# Purification, Characterization, and Activation of the Glucocorticoid-Receptor Complex from Rat Kidney Cortex†

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*Received September 27, 1984*

ABSTRACT: The unactivated molybdate-stabilized glucocorticoid receptor (GcR) was purified from rat kidney cortex cytosol ($RK_cC$) by using a modification of the procedure previously described by this laboratory for rat hepatic receptor. The purification includes affinity chromatography, gel filtration, and ion-exchange chromatography. The final preparation (approximately 1000-fold pure as determined from specific radioactivity) was used in subsequent physicochemical and functional analyses. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) showed a single heavily Coomassie-stained band at 90 kilodaltons. Density gradient ultracentrifugation indicated a sedimentation coefficient of 10.5 ± 0.05 S ($n$ = 2). Chromatography on an analytical gel filtration column produced a Stokes radius ($R_s$) of 6.4 ± 0.07 nm ($n$ = 5). The $R_s$ was unchanged when the molybdate-stabilized GcR was analyzed in the presence of 400 mM KCl or when analyzed in the unpurified (cytosolic) state. In contrast, the hepatic GcR was observed to exist as a larger form in cytosol (7.7 ± 0.2 nm). Following purification, or upon gel filtration analysis under hypertonic conditions, the $R_s$ was similar to that of the unpurified $RK_cC$ GcR. Following removal of molybdate from $RK_cC$ GcR and thermal activation (25 °C/30 min), DNA–cellulose binding increased 1.5–2-fold over the unheated control. Addition of $RK_cC$ or hepatic cytosol (endogenous receptors thermally denatured at 90 °C/30 min or presaturated with $10^{-7}$ M radioinert ligand) during thermal activation increased DNA–cellulose binding an additional 2–6-fold beyond the heated control. These data suggest that although the purified unactivated kidney cortex and hepatic forms of the GcR are similar, the presumably more native cytosolic, unactivated forms may differ in Stokes' radii which may relate this difference to the concept of receptor polymorphism.

Published data from this laboratory have indicated the presence of a glucocorticoid receptor, named binder IB, in kidney cortex cytosol that is similar to, yet distinct from, the traditional receptor (binder II) found in liver (Litwack, 1975, 1982; Markovic & Litwack, 1980; Markovic et al., 1980; Barnett & Ohl et al., 1982; Mayer et al., 1983a,b). Binder IB appears in a time-dependent manner in vivo (Markovic & Litwack, 1980) and after thermal activation in vitro (Markovic et al., 1980). This finding is consistent with the hypothesis that IB is the activated (DNA binding) form of the glucocorticoid receptor in kidney cortex. Binder IB differs from II in that it binds more tightly to DNA (Ohl et al., 1982), has a different specificity of binding to homodeoxypolymers (Ohl et al., 1982), does not bind to DEAE-Sephadex when generated in vitro or in vivo (Markovic et al., 1980; Barnett & Litwack, 1982; Mayer et al., 1983a) or DEAE-cellulose when generated in vivo (Mayer et al., 1983a,b), and is smaller than binder II (approximately 2.6 vs. 6.0 nm, respectively) (Markovic et al., 1980; Barnett & Litwack, 1982; Ohl et al., 1982; Bastl et al., 1984). It is the latter characteristic that leads most directly to the controversy of whether IB is an isomorphic form of II which is found predominantly in kidney and colon (Bastl et al., 1984) or whether it is a proteolytic product of II. This possibility cannot yet be eliminated totally despite the following data: (1) proteolytic inhibitors included in the homogenization buffer after in vivo or in vitro labeling of receptors have failed to prevent the appearance of IB (Ohl et al., 1982; Mayer et al., 1983); (2) mixing experiments using kidney cytosol and liver cytosol have failed to generate IB from II (Barnett & Litwack, 1982); thus, kidney cytosol does not contain a protease which converts II to IB, nor does liver cytosol contain an inhibitor which prevents formation of IB from II; (3) a chymotrypsin-derived receptor fragment differs from IB (Mayer et al., 1983a,b); thus, deliberate proteolysis does not yield IB. Thus, it appears that binder IB is generated in vivo and, by way of indirect studies, that proteolysis of receptors to generate IB does not occur during subsequent homogenization. This does not exclude the possibility that physiologically relevant proteolysis occurs in vivo.

If binder IB were a separate gene product from the traditional glucocorticoid receptor, i.e. binder II, it would most likely have a separate unactivated precursor. We have hypothesized this to be the case and have approached the problem in this context since the unactivated receptor forms tend to be more stable and hence somewhat easier to characterize. Therefore, in order to determine whether the concept of receptor polymorphism is valid and to eventually understand if different glucocorticoid responses are due to the existence of different receptor forms, we have purified and characterized the unactivated molybdate-stabilized glucocorticoid-receptor complex from rat kidney cortex where IB predominates in cytosol after activation.

## Materials and Methods

*Chemicals.* [6,7-$^3$H]Triamcinolone acetonide ([$^3$H]TA)[1] (33 Ci/mmol) was purchased from New England Nuclear.

† Supported by Research Grants BC 361B from the American Cancer Society, AM 13531 and AM 32870 from the National Institutes of Health, and PCM 7815767 from the National Science Foundation and by Core Grant CA 12227 from the National Cancer Institute to the Fels Research Institute.

‡ Trainee, supported by NIH Training Grant 5T32AM07162 to the Department of Biochemistry.

[1] Abbreviations: TA, triamcinolone acetonide [9α-fluoro-11β,21-dihydroxy-16α,17-[(1-methylethylidene)bis(oxy)]pregna-1,4-diene-3,20-dione]; SDS, sodium dodecyl sulfate; Mes, 2-($N$-morpholino)ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; DTT, dithiothreitol.

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Bio-Gel A-1.5m (100–200 mesh) agarose gel and hydroxylapatite (HTP-DNA grade) were obtained from Bio-Rad, DEAE-cellulose DE-52 was from Whatman, DNA–cellulose was from P-L Biochemicals, calibration kits for gel filtration and SDS–polyacrylamide gel electrophoresis and Sephadex G-25 PD-10 minicolumns were from Pharmacia, Liquiscint was from National Diagnostics, and Sterogel affinity resin was from G-K Biochemicals (Somerset, NJ). All other reagents were obtained from Fisher.

*Preparation of Cytosols.* Male Wistar rats (100–200 g) were adrenalectomized 2–5 days before use. Animals were anesthetized (1 mL of Nembutal/kg of body weight) and liver and kidneys perfused in situ via a portal vein cannula with 60 mL of ice-cold 0.9% saline followed by 20 mL of ice-cold homogenization buffer (50 mM potassium phosphate monobasic, 10 mM sodium molybdate, and 10 mM thioglycerol, pH 7.0 at 22 °C). The liver and kidneys were excised and the kidneys dissected in a 4 °C cold room to remove the medulla. Kidney cortices and livers were manually minced and then homogenized in homogenization buffer (1:1 w:v) with a Teflon-glass Potter–Elvehjem apparatus. Homogenates were centrifuged at 4000*g* for 10 min. The upper lipid layer was removed, and supernatants were subsequently centrifuged at 105000*g* for 60 min. These supernatants (cytosol) were stored in a liquid nitrogen tank under vapor phase until use.

*Receptor Purification.* All procedures were performed at 4 °C. Cytosol from kidney cortex or liver was incubated with packed affinity resin at a 3:1 volume ratio for 2 h under constant, slow stirring. This mixture was centrifuged at 10000 rpm for 10 min and the supernatant tested for specific binding. The affinity resin pellet was washed thoroughly with 10 mL of ice-cold homogenization buffer and recentrifuged. Washing was performed 8 times. Receptor was eluted from the affinity resin by incubating overnight with 2 $\mu$M [$^3$H]TA in a volume of buffer roughly equal to the volume of packed affinity resin and not greater than 5% of the Bio-Gel A-1.5m column bed volume. Following affinity chromatography the resin was regenerated for subsequent use; however, with repeated regenerations the recovery of receptor in the eluate decreased and recovery in the supernatant increased.

The eluate was collected under vacuum filtration and filtered on a preparative Bio-Gel A-1.5m column (100–200 mL bed volume) which had been preequilibrated with buffer. Fractions of 5–8 mL were collected and aliquots counted for radioactivity. The peak receptor fractions were combined and filtered on a DEAE-cellulose ion-exchange column as previously described (Sakaue & Thompson, 1977). Glucocorticoid-receptor complexes were eluted with a salt gradient of 50–500 mM potassium phosphate containing 10 mM sodium molybdate and 10 mM thioglycerol (pH 7.0 at 22 °C). Peak receptor fractions were combined and used in subsequent physicochemical analyses and activation studies.

*Assay of Glucocorticoid-Receptor Complexes.* Cytosol was incubated with 50–100 nM [$^3$H]TA in the absence (total binding) or presence (nonspecific binding) of a 1000-fold excess of radioinert TA for 2 h at 4 °C. Specific binding in cytosolic or purified samples was measured by adsorption on hydroxylapatite (Erdos et al., 1970). The buffer used was 50 mM potassium phosphate and 10 mM sodium molybdate, pH 7.0 at 22 °C.

*Gel Filtration Analyses.* Purified receptor samples (≈3% of column volume) were analyzed on Bio-Gel A-1.5m columns (0.9 × 42 cm) preequilibrated with buffer. Samples were run at a 0.5 mL cm$^{-2}$ min$^{-1}$ flow rate. Blue dextran and [$^3$H]TA were used to determine the void volume ($V_0$) and total volume ($V_t$), respectively. Analytical columns were calibrated with thyroglobulin [8.50-nm Stokes radius ($R_s$)], ferritin (6.10 nm), catalase (5.22 nm), aldolase (4.81 nm), albumin (3.54 nm), ovalbumin (3.05 nm), chymotrypsinogen A (2.09 nm), and ribonuclease (1.64 nm). The partition coefficient was calculated from $K_{av} = [(V_e - V_0)/(V_t - V_0)]$ where $V_e$ is the elution volume of standard or known. $R_s$ was calculated from the calibration curve of $[-\log (K_{av})]^{1/2}$ vs. $R_s$ (Siegel & Monty, 1966).

*Glycerol Gradient Ultracentrifugation.* Linear gradients were prepared as described previously (Stone, 1974). Three 1.5-mL layers of 4, 22, and 40% glycerol in 10 mM Mes, 10 mM sodium molybdate, 10 mM thioglycerol, and 40 mM KCl, pH 7.0 (22 °C), were gently pipetted into nitrocellulose tubes. Receptor samples (0.2 mL) were layered on the top of the gradient and centrifuged at 38000 rpm for 16 h at 4 °C in an SW50.1 rotor. Three-drop fractions were collected by piercing the bottom of the tube and either mixed with scintillation fluid to measure radioactivity (unknowns) or used for reading $A_{280nm}$ (standards). Catalase (11.2 S) and albumin (4.6 S), or ovalbumin (3.6 S) were run as external standards. Sedimentation coefficients were calculated as previously described (Martin & Ames, 1961).

*SDS–Polyacrylamide Gel Electrophoresis (SDS–PAGE).* SDS–PAGE was performed as previously described (O'Farrell, 1975) using 5% stacking and 10% separating slab gels of 1.5-mm thickness. Samples were concentrated by acid precipitation as previously described (O'Farrell, 1975; Grandics et al., 1984) and run against molecular weight standards of phosphorylase *b* ($M_r$ 97000), albumin ($M_r$ 67000), ovalbumin ($M_r$ 43000), carbonic anhydrase ($M_r$ 30000), and trypsin inhibitor ($M_r$ 20100). Gels were first fixed and stained with Coomassie Blue (0.24% w/v in distilled water) in 45% ethanol and 10% acetic acid, then destained with 45% ethanol and 10% acetic acid until the background became light, and finally stained with a silver stain as previously described (Irie et al., 1982; Morrissey, 1981).

*DNA–Cellulose Binding Assay.* This assay was used to distinguish unactivated and activated receptor forms (Lefevre et al., 1979). DNA–cellulose was prepared in 10 mM Tris-HCl–1 mM EDTA (pH 8.0 at 4 °C) (TE buffer). The assay was performed by adding 100 $\mu$L of sample to a DNA–cellulose pellet obtained by centrifugation of 200 $\mu$L of DNA–cellulose slurry. The mixture of sample and DNA–cellulose was incubated 45 min at 4 °C with frequent mixing. The reaction was stopped by addition of 2.0 mL of TE buffer, mixing, and centrifugation at 500*g* in a clinical centrifuge. The DNA–cellulose pellet obtained was washed 2 times with 2.0 mL of TE buffer and finally suspended in 1.0 mL of buffer. A 0.5-mL aliquot was taken for counting in 5.0 mL of scintillation fluid.

*Miscellaneous.* Protein concentration was determined by the method of Bradford (Bradford, 1976). Conductivity was measured on a Markson Model 10 conductivity meter. Radioactivity was measured in an Intertechnique SL 30 liquid scintillation spectrometer. Counting efficiency for tritium was 30–40%.

## Results

*Purification of Glucocorticoid Receptors from Rat Kidney Cortex Cytosol ($RK_cC$).* Specific binding in $RK_cC$ was 95–98%. Recovery of cytoplasmic glucocorticoid-receptor complexes in affinity resin eluate was 30–40% (36.5 ± 8.7%, $n = 4$) compared to 10–20% (14.7 ± 6.3%, $n = 4$) in affinity resin supernatant. Purification after the affinity step was 100–200-fold as measured by specific binding per milligram

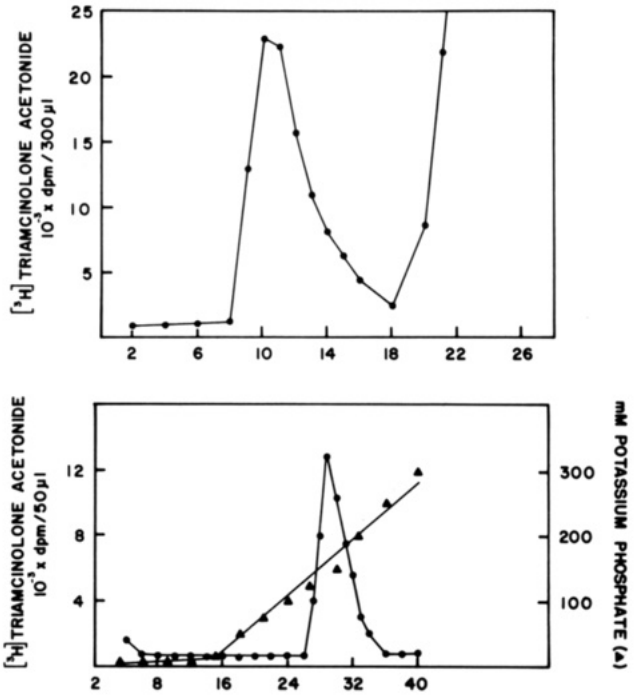


FIGURE 1: (Top) Gel filtration of affinity resin eluate on a preparative Bio-Gel A-1.5m column. The column was preequilibrated with 50 mM potassium phosphate, 10 mM sodium molybdate, and 10 mM thioglycerol. Peak glucocorticoid-receptor fractions (9–13) were combined and loaded on the DEAE-cellulose column. (Bottom) Ion-exchange chromatography on DEAE-cellulose of the Bio-Gel-eluted glucocorticoid-receptor fractions. Elution was performed with a linear 50–500 mM potassium phosphate gradient containing 10 mM sodium molybdate and 10 mM thioglycerol.

protein. The affinity resin eluate was then filtered on a preparative Bio-Gel A-1.5m column (Figure 1, top). Receptor-containing fractions were recovered in the first peak of radioactivity; the second peak contained free [$^3$H]TA. Recovery of cytosolic glucocorticoid receptor at this point was 20% (19.3 ± 9.9%, $n$ = 3) and purification increased another 3–4-fold. Receptor fractions from the Bio-Gel A-1.5m column were combined and chromatographed on a DEAE-cellulose ion-exchange column (Figure 1, bottom). Elution of bound glucocorticoid-receptor complexes was performed with a 50–500 mM potassium phosphate gradient. A single peak of radioactivity was observed at a salt concentration of 200–300 mM potassium phosphate. Final recovery of receptor was 5–10% (6.5 ± 1.5%, $n$ = 3) of cytosolic receptor. Purity increased another 2-fold to a final fold purification of approximately 1000. Samples from this step were analyzed further by physicochemical and functional properties.

*SDS–PAGE Analysis.* Figure 2 shows samples precipitated during the sequential purification of $RK_cC$ receptor and subsequently stained with Coomassie Blue or Coomassie Blue and silver, respectively. The double-stained gel clearly improves the appearance of protein bands at each level of purification. A heavily stained band at $M_r$ 90 000 is apparent in the Bio-Gel A-1.5m and DEAE-cellulose eluted radioactivity peaks by using Coomassie Blue or double staining and becomes more predominant as the purification progresses. Though other bands can be seen in the DEAE-cellulose eluate, they do not correspond with the pattern of radioactivity. Only the $M_r$ 90 000 band is clearly present in the [$^3$H]TA-containing fractions from the DEAE-cellulose column and absent in the non-[$^3$H]TA-containing fractions. The presence of other bands along with the $M_r$ 90 000 band was variable. That the $M_r$ 90 000 band was related to the receptor was confirmed by

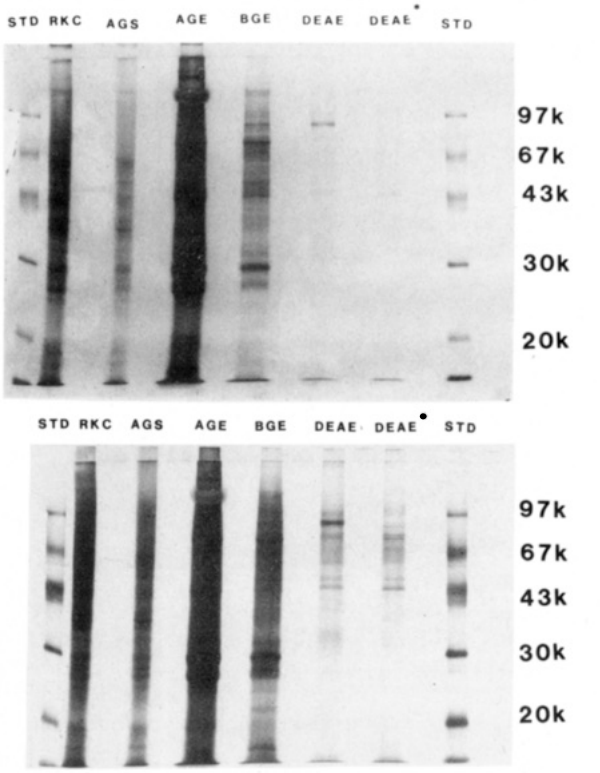


FIGURE 2: Electrophoretogram of samples during sequential purification steps and stained with Coomassie Blue (top) or Coomassie Blue and silver (bottom). Abbreviations: std, standard (97K, phosphorylase $b$, $M_r$ 97 000; 67K, albumin, $M_r$ 67 000; 43K, ovalbumin, $M_r$ 43 000; 30K carbonic anhydrase, $M_r$ 30 000; 20K, trypsin inhibitor, $M_r$ 20 100); RKC, rat kidney cortex cytosol; AGS, affinity gel resin supernatant; AGE, affinity gel resin eluate; BGE, Bio-Gel eluate; DEAE, DEAE-cellulose eluate from a peak radioactivity region of the column; DEAE*, DEAE-cellulose eluate from a nonpeak radioactivity region of the column.

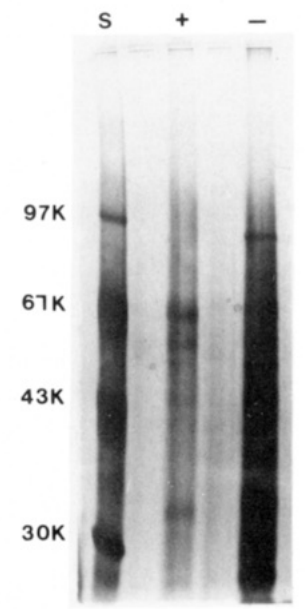


FIGURE 3: Electrophoretogram of DEAE-cellulose eluates from purified cytosols presaturated in the presence (+) or absence (–) of $10^{-7}$ M radioinert triamcinolone acetonide prior to incubation with affinity resin. S is the standard lane with abbreviations as in Figure 2.

presaturating cytosol with radioinert TA prior to incubation with the affinity resin. Side by side electrophoretic analysis of cytosols presaturated in the presence or absence of $10^{-7}$ M radioinert TA demonstrates the absence of the $M_r$ 90 000 band

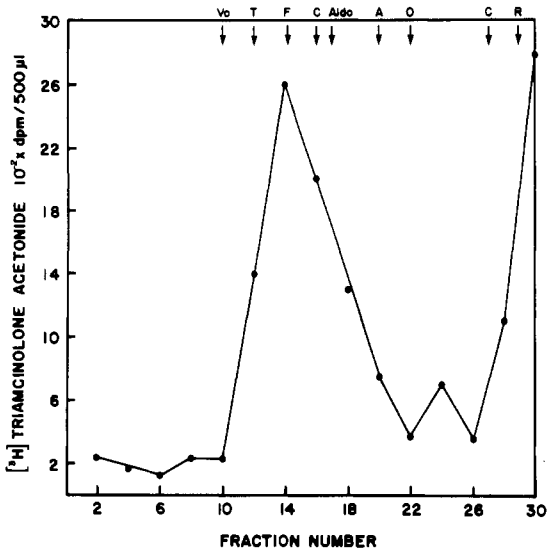


FIGURE 4: Gel filtration analysis of glucocorticoid-receptor complex. DEAE-cellulose-eluted complexes were rechromatographed on an analytical Bio-Gel A-1.5m column previously calibrated with the following protein standards: T, thyroglobulin ($R_s$ = 8.50 nm); F, ferritin ($R_s$ = 6.10 nm); C, catalase ($R_s$ = 5.22 nm); Aldo, aldolase ($R_s$ = 4.81 nm); A, albumin ($R_s$ = 3.55 nm); O, ovalbumin ($R_s$ = 3.05 nm); Ch, chymotrypsin ($R_s$ = 2.09 nM); R, ribonuclease ($R_s$ = 1.64 nm). Elution was performed with 50 mM potassium phosphate, 10 mM sodium molybdate, and 10 mM thioglycerol, pH 7.0 at 4 °C, buffer.

Table I: Summary of Gel Filtration Analyses of Stokes' Radius in Purified or Cytosolic Unactivated Preparations of Kidney and Liver Glucocorticoid Receptor

| | conditions of chromatography | |
|---|---|---|
| | hypotonic[a] (nm) | hypertonic[b] (nm) |
| purified kidney receptor | 6.4 ± 0.07 ($n$ = 5)[c] | 6.5 |
| cytosolic kidney receptor | 6.8 ± 0.06 ($n$ = 3) | 6.4 ± 0.10 ($n$ = 2) |
| purified liver receptor | 6.4 ± 0.05 ($n$ = 2) | |
| cytosolic liver receptor | 7.7 ± 0.20 ($n$ = 6) | 6.3 ± 0.06 ($n$ = 3) |
| partially purified kidney receptor ($DEAE_c$ only) | | 6.5 |
| partially purified liver receptor ($DEAE_c$ only) | 6.8 ± 0.01 ($n$ = 2) | 6.2 |

[a] Elution buffer was 50 mM potassium phosphate, 10 mM sodium molybdate, and 10 mM thioglycerol, pH 7.0 at 4 °C. [b] Elution buffer was 50 mM potassium phosphate, 10 mM sodium molybdate, and 10 mM thioglycerol, pH 7.0 at 4 °C, buffer containing 400 mM KCl (cytosolic samples) or 150 mM KCl (purified samples). [c] Values are means ± SEM with number of experiments in parentheses.

in the presaturated cytosol (Figure 3).

*Physicochemical Characterization.* Rechromatography of purified molybdate-stabilized receptor from DEAE-cellulose eluate on analytical Bio-Gel A-1.5m columns demonstrated the appearance of a single peak of radioactivity that eluted with a $R_s$ of 6.4 ± 0.07 nm ($n$ = 5) (Figure 4). Analysis under hypertonic conditions (400 mM KCl added to the buffer) did not alter the $R_s$, nor did analysis of unpurified (cytosolic) receptor reveal a different $R_s$ (6.9 ± 0.05 nm, $n$ = 2). Interestingly, a substantial difference in $R_s$ was observed between purified and cytosolic forms of liver receptor (6.4 vs. 7.7 nm, respectively). These data are summarized in Table I. Purified kidney receptor from DEAE-cellulose chromatography sedimented at 10.5 S under low-salt conditions (Figure 5). This was similar to the sedimentation coefficient obtained for purified liver receptor (10.3 S). By use of the $R_s$ and sedimentation coefficient obtained under low-salt conditions, purified kidney receptor has a calculated apparent $M_r$ 294 000, and purified liver receptor has a calculated apparent $M_r$ 288 000.

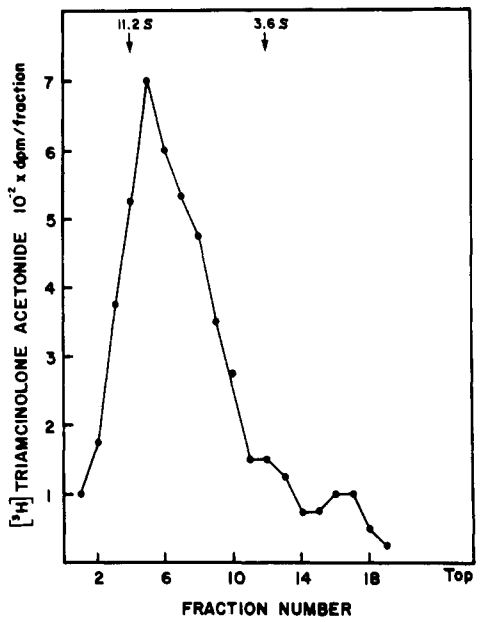


FIGURE 5: Density gradient ultracentrifugation of purified glucocorticoid-receptor complex. DEAE-cellulose-eluted complexes were layered onto linear glycerol gradients made in 10 mM potassium phosphate, 10 mM sodium molybdate, 10 mM thioglycerol, and 40 mM potassium chloride. External standards were catalase (11.2 S) and ovalbumin (3.6 S).

Table II: Activation of Purified Glucocorticoid-Receptor Complexes from Rat Kidney Cortex Cytosol[a]

| treatment | expt | hydroxyl-apatite bound (dpm) | DNA-cellulose bound (dpm) | % binding to DNA-cellulose | change ($x$-fold) |
|---|---|---|---|---|---|
| 0 °C, 30 min | 1 | 3803 | 219 | 5.8 | 1.0 |
| | 2 | 7141 | 364 | 5.1 | 1.0 |
| | 3[b] | 3494 | 470 | 13.5 | 1.0 |
| 25 °C, 30 min | 1 | 2083 | 274 | 8.9 | +1.6 |
| | 2 | 5169 | 418 | 8.1 | +1.5 |
| | 3[b] | 3870 | 1056 | 27.3 | +2.0 |
| 25 °C, 30 min, +10 mM sodium molybdate | 2 | 4638 | 331 | 7.1 | −1.1 |
| | 3[b] | 4004 | 757 | 18.9 | −1.4 |
| 25 °C, 30 min, $+RK_cC$[c] | 1 | 2204 | 498 | 21.6 | +2.4 |
| | 3[b] | 3857 | 1295 | 33.6 | +1.2 |
| 25 °C, 30 min, +RLC[d] | 1 | 2923 | 627 | 21.4 | +2.4 |
| | 3[b] | 4397 | 1666 | 37.9 | +1.4 |
| 25 °C, 30 min, +90 °C treated $RK_cC$ | 1 | 3348 | 842 | 25.1 | +2.8 |
| | 3[b] | 3733 | 2115 | 56.7 | +2.1 |
| 25 °C, 30 min, +90 °C treated RLC | 1 | 3508 | 1051 | 30.0 | +3.4 |
| | 2 | 5203 | 2836 | 54.5 | +6.7 |
| | 3[b] | 3737 | 1939 | 51.9 | +2.0 |

[a] Glucocorticoid-receptor complexes were passed through a gel filtration column prior to treatment to remove sodium molybdate. Unheated cytosols were presaturated with $10^{-7}$ M TA for 2 h at 4 °C prior to incubation with purified receptor complexes. [b] Denotes receptor purified through Bio-Gel A-1.5M chromatography stage. [c] $RK_cC$, rat kidney cortex cytosol. [d] RLC, rat liver cytosol.

*In Vitro Activation of Purified Receptor from $RK_cC$.* Prior to attempted activation of purified receptor, sodium molybdate was removed by filtering the DEAE-cellulose eluted receptor through a Sephadex G-25 or G-75 column preequilibrated in 10 mM Mes, 50 mM KF, 0.5 mM EDTA, and 0.5 mM DTT, pH 6.5, buffer ± 2 mg/mL BSA, respectively. Activation was assessed by the extent of binding of glucocorticoid-receptor complexes to DNA–cellulose (Table II). At 0 °C DNA–cellulose binding was low, indicating the presence of unactivated glucocorticoid receptor. Binding to DNA–cellulose

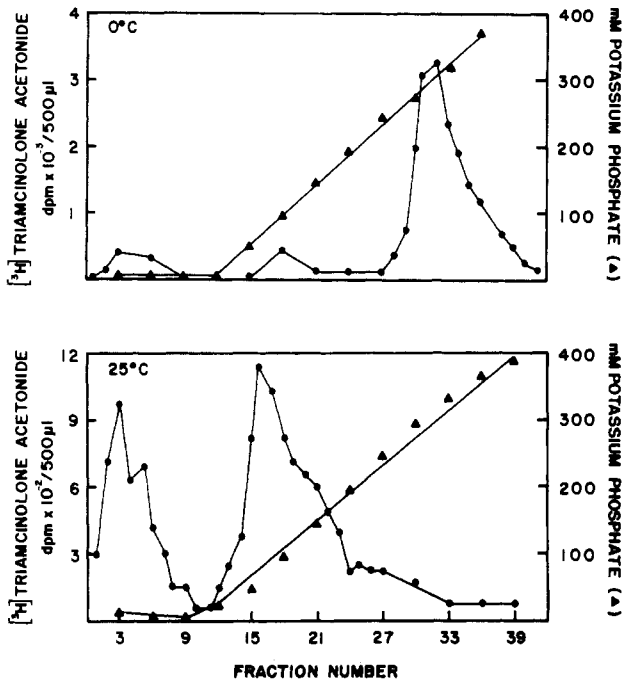


FIGURE 6: DEAE-cellulose chromatography of thermally activated glucorticoid-receptor complex. DEAE-cellulose-eluted complexes were filtered through a Sephadex G-25 column to remove sodium molybdate and subsequently were treated at 0 (top) or 25 °C (bottom) for 30 min. Samples were then rechromatographed on DEAE-cellulose and eluted with a 5–500 mM potassium phosphate buffer containing 10 mM sodium molybdate and 10 mM thioglycerol. The Sephadex G-25 column buffer was 10 mM Mes, 0.5 mM EDTA, and 0.5 mM dithiothreitol with 5 mg of albumin/mL.

approximately doubled upon heating (25 °C, 30 min), and this effect was inhibited by the addition of 10 mM sodium molybdate prior to heat treatment. Verification of activation was obtained by rechromatographing glucocorticoid-receptor complexes on DEAE-cellulose to observe the salt elution profile. Glucocorticoid-receptor complexes obtained following Sephadex G-25 or G-75 chromatography and maintained at 0 °C eluted at a potassium phosphate concentration of approximately 250 mM (Figure 6). Corresponding DNA–cellulose binding was 5.8%. Heat treatment caused a shift in the elution profile to a salt concentration of approximately 5 and 50 mM (Figure 6). DNA–cellulose binding was 8.9%. Although binding to DNA–cellulose after heating approximately doubled compared to the unheated control, it is substantially less than that seen with unpurified (cytosolic) receptor (approximately 40–50%). Binding of the purified, heat-treated receptor to DNA–cellulose could be increased to the level observed with cytosol by the addition of presaturated ($10^{-7}$ M TA) or boiled (90 °C, 30 min) cytosol from kidney or liver.

## Discussion

Rat kidney cortex glucocorticoid receptor was purified in the unactivated state by using a procedure previously described in this laboratory for hepatic glucocorticoid receptor (Grandics et al., 1984). All purification steps included 10 mM sodium molybdate to minimize degradation and increase stability of the receptor (Hazato & Murayama, 1981; Leach et al., 1983; Weatherill & Bell, 1982; Vedeckis, 1983). Nevertheless, substantial steroid dissociation or receptor loss occurred during the gel filtration and ion-exchange chromatography steps leading to the relatively low fold purification based on specific activity (1000-fold) of $RK_cC$ GcR compared to that of hepatic GcR (4000-fold). Since the purification based on protein concentration was almost 5000-fold, which is similar to that in the hepatic system, it is likely that steroid dissociation accounted for the low fold purification when measured by specific activity. In the final preparation the receptor appeared as a predominant 90 kilodalton (kDa) band with Coomassie staining of SDS–polyacrylamide gels. Double staining with the sensitive silver technique revealed several other bands along with the 90-kDa band in the final preparation. This indicates that Coomassie staining alone is not sufficient to judge the purity of the receptor preparation and that further purification of the kidney receptor is necessary. To determine which band(s) present on the SDS–polyacrylamide gel was (were) dependent on the presence of specific ligand, the purification was performed with cytosol that had been presaturated with $10^{-7}$ M radioinert TA prior to incubation with affinity resin. Such a concentration of TA saturates the receptor, preventing its association with the affinity resin. Under these conditions the 90-kDa band did not appear, which indicated that it is the glucocorticoid binding moiety. The identity of the bands other than the 90 kDa band is presently unknown, but it is unlikely that they are receptor fragments since they still appear when the cytosol was presaturated with radioinert TA. If they were large proteolytic fragments of receptor produced during homogenization or purification, then they would likely still contain the steroid binding site, and preincubation with radioinert TA should preclude their appearance. Supportive evidence that the 90-kDa band is the steroid binding moiety is the absence of a 90-kDa band in the nonpeak radioactivity region of the DEAE-cellulose column. That the 90-kDa band is the glucocorticoid binding moiety in the kidney is consistent with previous reports on the glucocorticoid receptor from rat hepatic tissue (Grandics et al., 1984; Wrange et al., 1984) and cultured rat hepatocytes (Simons & Thompson, 1984; Simons et al., 1983). We have previously hypothesized the liver receptor to be an oligomer of 90-, 41-, 40-, and 24-kDa subunits (Grandics et al., 1984). Physiologically relevant receptor subunits would be expected to exist with the steroid binding subunit in a stoichiometric relationship. At present the variable appearance of other bands along with the 90-kDa band precludes judgement regarding the association of other protein bands with the 90-kDa subunit. It may be that more purified preparations containing greater receptor concentrations will be necessary to resolve the question of subunit structure.

Gel filtration analysis of the rat kidney glucocorticoid receptor was performed under conditions which optimized receptor stabilization during rechromatography. Specifically, 10 mM sodium molybdate was used in the column buffer, a load volume of 3.0% of the column bed volume was employed in order to increase the receptor concentration while maintaining high resolution, and a small analytical column (0.9 × 42 cm) was used to decrease running time of the column. Under these conditions purified kidney receptor samples ran as a single, discrete peak of $R_s$ = 6.3–6.5-nm in the presence or absence of 400 mM KCl. Since unpurified receptor from $RK_cC$ ran at a similar position ($R_s$ = 6.8–6.9 nm), inclusion of an inert protein, as was previously the case with the liver (Grandics et al., 1984), to stabilize the system was not deemed necessary. The similarity in $R_s$ values between purified and cytosolic kidney glucocorticoid receptor samples run under hypertonic and hypotonic conditions indicates that proteolysis or dissociation of subunits during the purification procedure did not occur. Proteolysis or dissociation cannot be ruled out entirely, however, as it may have occurred prior to purification, i.e., during preparation of cytosol or thawing of frozen cytosol. The latter possibility was previously tested in this laboratory

by comparing fresh vs. frozen kidney cytosol and was eliminated when no differences were found (Mayer et al., 1983b). Therefore, the only possibility for proteolysis or dissociation to have occurred, and thereby to have influenced the sizing data, was during preparation of cytosol. Sherman et al. (1983) have proposed the existence of a membrane-bound, lysine-specific enzyme which is a factor in receptor degradation in cytosol from rat kidney. Despite the suggestion by the same investigators that the primary physiological role of this enzyme is not the degradation of steroid hormone receptors, the possibility remains that this or another yet to be described enzyme may have proteolyzed the receptor.

The substantial difference in $R_s$ observed between purified (4000-fold) and cytosolic liver glucocorticoid receptor can be interpreted two ways: (1) proteolysis of liver receptor occurred during purification, and (2) liver receptor possesses a subunit which dissociates during purification that is not present on the kidney receptor. The former possibility seems unlikely as it was not the case in the kidney, a tissue with great proteolytic activity (Sherman et al., 1983). The latter possibility seems more likely since the smaller $R_s$ form of the liver receptor can be generated after passage of cytosol through a DEAE-cellulose column or by running cytosol on a sizing column in hypertonic buffer (Table I). These data are suggestive of subunit differences between unactivated liver and kidney receptor when examined as they would exist in cytosol. Prior data from this laboratory have indicated that the purified hepatic GcR had a $R_s$ of approximately 7–8 nm (Grandics et al., 1984) i.e., similar to that of unpurified hepatic receptors. This differs from the present report in which the purified hepatic receptors $R_s$ was observed to be 6.4 nm. Reasons for this variation are unknown but may be due to differences in conditions of analysis or possibly to dissociation of receptor complexes on the sizing column. If the latter did occur and the liver receptor $R_s$ is larger than the kidney receptor in the purified state, then this would be consistent with previous evidence of differences in receptor structure in these tissues (Markovic et al., 1980; Barnett & Litwack, 1982; Mayer et al., 1983a,b).

Purified receptor from kidney was found to sediment at 10.5 S and purified liver receptor at 10.3 S. This is similar to values previously reported (9–10 S) for unpurified kidney and liver receptor (Sherman et al., 1983) and purified liver receptor (Grandics et al., 1984). Furthermore, preliminary data indicate that thermal activation of purified kidney receptor in the presence of boiled cytosol resulted in the conversion of the 10S sedimenting form to a 4–5 S sedimenting form. An intermediate form of 7 S was observed upon thermal activation alone. These results are in agreement with recent reports (Eastman-Reks et al., 1984; Tymoczko et al., 1984) of an intermediate hepatic receptor form which is capable of binding to DNA–cellulose.

This is the first report on in vitro activation of purified kidney receptor and is further evidence of purification of the unactivated receptor. As with purified liver glucocorticoid-receptor complex (Grandics et al., 1984) thermal activation of purified kidney receptor increased binding to DNA–cellulose to a lesser extent than that normally seen in an unpurified system (Lefevre et al., 1979; Simons et al., 1983; Sakaue & Thompson, 1977). Reconstituting the purified system by adding unheated TA-presaturated cytosol or boiled cytosol from kidney or liver during thermal activation raises the DNA–cellulose binding to that seen in crude systems. This effect is not due to protein stabilization of the GcR since (1) a buffer control with 30 mg/mL albumin did not produce the same increase in DNA–cellulose binding, and (2) the protein concentrations of unheated (30 mg/mL) and boiled (2 mg/mL) cytosol are very different. We interpret this to mean that a heat-stable factor (stimulator) is present in both tissues that is involved in the activation process. Further details on the mechanism of activation and on characteristics of cytosolic stimulator have been reported elsehwere (Schmidt et al., 1984) and are the subject of a forthcoming paper. Sodium molybdate readdition inhibits both thermal and thermal plus stimulator activation. The extent of inhibition is variable and is likely dependent on the number of activated complexes present before readdition. Interaction of sodium molybdate directly with the purified receptor seems apparent though it is possible that its mechanism of action is via an inhibitor of activation, previously termed "modulator" (Sekula et al., 1981), which copurifies or is tightly associated with the receptor. Purified unactivated kidney receptor is completely shifted by thermal activation from a 200 mM salt position to a 50 mM salt position on DEAE-cellulose chromatography. The identity of this peak is presently under investigation but is reminiscent of in vitro generated binder IB (Mayer et al., 1983a,b) and binder II (Grandics et al., 1984; Sakaue & Thompson, 1977). Identification of the 50 mM peak as binder II or IB can be achieved by chromatographing purified activated receptor on DEAE-Sephadex (Markovic et al., 1980). The radioactivity peak seen in the prewash is the position that free steroid would be expected; thus, it is likely that this radioactivity peak is not receptor bound. Mayer et al. (1983b) have observed binder IB in the prewash of DEAE-cellulose chromatograms only when generated in vivo.

In summary, these data indicate that the purified unactivated form of the GcR from $RK_cC$ has physicochemical characteristics similar to those observed for the purified unactivated hepatic GcR. This is not unexpected since if a different form of the GcR arose during evolution, it would likely be through gene duplication and may not involve drastic alterations in primary amino acid sequence and hence tertiary structure. Thus, these data should not be interpreted to mean that these tissue receptors are identical especially in light of the observation that the unpurified unactivated kidney and hepatic GcR have different Stokes' radii. Existence of different unactivated precursors in the cell is consistent with previous data on different tissue-specific activated receptor forms and supportive of the concept of polymorphism. On the basis of previous data showing a different specificity for homodeoxypolymers (Ohl et al., 1982) and the concept of isoreceptors as an explanation for different physiologic responses to the same hormonal stimulus, it seems reasonable that differences in receptor structure, if they do exist, would involve the DNA binding site. We suspect that the steroid binding site would be a highly conserved region of the molecule while preliminary immunologic data (N. Robertson, W. Kusmik, and G. Litwack, unpublished data) suggest that the immunologic domains of the molecule are also conserved. In contrast, if the unactivated precursors are similar, as the bulk of the present physicochemical data indicates, yet IB appears smaller than II (gel filtration analysis of $R_s$) (Barnett & Litwack, 1982; Ohl et al., 1982; Mayer et al., 1983a) in the apparent absence of proteolysis, then an explanation is less obvious. Kidney- and colon-activated forms (IB) may appear smaller than II because of differences in tertiary structure and therefore amino acid sequence. Evidence of this can be obtained by activating purified unactivated receptor from kidney and liver and analyzing the activated receptor forms on SDS–PAGE and by tryptic digests. Further investigation is under way to study

the structure of unactivated and activated forms of the glucocorticoid receptor in kidney and liver. Ultimately the question of the physiologic relevance of IB as a receptor, regardless of its mechanism of generation, may have to come from experiments in intact cells using fluorescently labeled monoclonal antibodies specific to IB and unreactive with II.

ACKNOWLEDGMENTS

We acknowledge the technical expertise of Mel Paden, Vincent Pearson, Noreen Robertson, and Cassandra Wooten and appreciate the critical input from Dr. Carol Barnett, Peter Bodine, and Dr. Tom Schmidt.

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