were incubated at 22 °C for 1 h with 2 pmol of partially purified receptor-triamcinolone complex. The final volume was 0.2 mL and the salt concentration was adjusted with 2 M NaCl (see Methods). At the end of the incubation, the samples were applied to linear sucrose gradients (5–20%) and centrifuged for 5 h at 60 000 rpm and 0 °C in the SW 60 Beckman rotor. The absorbance at 260 nm (dashed line) and the radioactivity in each fraction (solid line) are indicated. (a) Rat liver nucleosome monomers in sucrose gradients prepared in buffer of 0.1 ionic strength. (b) Chicken erythrocyte nucleosome monomers run as in a. (c) Rat liver nucleosome monomers in sucrose gradients prepared in buffer of 0.01 ionic strength. (d) Chicken erythrocyte nucleosome monomers run as in c.

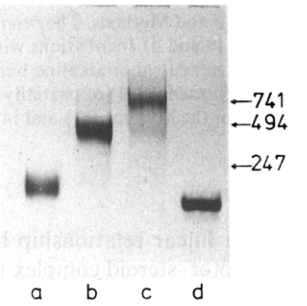

FIGURE 7: Polyacrylamide gel electrophoresis of nucleosomal DNA. DNA was extracted from the chromatin subunits prepared from rat liver, as described under Materials and Methods. Ten micrograms of DNA was submitted to electrophoresis in 5% polyacrylamide gel slabs (Loening, 1969). The gels were stained with stains all (Serva, Heidelberg) and photographed after destaining in water. (a) Native nucleosome monomers. (b) Native nucleosome dimers. (c) Native nucleosome trimers. (d) Core nucleosome monomers prepared with 0.6 M NaCl and micrococcal nuclease. The arrows indicate the electrophoretic mobility and number of base pairs of marker DNA fragments obtained by digestion of mouse satellite DNA with *Eco*RII restriction enzyme (kindly provided by Drs. H. G. Zachau and W. Hörz, München). The number of base pairs in the nucleosomal DNA was calculated by plotting the number of base pairs in the marker DNA against the square root of the electrophoretic mobility (Williamson, 1970).

either in a higher order conformation of the chromatin or in that part of the chromatin genome which is not organized as nucleosomes.

In order to study the structural factors of the chromatin subunits which are responsible for glucocorticoid receptor binding, we compared the binding of the receptor to native rat liver nucleosomes and to monomers which had been treated with either 0.6 M NaCl or tRNA to remove histone H1 (Ohlenbusch et al., 1967; Ilyin et al., 1971). Histone H1 deprived monomers were subjected to further digestion with nuclease in order to remove the terminal base pairs, and the resulting "core" monomers were also used for the receptor-binding studies. The results of these experiments are shown in Figures 7, 8, and 9. First, it is evident that treatment with nuclease after removal of H1 histone results in a shortening of the DNA of the monomers, as demonstrated by polyacrylamide gel electrophoresis of the extracted DNA (Figure 7). These findings confirm previous reports (Whitlock and Simpson, 1976) that the DNA in the native rat liver nucleo-

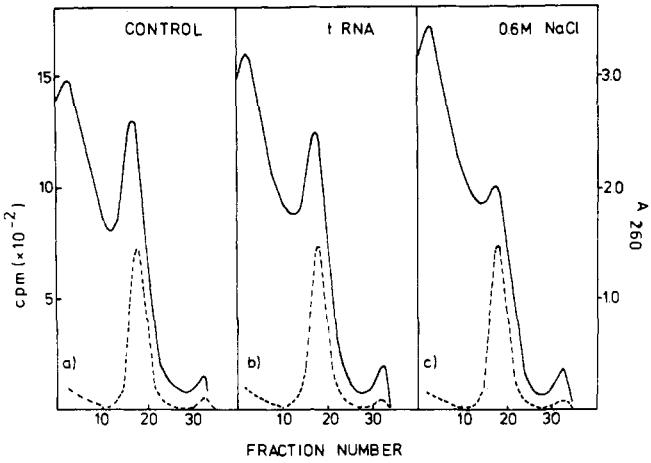

FIGURE 8: Interaction of the receptor–triamcinolone complex with native and histone H1 depleted nucleosomes. Native nucleosome monomers as well as monomers deprived of histone H1 (2.0 A_{260} units each) were incubated at 22 °C for 1 h with 2 pmol of partially purified receptor. The final volume of the assays was 0.2 mL and the salt concentration was adjusted to 90 mM NaCl with concentrated salt. After incubation, the samples were applied to linear sucrose gradients (5–20%) containing 10 mM Tris-HCl, pH 7.5, 0.2 mM Na_2EDTA, and 90 mM NaCl. The samples were centrifuged for 5 h at 60 000 rpm and 0 °C in the SW 60 Beckman rotor. The absorbance at 260 nm (dashed line) and the radioactivity in each fraction (solid line) are indicated. (a) Native nucleosome monomers. (b) Nucleosome monomers deprived of histone H1 by treatment with tRNA. (c) Nucleosome monomers deprived of histone H1 by treatment with 0.6 M NaCl.

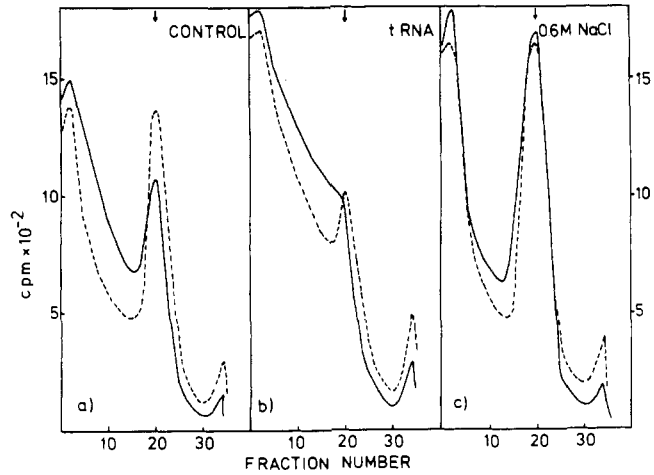

FIGURE 9: Interaction of the receptor–triamcinolone complex with native and core nucleosomes. Native nucleosome monomers as well as core monomers deprived of histone H1 and the terminal base pairs (1.5 A_{260} units each) were incubated at 22 °C for 1 h with 2 pmol of partially purified receptor. The final volume of the assays was 0.2 mL and the salt concentration was adjusted with 2 M NaCl. After incubation the samples were applied to linear sucrose gradients (5–20%) containing 10 mM Tris-HCl, pH 7.5, 0.2 mM Na_2EDTA, and, when indicated, 90 mM NaCl. The samples were centrifuged for 5 h at 60 000 rpm and 0 °C in the SW 60 Beckman rotor. The figure represents the radioactivity in each fraction (three drops), collected from the top of the gradient. The arrows indicate the position of the monomers as determined by their absorbance at 260 nm. The dashed lines represent the radioactivity in gradients without NaCl, and the full lines the radioactivity in gradients with 90 mM NaCl. The receptor–triamcinolone complex was incubated with (a) native nucleosome monomers, (b) core monomers prepared with tRNA, and (c) core monomers prepared with 0.6 M NaCl.

somes is around 200 base-pairs long, whereas after removal of histone H1 and further treatment with nuclease the nucleosomal DNA fragment is only 140 base-pairs long. Incubations of these different classes of nucleosomes with partially purified receptor show that removal of histone H1 with tRNA or 0.6 M NaCl leads to a diminution of the amount of receptor bound per nucleosome (Figure 8). Further digestion of tRNA-treated nucleosomes with the nuclease almost completely inhibits receptor binding (Figure 9b). Removal of histone H1 with 0.6 M NaCl followed by nuclease digestion results in core nucleosomes with increased receptor-binding capacity (Figure 9c). In addition, whereas the binding of receptor to native nucleosomes and to core nucleosomes prepared with tRNA is markedly depressed by salt, the binding to core nucleosomes prepared with 0.6 M NaCl is quite insensitive to ionic strength within the physiological range (Figure 9).

Discussion

The basic principle of the purification of the glucocorticoid receptor used in our studies has also been employed in other laboratories with minor differences (Colman and Feigelson, 1976; Simons et al., 1976; Atger and Milgrom, 1976; Eisen and Ginsmann, 1976). It offers the advantage of being amenable to a large-scale preparation, but also implies certain limitations, the most significant one being the fact that only the activated complex of receptor and triamcinolone acetonide is partially purified, whereas the nonactivated complex is lost during the purification. The procedure used by Failla et al. (1975), which is based on affinity chromatography, appears to circumvent this limitation but is considerably more complicated and time consuming. Other reports involving affinity chromatography probably result in the purification of transcortin-like proteins (Wong et al., 1973).

The partially purified receptor–triamcinolone complex used in our experiments is rather stable and can be stored for several weeks at −75 °C in the presence of 1 mg/mL bovine serum albumin without losing activity. In fact, the degree of stability of the partially purified receptor reflects the dissociation rate of the triamcinolone from the complex, and this is a very slow process at −75 °C. This explains why the complex of receptor and dexamethasone is much less stable under the various storage conditions tested, since dexamethasone dissociates faster from the receptor than triamcinolone (King and Mainwaring, 1974).

The complex pattern obtained upon Sephadex gel filtration of crude cytosol has also been observed by other authors using natural glucocorticoids (Snart et al., 1970). We interpret these findings as reflecting different stages of aggregation of the receptor, either with itself or with other proteins of the cytosol. After partial purification, however, the receptor does not aggregate at physiological ionic strength.

The partially purified receptor prepared by this procedure exhibits a lower sedimentation constant than the values reported for other glucocorticoid receptors (Cake and Litwack, 1975). Although it appears that after partial purification the sedimentation coefficient of the receptor is slightly lower than in the original cytosol preparation, this difference is not very pronounced and the values are scattered around $s_{20,w} = 3.0$. This sedimentation behavior is also compatible with the results of Sephadex gel-filtration experiments according to which the partially purified receptor has a Stokes' radius of 26.5 Å. Using these two parameters, a molecular weight of 33 500 is found for the partially purified receptor, and the frictional ratio is 1.14, indicating that it has a slightly elongated shape. This form of the receptor could represent a subunit of the previously described 4S form, which exhibited a molecular weight of 66 000 and a frictional ratio of 1.35 (Beato and Feigelson, 1972; Koblinsky et al., 1972). A glucocorticoid-binding protein, glucocorticoid binder II, with very similar properties has been

described by Litwack et al. (1973), with a molecular weight of 67 000. Litwack and Rosenfield (1975) have also reported the existence of a small glucocorticoid binder of molecular weight 30 500 and Stokes' radius of 20 Å. However, some of the properties of this binder are very different compared with those exhibited by the partially purified receptor obtained by our procedure. For instance, the binder reported by Litwack and Rosenfield is relatively stable in the absence of the steroid, and the radioactive hormone can be displaced by various nonradioactive steroids, including 17β-estradiol. A more detailed analysis of the ability of this binder to interact with chromatin and DNA will certainly help to clarify its relationship to the glucocorticoid receptor.

Recently, Sherman et al. (1974, 1976) have reported the dissociation of the progesterone receptor of chick oviduct into a small subunit with a sedimentation coefficient of 2.6 S, induced by various cations. A cytosol receptor for androgens with a sedimentation coefficient of 3 S has also been isolated from rat prostate (Hu et al., 1975). Similarly, the estrogen receptor of calf uterus appears to consist of monomers with a sedimentation coefficient of 2.8 S, which exhibit a Stokes' radius of 28 Å and a molecular weight around 30 000 (Sica et al., 1976; Erdos and Fries, 1974). It is therefore conceivable that most, if not all, the steroid hormone receptors of mammalian tissues contain a relatively small steroid-binding subunit of molecular weight around 30 000, which, through aggregation, gives rise to the different forms of the receptor. In principle, this subunit could contain the steroid and the DNA-binding sites, or act in combination with another DNA-binding subunit to build the complete functional receptor. In the case of glucocorticoids, the first possibility appears to be more plausible, since the partially purified receptor interacts with DNA and chromatin in the same way as the receptor in crude cytosol (Bugany and Beato, 1977).

As most of the DNA in chromatin is structured in the form of repeating subunits, called nucleosomes, it was of interest to study the interaction of the receptor with the isolated chromatin subunits. The receptor binds equally well to monomeric and polymeric subunits of chromatin, and this binding is very sensitive to elevation of the ionic strength. In addition, the structural integrity of the whole nucleosomes is important for receptor binding, as treatment with high salt or tRNA, which removes the histone H1, leads to marked impairment of the ability of the subunits to bind the receptor. This effect is reminiscent of the disappearance of high-affinity sites for ethidium bromide binding, which is observed after removal of H1 histone (Lawrence and Daune, 1976), and suggests that H1 maintains the accessibility of certain DNA loops in chromatin (Rosenberg, 1976). Removal of H1 histone by high salt, followed by further nuclease digestion, results probably in structural change of the nucleosome arrangement (Altenburger et al., 1976). As a consequence, higher receptor binding is observed and the binding is insensible to elevation of the ionic strength. In this respect, it should be kept in mind that 0.6 M NaCl not only extracts histone H1 but also some nonhistone proteins of chromatin (Whitlock and Simpson, 1976).

Similarly to what is found with unfractionated chromatin (Bugany and Beato, 1977), no saturation of the receptor binding activity of the chromatin subunits was detected up to a concentration of receptor–steroid complex in the order of 10 nM. However, whereas unfractionated liver chromatin and erythrocyte chromatin have quite different ability to bind the receptor (Bugany and Beato, 1977), the native nucleosomes derived from both sources behave similarly. We can conclude that the higher capacity of liver chromatin to bind the glucocorticoid receptor should reside in either a higher order structure of the chromatin subunits or in that part of the chromatin which is not organized in the form of nucleosomes. A study of a differential influence of various nucleases, such as deoxyribonuclease I or micrococcal nuclease, on the receptor binding in liver and erythrocyte chromatin will probably provide further important information on the structural organization of the receptor-binding sites in the genome of target tissues.

Acknowledgments

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Intramitochondrial Intermembranal Reversible Translocation of Aspartate Aminotransferase and Malate Dehydrogenase through the Inner Mitochondrial Membrane†

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ABSTRACT: The translocation of aspartate aminotransferase, malate dehydrogenase, and bulk protein from the rat liver inner mitochondrial membrane and matrix toward the intermembranal space induced by certain organic acids (movement effectors) has been studied. Experiments involving a two-stage dissolution of the mitochondrial membranes by the use of detergents strongly suggest that enzymes like aspartate aminotransferase can cross the inner mitochondrial membrane providing exogenous movement effector was present. Experiments which measured the changes in membranal distribution of malate dehydrogenase induced by the movement effectors also suggested the occurrence of a similar phenomenon for this enzyme in intact mitochondria. Control experiments revealed that under our experimental conditions, the inner mitochondrial membrane remained impermeable to small molecules, e.g., sucrose, and that the release of aspartate aminotransferease, malate dehydrogenase, insocitrate dehydrogenase, and bulk protein into the intermembranal space in the presence of succinate occurred at a much lower concentration of digitonin than that required to disrupt the inner mitochondrial membrane.

Lateral protein diffusion in biological membranes could be facilitated by the high degree of molecular mobility of the membranal lipids (Gitler, 1972). Thus, the fluid character of a membrane would be the result of the spatio-temporal interactions of its lipid–protein constituents. Changes in these interactions could be caused by modifications to the chemical composition of the membrane itself or by changes in the environment surrounding such a membrane (Branton et al., 1972).

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