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Evidence for a Physiological Role of Corticosteroid Binder IB†

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ABSTRACT: Glucocorticoid binding proteins in liver and kidney of adrenalectomized rats have been analyzed by rapid ion-exchange chromatography, gel filtration, and specific interactions with antibodies against purified liver cytosol receptor and purified transcortin. In both tissues, the unactivated form of the [^{3}H]triamcinolone acetonide–receptor complex is eluted by 0.4–0.5 M KCl from DEAE-Sephadex minicolumns. After heat activation, the major form in liver cytosol, corticosteroid binder II, is eluted by 0.2 M KCl from the DEAE minicolumn; a minor component in the buffer wash is identified as corticosteroid binder IB. Binders II and IB are shown to be DNA-binding proteins whereas the unactivated forms do not bind to DNA. In contrast, the major activated form in kidney cytosol has the properties of corticosteroid binder IB (e.g., it is eluted in the buffer wash of the DEAE minicolumn, is a DNA-binding protein, and has a Stokes radius of 20–26 Å). Evidence is presented which suggests that IB is not a proteolytic product of II. Antibody to highly purified rat liver glucocorticoid receptor was immobilized on Sepharose 4B-CL. The major unactivated and activated [^{3}H]triamcinolone acetonide–receptor complexes from rat liver are adsorbed by the immunoaffinity column. Liver binder IB, however, is not adsorbed. The major unactivated and activated forms in kidney are not adsorbed by the antibody column; thus, both rapid ion-exchange and immunoaffinity chromatography suggest that the kidney form is similar to liver IB. Kidney cortex was separated from the medulla. The medulla contained corticosteroid binder II as defined by ion-exchange and immunoreactivity. This small quantity of medulla binder II is masked when cytosols are prepared from the whole kidney. In cytosols from heart, thymus, brain, and muscle, the receptor is similar to the liver and kidney medulla receptors. Further analysis of corticosteroid-binding proteins in kidney was carried out by using an antibody to purified serum transcortin. The kidney contains a large quantity of transcortin-like protein that binds [^{3}H]corticosterone. After removal of this protein by immunoprecipitation, the remaining receptor in kidney has the properties of corticosteroid binder IB. Taken together, these results show that binder IB may be a second glucocorticoid receptor in liver and the only glucocorticoid receptor in kidney cortex.

Glucocorticoids regulate gene expression in eukaryotic cells via a two-step mechanism common for all steroid hormones (Litwack et al., 1973; Kalimi et al., 1973; Rousseau et al., 1973). A complex of steroid with a specific receptor protein forms in the cytoplasm of the target cell (Litwack et al., 1965; Baxter & Tomkins, 1971; Beato et al., 1971). The hormone–receptor complex must undergo activation to enable translocation to the nucleus (Litwack et al., 1973; Kalimi et al., 1973; Baxter et al., 1972; Higgins et al., 1973; Beato et al., 1973; Wira & Munck, 1974; Atger & Milgrom, 1976) and binding to chromatin (Simons et al., 1976) or in vitro to purified DNA (Baxter et al., 1972; Wira & Munck, 1974; Milgrom et al., 1973; Cake et al., 1978).

Although numerous reports exist on specific glucocorticoid receptors in various target tissues, the few comparative studies which have been done (Ballard et al., 1974; Acs et al., 1975; Lippmann & Thompson, 1974; Feldman et al., 1978) suggest that receptors in different target organs are similar. Deciding whether receptors from different targets are identical is important to the understanding of the general mechanism of steroid hormone action. Kidney was selected in addition to the liver, which is a well characterized target tissue for glucocorticoids (Litwack et al., 1965). Besides its role as a target tissue for mineralocorticoids, the kidney displays gluconeogenic activity (Krebs et al., 1963) which is responsive to administered glucocorticoids (Londau, 1960). Specific glucocorticoid receptors have been identified in kidney cytosol (Rousseau et al., 1972; Funder et al., 1973). The results here indicate the existence of a distinctive and identical glucocorticoid receptor type in all the tissues studied except for kidney cortex, whose

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major receptor is similar to a second form in liver cytosol, corticosteroid binder IB (Litwack & Rosenfield, 1975).

Materials and Methods

Animals. Adrenalectomized male rats weighing 150–175 g of the Sprague-Dawley strain were used 4–8 days following surgery. They were fed a normal diet and given 0.9% NaCl to drink.

Isotopes and Chemicals. [1,2,4-$^3H_3$]Triamcinolone acetonide (37.4 Ci/mmol), [1,2-$^3H_2$]cortisol (40 mCi/mmol), and [1,2-$^3H_2$]corticosterone (53.6 Ci/mmol) were obtained from New England Nuclear Corp. Activated charcoal and triamcinolone acetonide were obtained from Sigma Chemical Corp. DNA–cellulose (1.0 mg of native calf thymus DNA/mL of cellulose) was prepared by the method of Alberts & Herrick (1970). DEAE-Sephadex A-50, Dextran T-500, Sephadex G-100 and G-150, and Blue Dextran 2000 were obtained from Pharmacia (Upsala).

Preparation of Cytosol. Animals were killed by decapitation, and liver, kidney, and heart were perfused in situ with 30–40 mL of cold 0.145 M NaCl. Brain, skeletal muscle, and thymus were sectioned out without perfusion. The tissues were removed and homogenized in an equal volume of ice-cold homogenization buffer (50 mM Tris-HCl, pH 7.5, 0.25 M sucrose, and 3 mM $MgCl_2$), and cytosol was prepared as previously described (Parchman & Litwack, 1977).

Steroid Labeling of Cytosol. Cytosols were labeled in vitro by incubation for 90–120 min at 4 °C with [^{3}H]triamcinolone acetonide ([^{3}H]TA) or [^{3}H]corticosterone at a final concentration of 3×10^{-8} M except for kidney, where a 5×10^{-8} M concentration of hormone was used.

For in vivo labeling each animal received a single intraperitoneal injection of 25 μCi/100 g body weight of [^{3}H]TA dissolved in 0.145 M NaCl and was killed by decapitation after 15 min. The homogenization buffer described above was used with a 100-fold excess of unlabeled triamcinolone acetonide added to prevent binding of any free labeled steroid during the preparative procedure.

Activation of cytosol and receptor complexes, DNA–cellulose binding assays, and determination of bound steroid were performed as previously described (Cake et al., 1978).

Ion-Exchange Chromatography. DEAE-Sephadex A-50 minicolumns (1.4 × 7 cm) were equilibrated with 20 mM potassium phosphate buffer (pH 6.8). All columns included dextran-coated charcoal at the bottom to remove free steroid. Protein concentration of eluates in tissue cytosol was sufficient to prevent losses by nonspecific adsorption to charcoal. Chromatography was carried out as previously reported (Parchman & Litwack, 1977), and a linear KCl gradient elution was used. A 0.5-mL amount of labeled cytosol was applied to the minicolumn, and after passage of 16 mL of initial buffer (first eight fractions, 2 mL each) elution was begun by a linear salt gradient formed from 25 mL of 20 mM potassium phosphate buffer (pH 6.8) and 25 mL of the same buffer containing 1 M KCl. One hundred fractions of 0.5 mL were collected at a rate of 1 mL/min and were analyzed for radioactivity, protein, and salt concentration.

Determination of Stokes' radius of activated rat liver and kidney receptor forms was performed by the method of Andrews (1964) and Siegel & Monty (1966) using column chromatography on Sephadex G-100 or G-150 (0.5 × 10 cm) equilibrated with 20 mM KCl. The flow rate was maintained at 15 mL/h, and 0.24-mL fractions were collected directly into the scintillation vials and analyzed for radioactivity. Following the ion-exchange chromatography, 0.5 mL of the sample containing IB-like binding protein or the major activated liver and medulla form eluted by 0.20–0.22 M KCl was applied to a Sephadex G-100 or G-150 column, respectively, and elution was performed with the same buffer used for equilibrating the column. The following standard proteins were used for calibrating the columns: cytochrome *c* (from horse heart), ovalbumin (grade V), albumin (bovine serum fraction V), γ-globulin (all obtained from Sigma Chemical Corp.), and myoglobin (whole skeletal, A grade purchased from Calbiochem. Corp.). Void volume was determined by using Blue Dextran 2000.

Purification of Rat Serum Transcortin and Preparation of Anti-Rat Transcortin. Eleven male adrenalectomized rats each weighing 175 g were used for every preparation. Animals were decapitated, blood was collected in a beaker, and clotting ensued on ice for at least 1 h. After centrifugation at 5000 rpm in the Sorvall RC-2 centrifuge for 10 min, 0.5 nM [1,2-$^3H_2$]cortisol (New England Nuclear, 40 mCi/mmol) was incubated in 20 mL of serum for 90 min on ice with occasional swirling. The mixture was loaded onto a 3 × 66 cm Sephadex G-75 column and eluted with 50 mM Tris-HCl, pH 7.5, in the cold. Radioactivity (Weinstein et al., 1967) and absorbancy at 280 nm were measured on the eluates. Three protein peaks and a single major included radioactive peak were observed. After being pooled (>80 mL), the radioactive peak was concentrated by lyophilization to 13 mL, loaded on a 3 × 80 cm DEAE-Sephadex A-50 column, and eluted with a linear gradient of 50 mM Tris, pH 7.5, to 50 mM Tris with 1 M KCl at pH 7.5. Eluates were assayed for radioactivity and absorbancy at 280 nm as described above. The single major radioactive peak was pooled, lyophilized, and dialyzed against 50 mM Tris, 1% glycine, and 0.5 nM [^{3}H]cortisol, pH 7.5, overnight and, if necessary, stored frozen. This material (0.5 mL at a time) was subjected to preparative isoelectrofocusing on an LKB 110-mL isoelectrofocusing column at 4 °C. Voltage was set at 100 V for 4.5 h and then increased to 300 V to completion. Two-milliliter fractions were collected at 4 °C. A major peak with a p*I* at 4.4–4.5 was obtained in several experiments. This was chromatographed on a Sephadex G-100 column (2 × 60 cm) after the radioactive peak was concentrated sixfold by lyophilization to 9–10 mL. This fraction was estimated to contain about 10 mg of protein/mL. The major radioactive peak eluted at a position corresponding to a molecular weight of about 61 000. Normalization of bound radioactivity an absorbance at 280 nm indicated that a high degree of purification had been achieved, and subsequent isoelectrofocusing confirmed this. Polyacrylamide gel electrophoresis was attempted, but the ligand dissociated under these conditions.

New Zealand white rabbits (Hilltop Laboratories, Scottsdale, PA) weighing 2.5 kg were used. Purified serum transcortin (0.4 mg) in 2 mL of 50 mM sodium phosphate buffer, pH 7.4, was combined with 2 mL of complete Freund's adjuvant, vortexed for 15 s until white and cloudy, and injected intramuscularly. Rabbits were bled from the ear vein before each injection. A total of eight weekly bleedings and injections were done. A positive Ouchterlony precipitin line appeared after the second bleeding, and the potency increased until the fifth bleeding when the antiserum could be diluted 10-fold and still showed a strong precipitin line. The IgG fraction was purified from collected antisera by the method of Livingston (1974). This preparation (in concentration, 10 mg/mL) was used in precipitation of transcortin by incubation of kidney cytosol for 12–16 h at 4 °C. The equivalence point was determined, and 122 μL of antibody preparation was used for

100 μL of kidney cytosol or serum (diluted 1:50 with TSM buffer) used in control experiments. Following incubation and centrifugation (10 min at 2000 rpm), the resulting supernatant fraction was incubated for 50 min at 4 °C with [^{3}H]corticosterone (final concentration of 25–30 nM), and after an aliquot was submitted to heat activation, both unactivated and activated cytosols were analyzed by ion-exchange chromatography.

Antibody to Highly Purified Liver Glucocorticoid Receptor. The preparation of this antibody and the antibody–Sepharose 4B column has been described in a separate manuscript (Eisen, 1980). The antigen was the activated form of the [^{3}H]-triamcinolone–receptor complex obtained from rat liver. The receptor was purified 10 000-fold by a two-stage procedure using DNA–cellulose (Eisen & Glinsmann, 1978). Further details of specificity and properties of the rabbit antibody are presented by Eisen (1980). Preimmune serum and serum from other control rabbits did not react with the [^{3}H]triamcinolone–receptor complex. The antiserum does not react with [^{3}H]corticosterone–transcortin complexes or with an estrogen-binding protein present in rat liver.

Analysis of Liver and Kidney Receptors by Specific Interaction with Antibody against Liver Cytosol Receptor. Antibody to homogeneous rat liver glucocorticoid receptor was immobilized on a Sepharose 4B minicolumn (0.5 × 2 cm). A 0.5-mL amount of cytosol was applied to a column, and 10 fractions of 1 mL each were collected by washing the column with 20 mM potassium phosphate buffer (pH 6.8). When all unbound radioactivity was removed, the specifically adsorbed activity by antibody was washed out by 0.1 M acetic acid.

Results

Resolution of Unactivated and Activated Receptor Forms by DEAE-Sephadex A-50 Ion-Exchange Chromatography. A 0.5-mL amount of rat liver or kidney cytosol, labeled in vitro with [^{3}H]TA, was applied to a minicolumn of DEAE-Sephadex A-50. The elution profiles are shown in parts A and E of Figure 1. The major binding protein was eluted with 0.4–0.5 M KCl. After activation by heat (30 min at 25 °C), liver cytosol radioactivity was largely associated with the less acidic fraction eluted with 0.2–0.25 M KCl, corticosteroid binder II (Litwack et al., 1973). The radioactivity bound to the protein fraction in the buffer wash is identified as the previously characterized (Litwack & Rosenfield, 1975) corticosteroid binder IB (Figure 1C). Following heat activation, almost all radioactivity from kidney cytosol was bound to the protein which is eluted in the position of IB (Figure 1G). The two major protein fractions from liver cytosol separated by ion-exchange chromatography and their redistribution after heat activation were previously described by using step elution gradients (Parchman & Litwack, 1977). These two fractions correspond to unactivated and activated forms of receptor, confirmed by the inability or ability to bind to DNA–cellulose. If the liver or kidney unactivated cytosol was treated with DNA–cellulose and applied to the ion-exchange column, the unactivated receptor form was recovered completely (parts B and F of Figure 1). After heat activation, on the other hand, the activated forms of both tissues were removed by prebinding to DNA–cellulose (parts D and H of Figure 1).

The unactivated forms of receptor from liver and kidney cytosols elute at the same position from DEAE-Sephadex A-50 minicolumns. Heat activation produces a shift in the position of the receptor in the chromatogram to a less acidic form capable of binding to DNA–cellulose. The redistribution from unactivated to activated forms in liver is different from whole kidney cytosol, implying the existence of different activated forms in the two tissues.

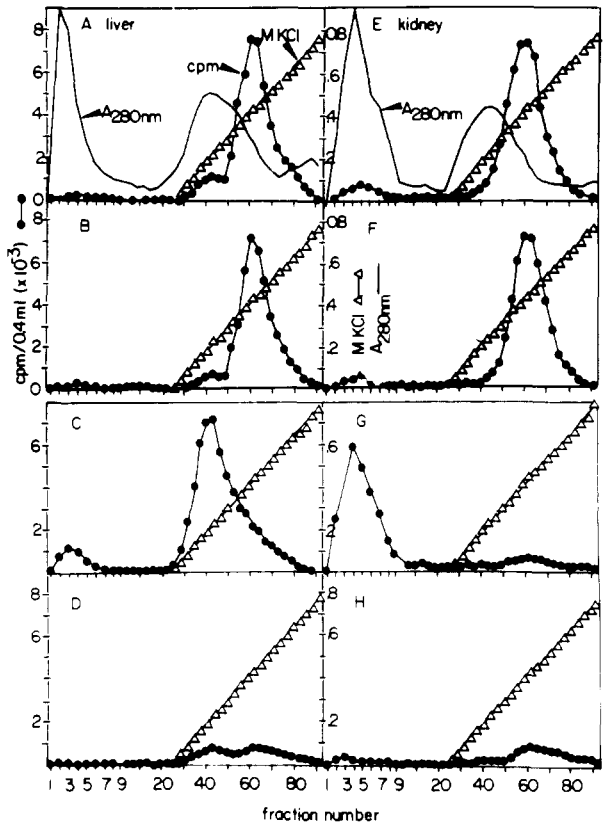

FIGURE 1: DEAE-Sephadex chromatography of unactivated and activated liver and kidney cytosols labeled in vitro. Cytosols were labeled for 90 min at 4 °C with 30 nM [^{3}H]TA. 0.5 mL of labeled cytosol was applied to the DEAE-Sephadex A-50 minicolumn and eluted as described under Materials and Methods after the following treatments. (A and E) Unactivated liver or kidney cytosol (controls). (B and F) Unactivated liver or kidney cytosol was incubated with DNA–cellulose as described elsewhere (Cake et al., 1978) for 45 min at 4 °C. After centrifugation for 10 min at 800*g*, 0.5 mL of the resulting supernatant fraction was applied to the ion-exchange minicolumn. (C and G) Liver or kidney cytosol was activated by heat for 30 min at 25 °C. (D and H) Following activation by heat, liver or kidney cytosol was treated as described under (B) and (F). The A_{270nm} profiles of all chromatograms were similar to the ones shown in (A) and (E).

In Vivo Labeling of Liver and Kidney Receptors. The hormone–receptor complexes formed in vivo from liver and kidney cytosol were analyzed by using the same procedures (Figure 2). Binders II and IB are present in both tissues, although in kidney the amount of the binder II form is much smaller than the amount of IB (Figure 2). The amount of IB labeled in vivo is the same as that labeled in vitro (compare Figures 2A and 1C), but there is substantially less binder II after in vivo labeling. DNA–cellulose pretreatment shows that both IB and II are DNA-binding proteins (compare Figure 2C to parts A and B of Figure 2).

Ion-exchange chromatography also was used to analyze receptor forms of heart, thymus, brain, and skeletal muscle in vitro before and after activation. The percentages of radioactivity bound to different forms of receptors are summarized in Table I. The receptor distribution in all the tissues studied resembles that in the liver.

Stokes' Radius of Activated Receptors of Liver and Kidney Cytosols. The Stokes radius of different receptor forms was determined by chromatography on Sephadex G-100 and G-150. Unactivated receptor forms elute in the void volumes of these columns. The presence of 1–10 mM EDTA and KCl in different concentrations (0.1–0.2 M KCl) did not prevent aggregation. The Stokes radius of IB in both liver and kidney

Table I: Distribution of Various Forms of the Glucocorticoid Receptor in Tissues Other Than Liver and Kidney Assayed by the DEAE-Sephadex Minicolumn (Figure 1)[a]

tissue cytosol		0.4 M form (%)	binder II (%)	binder IB (%)	[^{3}H]TA concn (final) (nM)	% bound (charcoal-dextran method)	DNA (% of steroid bound)
heart	unact 4 °C	70.3	29	0.7	30	6.7	3.3
	act. 25 °C	27.3	63.3	9.4		7.4	37.6
thymus	unact 4 °C	83	15.5	1.5	42	11.3	1.4
	act. 25 °C	27.6	69.7	2.7		16.4	20.9
brain	unact 4 °C	85.1	14.2	0.7	22	25.7	1.3
	act. 25 °C	41.5	56.5	2		26.2	26.4
muscle	unact 4 °C	67.6	23.4	9	27.7	7.4	
	act. 25 °C	31	46	23			

[a] The ion-exchange chromatography was performed with the cytosol of all tissues shown labeled in vitro with [^{3}H]TA (at final concentration between 22 and 42 nM) as described under Materials and Methods and in parts A and C of Figure 1. Radioactivity associated with different receptor forms is calculated as a percentage of total recovered radioactivity from the minicolumn either before activation (4 °C) or after activation (25 °C). The 0.4 M form corresponds to the unactivated receptor. Binder II is represented by the form eluted by 0.2 M KCl. Binder IB receptor corresponds to the protein which is excluded from the column. All cytosols were assayed with the charcoal–dextran method (Sherman et al., 1979), and the percentage of specifically bound hormone is given. Also, the results of DNA–cellulose binding before and after heat activation are presented (except for muscle).

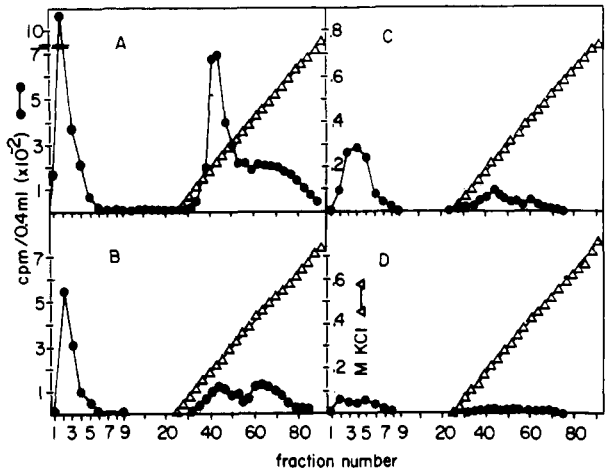

FIGURE 2: DEAE-Sephadex chromatography of rat liver or kidney cytosol labeled in vivo with [^{3}H]TA, as indicated under Materials and Methods. Details of ion-exchange chromatography are as described in Figure 1. (A) Liver cytosol; (B) kidney cytosol; (C) liver cytosol treated before chromatography with DNA–cellulose, as in Figure 1B; (D) kidney cytosol treated as in (C).

is 20–26 Å. The major liver form (0.2 M peak after DEAE-Sephadex A-50 chromatography) activated in vitro has a Stokes radius in the range of 50–60 Å and is eluted from Sephadex G-150 columns close to γ-globulin. Since IgG has been shown to have a Stokes radius of 51 Å (Tanford et al., 1974), this is probably identical to the 61-Å form of Wrange & Gustafsson (1978).

Appearance of IB in Vitro Is Probably Not Due to Protease Action on II. Wrange & Gustafsson (1978) have studied the proteolytic cleavage products of the liver glucocorticoid receptor. The most native form is a 61-Å receptor (analogous to binder II in our experiments). The first product is 36 Å and retains its ability to bind to DNA. The final degradation product is 19 Å and no longer contains the DNA binding domain. This last product is the "meroreceptor" of Sherman et al. (1979). IB, with a Stokes radius of 20–26 Å, must be compared to the meroreceptor. Unpublished experiments show that generation of this material from binder II by proteolytic action yields a 20-Å product which is tightly bound to DEAE-Sephadex A-50 (J. J. Diaz-Gil and G. Litwack, unpublished results). This agrees with Sherman's p*I* for the rat kidney meroreceptor of 5.9 (Sherman et al., 1979). From the position of IB in DEAE chromatograms, we estimate the p*I* of this protein in a range from 8.5 to 6.5. Its elution position is vastly different from that of the meroreceptor. The meroreceptor does *not* bind to DNA, and evidence presented here shows that IB is a DNA-binding protein.

Because kidney cortex activated form appears to be IB, exclusively, we decided to test the possibility that this tissue contains an active protease capable of converting binder II to IB. Kidney cytosol was incubated for 30 min at 37 °C to destroy endogenous glucocorticoid receptor and was then incubated with 50 nM [^{3}H]TA for 90 min at 0 °C. Liver cytosol was directly bound with radioactive TA in the cold under the same conditions. The treated kidney cytosol, the liver cytosol, and an equivolume mixture of the two were activated for 30 min at 25 °C. The mixture and the liver cytosol were examined separately on DEAE-Sephadex minicolumns. The kidney cytosol alone contained only about 2000 cpm bound, most of the glucocorticoid receptor binding activity having been destroyed by the initial heat treatment. Comparison of chromatograms for liver cytosol and the mixture shows that the heated kidney cytosol was unable to convert binder II to IB. In order to determine if a putative kidney protease had been destroyed in the initial heating in the experiment described above, we carried out a different experiment. Unheated kidney cytosol was incubated with excess unlabeled TA. The protocol was identical with the one described above except the initial heating of kidney cytosol was not done. The result was the chromatographic patterns of [^{3}H]TA-labeled liver cytosol were unchanged. The converse experiment where liver cytosol was incubated with excess unlabeled TA and combined with [^{3}H]TA-labeled kidney cytosol resulted in an unaltered chromatogram, for example, as shown in Figure 1G. From these experiments, we conclude that the appearance of IB is not the result of proteolysis of binder II. This result is consistent with the data of Wrange & Gustafsson (1978) and of Sherman et al. (1979), who showed that the ~20-Å proteolytic degradation product of binder II is not a DNA-binding protein.

Addition of various protease inhibitors, including 5 mM leupeptin, does not affect the appearance of IB in kidney cytosol.

Analysis of Receptor Forms by Rat Liver Glucocorticoid Receptor Antibody. By use of antibody to rat liver glucocorticoid receptor immobilized on a Sepharose 4B column, liver and kidney unactivated and activated forms were analyzed. Following the DEAE-Sephadex A-50 minicolumn chromatography of activated liver cytosol labeled with [^{3}H]TA in

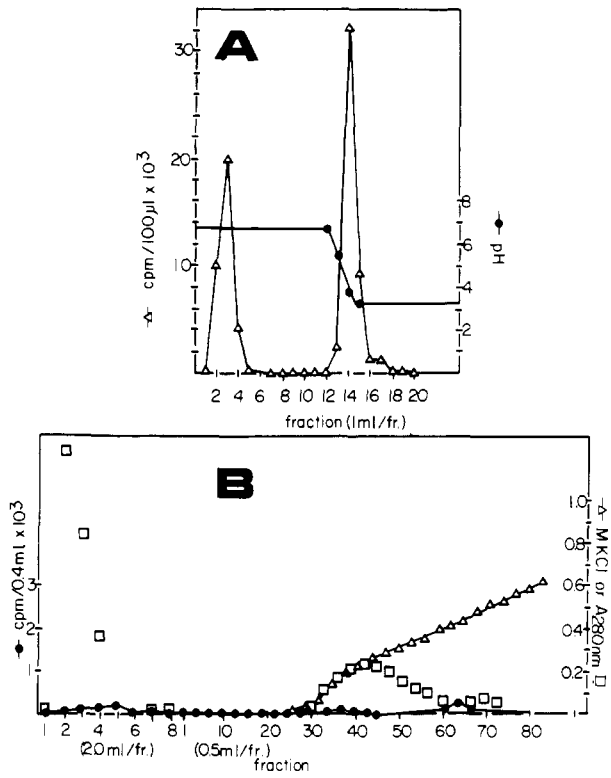

FIGURE 3: Analysis of liver activated cytosol by a liver glucocorticoid receptor antibody–Sepharose 4B minicolumn. (A) 1.0 mL of heat-activated cytosol labeled with 54 nM [^{3}H]TA in vitro was applied to the antibody–Sepharose 4B column, and elution was performed as indicated under Materials and Methods. (B) DEAE-Sephadex A-50 chromatography of the unadsorbed (first) peak from the antibody column. 0.9 mL of peak fraction eluted by phosphate buffer (pH 6.8, Figure 6A) was applied to the ion-exchange minicolumn and analyzed as before.

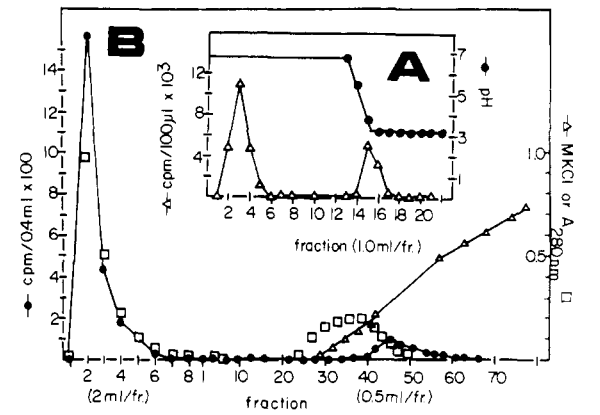

FIGURE 4: Analysis of liver cytosol labeled in vivo by a glucocorticoid receptor antibody–Sepharose 4B minicolumn. (A) 0.5 mL of the cytosol labeled in vivo for 10 min with [^{3}H]TA was analyzed by the antibody–Sepharose 4B minicolumn. Unadsorbed radioactivity (the first peak) was applied immediately to an ion-exchange minicolumn (B). Details are provided in Figure 3 and under Materials and Methods.

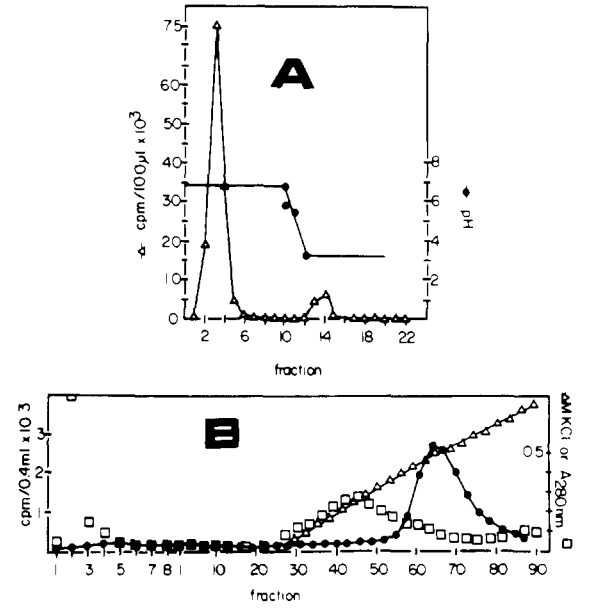

FIGURE 5: Analysis of whole kidney unactivated cytosol by an antibody–Sepharose 4B minicolumn. (A) 0.5 mL of kidney cytosol labeled in vitro was analyzed by the antibody–Sepharose 4B minicolumn. (B) A portion (0.4 mL) of the first, unadsorbed peak from the antibody–Sepharose 4B minicolumn was applied immediately to the DEAE-Sephadex minicolumn.

vitro, the major activated form (binder II) was separated. At the same time 0.5 mL of the same cytosol was applied to the antibody column (Figure 3A). The first peak of radioactivity corresponds to unadsorbed activity and is eluted by buffer alone. The radioactivity bound to the antibody was recovered by washing the column with 0.1 M acetic acid. When the first, unbound peak was analyzed subsequently by rapid ion-exchange chromatography, almost no radioactivity was recovered (Figure 3B). Therefore, this peak represents mostly free steroid adsorbed by the charcoal–dextran mixture at the bottom of the ion-exchange column, while the activated receptor form was removed previously by adsorption to the antibody column. Nearly all of the major liver activated receptor cross-reacted with the antibody.

For determination of the cross-reactivity of IB-like protein with antibody against major liver glucocorticoid receptor, the receptor was bound with ligand by injection of animals with 25 μCi of [^{3}H]TA/100 g body weight. Both unactivated and activated forms of binder II are adsorbed by the antibody column, while the IB-like protein does not cross-react with antibody (Figure 4). IB labeled in vivo does not cross-react with antibody similarly to IB labeled in vitro.

Analogous experiments were done with kidney. Kidney unactivated cytosol was applied to DEAE-Sephadex A-50 columns as a control. At the same time, reaction with the antibody column (Figure 5A) showed that the amount of bound radioactivity cross-reacting with antibody is very small. Analysis of the unbound peak by ion exchange shows a correspondence to the unactivated kidney form (Figure 5B). If an aliquot of this peak is activated by heat and then chromatographed on the exchange resin, the unactivated receptor is converted to the activated form and is eluted in the column wash. When kidney cytosol was first heat activated and then applied on the antibody column, similar results were obtained, suggesting that kidney receptor is very different from the receptor of liver and does not cross-react with antibody against major liver receptor.

Analysis of Kidney Cortex and Medulla. Other tissues, except kidney, have the same kind of receptor as liver (Table I). A small but significant amount of radioactivity from whole kidney cytosol was always bound to the antibody column (Figure 5A), and some binder II like protein could be seen in whole kidney cytosol under in vivo conditions (Figure 2B). When kidney cortex cytosol alone, labeled in vitro with [^{3}H]TA, was applied to ion-exchange columns, the general patterns of radioactivity were the same as those for entire kidney (compare parts A and B in Figure 6 and parts E and G in Figure 1). After unactivated kidney cortex cytosol was

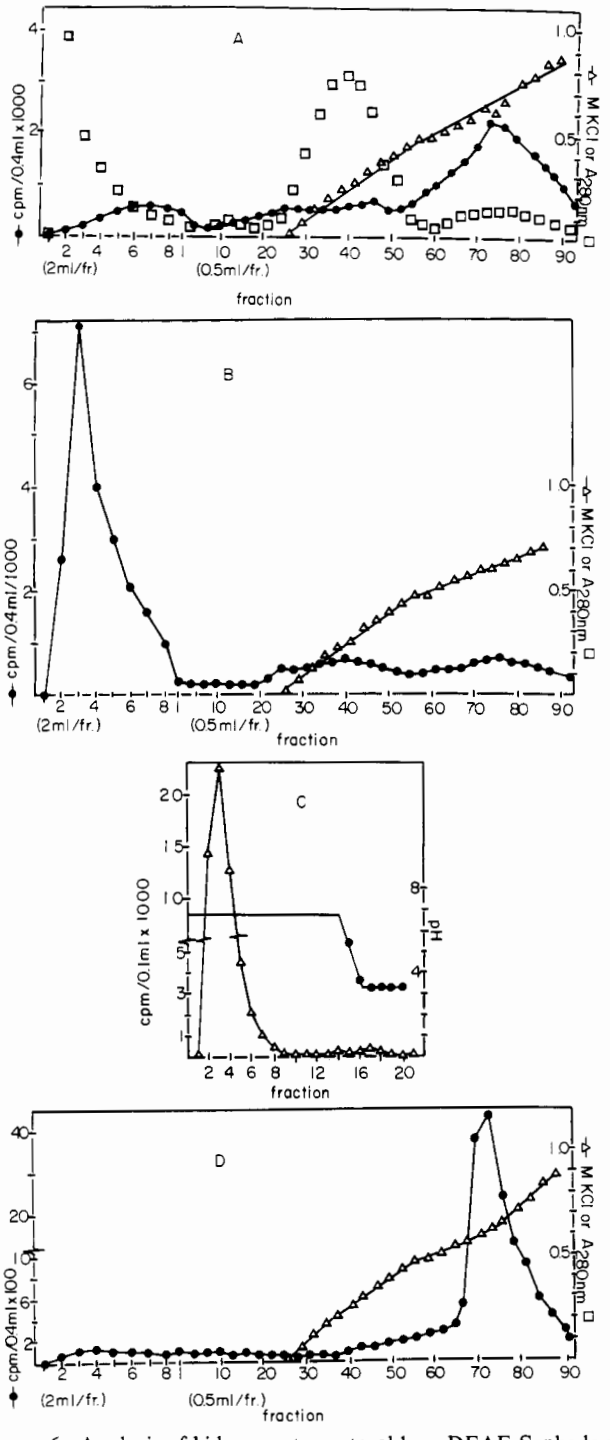

FIGURE 6: Analysis of kidney cortex cytosol by a DEAE-Sephadex minicolumn and by antibody–Sepharose 4B chromatography. 0.5 mL of cortex cytosol labeled in vitro with [^{3}H]TA was applied to an ion-exchange minicolumn before (A) and after (B) heat activation and analyzed as explained under Materials and Methods. 0.5 mL of unactivated cytosol was applied to an antibody–Sepharose 4B minicolumn (C). (D) Unadsorbed radioactivity of the first peak was analyzed by a subsequent DEAE-Sephadex A-50 minicolumn as described (Materials and Methods).

applied to the antibody column (Figure 6C), no adsorption was found. The unbound peak from the antibody column after ion-exchange chromatography corresponded to unactivated cortex receptor. With medulla cytosol, quite different results were obtained (Figure 7). Unactivated and activated forms of medulla receptor are eluted from the ion-exchange minicolumn at the same position as those in liver or any of the other tissues (parts A and B of Figure 7). After passage through

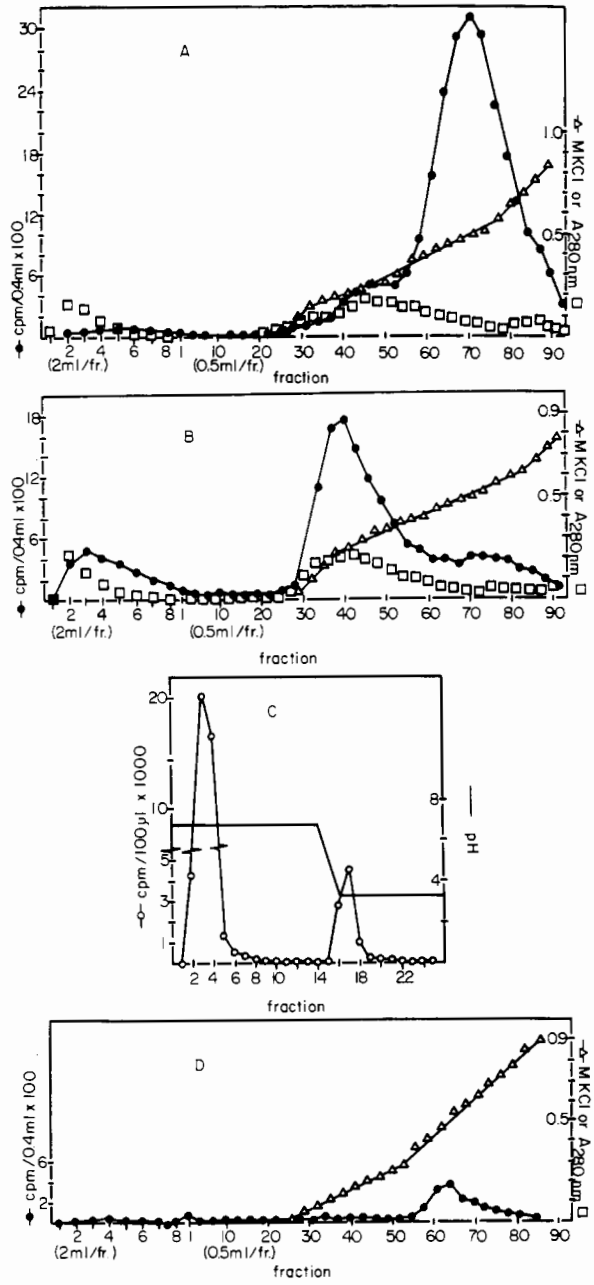

FIGURE 7: Analysis of kidney medulla cytosol by a DEAE-Sephadex A-50 minicolumn and by antibody–Sepharose 4B chromatography.

the antibody column a significant amount of radioactivity was bound (Figure 7C). The first unbound peak submitted to ion exchange represents a small amount of unactivated receptor (Figure 7D). The receptor–hormone complex had been removed almost entirely by the antibody column in the previous step.

Analysis of the Kidney Cytosol Glucocorticoid Receptor As Revealed by Pretreatment with Antitranscortin. Binding of [^{3}H]corticosterone to whole kidney cytosol followed by ion-exchange chromatography DEAE minicolumns results in a large peak of bound hormone eluting at about 0.3 M KCl (Figure 8A) with two small peaks of bound hormone in the wash through. All of these peaks appear to derive from serum as shown in Figure 8D. When serum bound with [^{3}H]-corticosterone is treated with antitranscortin and the precipitin removed prior to chromatography (Figure 8E), most of the peak eluting at 0.28 M KCl is lost, indicating that the major peak in Figure 8A is transcortin-like. The peak of bound radioactivity in the wash through in Figure 8E seems to derive

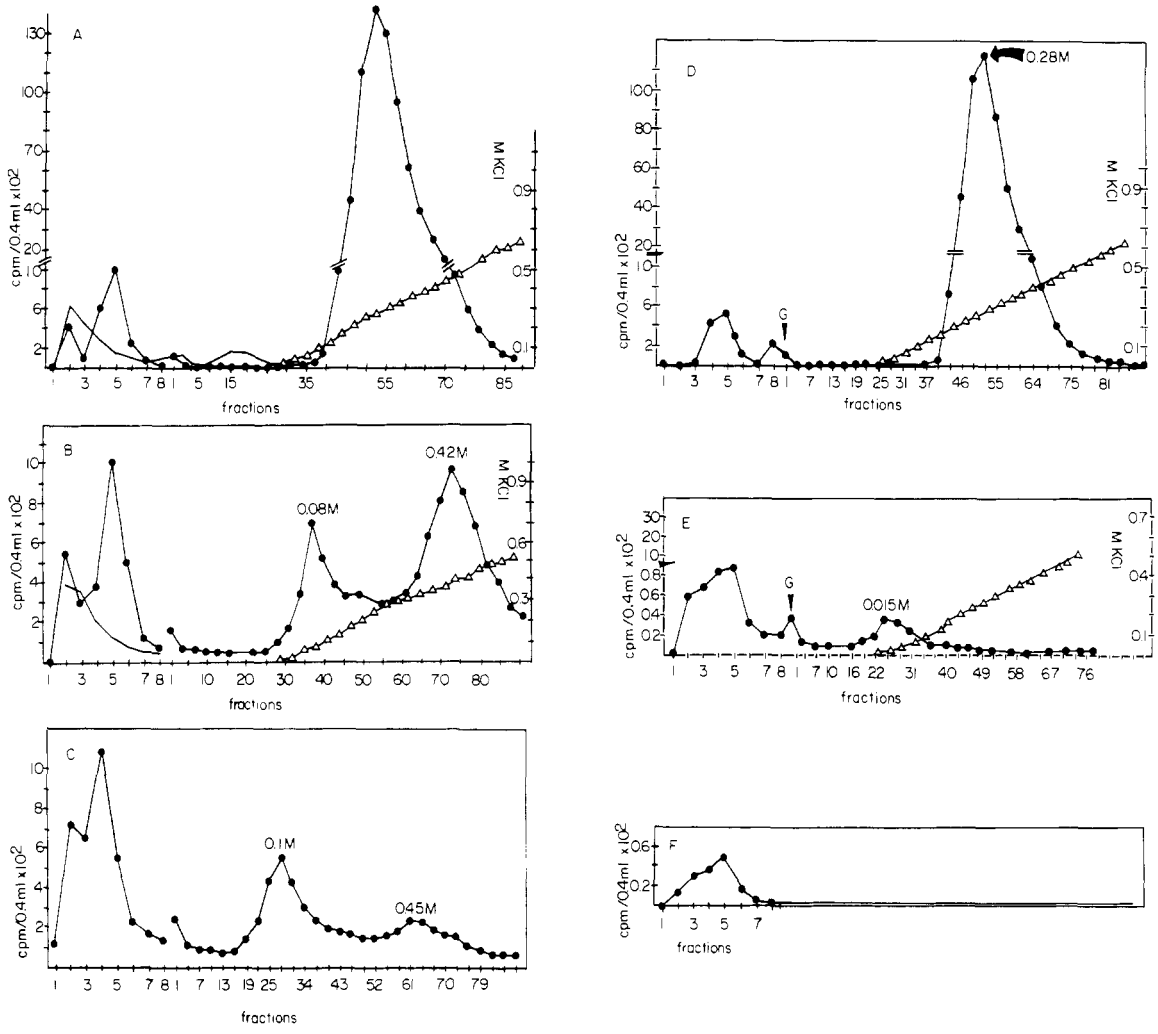

FIGURE 8: DEAE-Sephadex chromatography of kidney cytosol labeled in vitro with [^{3}H]corticosterone. (A) 0.5 mL of kidney cytosol incubated 90 min at 4 °C with [^{3}H]corticosterone was analyzed by ion-exchange chromatography. Chromatography of unactivated (B) and heat activated (C) kidney cytosol labeled in vitro with [^{3}H]corticosterone after transcortin was removed by specific transcortin antibody. (D) Control chromatography of serum incubated with [^{3}H]corticosterone in vitro. Fresh serum was prepared, diluted 1:50 with Tris buffer (pH 7.55), and incubated with [^{3}H]corticosterone under the same conditions as kidney cytosol. (E) Control chromatography of serum incubated with [^{3}H]corticosterone after transcortin was removed by specific transcortin antibody. (F) Control chromatography of specific transcortin antibody incubated with [^{3}H]corticosterone in vitro. Details are given under Materials and Methods.

from the antibody preparation (Figure 8F). When kidney cytosol is pretreated with antibody and the resulting precipitin removed prior to ion-exchange chromatography, the activation mechanism of the non-transcortin-like steroid receptor can be visualized (parts B and C of Figure 8) whereas the reaction is totally masked by the major peak of transcortin-like material if it is not first removed. When the unactivated system is chromatographed (Figure 8B), there are four peaks at 0.42 M, 0.08 M, and wash through. The heat-activated system (Figure 8C) shows a reduction of the 0.42–0.45 M peak, indicating this to be the major unactivated form with [^{3}H]-corticosterone bound to receptor. Since the amount of intermediate form eluting at 0.08–0.1 M does not change and there is a slight shift and increase in the wash-through peaks, it appears that the activated form is in this region (parts B and C of Figure 8). These results with bound [^{3}H]corticosterone after antitranscortin treatment during activation align well with the results with the synthetic fluorinated glucocorticoid, for example, parts E–G of Figure 1, and suggest that the "type III" corticosterone receptor described by the Edelman group (Feldman et al., 1973, 1978; Funder et al., 1973) is a transcortin-like protein. We should, however, consider the possibility that there exists a small amount of this fraction which does translocate to the nucleus but which we could not observe on rapid ion-exchange chromatography due to excessive lability of the complex. When [^{3}H]corticosterone-bound cytosol or serum under conditions of parts A or D of Figure 8 is heated, there is no reduction of the 0.28 M peak or appearance of an activated form in the wash-through fractions (data not presented).

Discussion

We have previously shown that rapid anion-exchange chromatography can be used to resolve activated and unactivated forms of the rat liver glucocorticoid receptor (Parchman & Litwack, 1977). Antibodies to the major rat liver glucocorticoid receptor and to serum transcortin provide novel probes for discriminating between various forms of glucocorticoid binding proteins. The combined approaches appear to be particularly useful in analyzing the glucocorticoid binding proteins in kidney.

In liver and kidney, the unactivated forms of [^{3}H]triamcinolone acetonide–receptor complexes have similar characteristics on anion exchange (eluted at 0.4–0.5 M KCl). Following heat activation, important differences are noted. The major activated form in liver is eluted by 0.2 M KCl, whereas the kidney form is eluted in the buffer wash. The major activated form in liver is identified as binder II and has

a Stokes radius (S_r) of 50–60 Å; the major kidney activated form is identified as binder IB and has a S_r of 20–26 Å. Both activated forms are DNA-binding proteins and, consequently, by this biochemical criterion are glucocorticoid receptors.

The antibody to the liver receptor adsorbs both the activated and unactivated forms of binder II. Additional experiments show that this antibody also reacts with ligand-free binder II. In contrast, the major kidney form, unactivated or activated, does not react with the antibody. A small quantity of liver-like binder II is found in the kidney, but this is shown to be localized in the medulla. Since binder IB in kidney cortex and liver has a smaller Stokes radius than binder II, it is important to determine if binder IB is a proteolytic product of binder II. The mixing experiments described in this report do not result in the formation of IB from binder II. Extensive studies with endogenous (lysosomal) and exogenous proteases (Wrange & Gustafsson, 1978) indicate that the forms generated by proteolysis of binder II are considerably different from binder IB. Thus, the 20-Å fragment of binder II (the meroreceptor) is an acidic protein and does not bind to DNA. Binder IB is a DNA-binding protein, is more basic than the meroreceptor, and hence does not bind to the DEAE-Sephadex minicolumn. The most direct explanation of these results is that binder IB is derived from an unactivated form that is immunochemically distinct from binder II and therefore is a distinct moiety. Alternatively, binder IB could represent a cleavage fragment of binder II, intermediate in size between the 20- and 40-Å forms described by Gustafsson. The mechanism by which such a putative cleavage fragment could be generated is currently unknown. A critical question, which cannot be answered with current methods, is the S_r of the unactivated form of the kidney cortex receptor. On gel filtration, both unactivated liver and kidney forms are aggregated. The presence of 100, 150, or 200 mM KCl in the elution buffer recommended by Wrange & Gustafsson (1978), or 1–10 mM EDTA demonstrated by Leseney et al. (1979) as a substance which prevents aggregation, or both agents together did not prevent aggregation in our experiments. The aggregation is not the consequence of dilution by ion-exchange chromatogrphy since the same results were obtained by analyzing total undiluted cytosol which was not exposed to activation.

The kidney is a site of action of glucocorticoids as well as mineralocorticoids. The kidney appears to contain three distinct forms of glucocorticoid receptors. These forms have been characterized kinetically in terms of interaction with aldosterone, corticosterone, and fluorinated glucocorticoids. Aldosterone binds to two kinetically distinct receptors: type I (high affinity for aldosterone) and type II (lower affinity for aldosterone). Type I and type II receptors cannot be distinguished on the basis of kinetics using fluorinated glucocorticoids. The evidence presented in this report suggests that the major kidney form is immunochemically distinct from the major form of glucocorticoid receptor found in other tissues. It should be possible to use the rapid anion-exchange separation and immunoaffinity chromatography to study aldosterone binding, and such studies would help to define the difference between mineralocorticoid and glucocorticoid receptors. It is possible that binder IB is actually the type I mineralocorticoid receptor and that the type II receptor represents binder II in the medulla. Kidney contains large quantities of transcortin-like protein that binds corticosterone. This protein can be removed by immunoprecipitation, and the remaining proteins that bind corticosterone appear to be similar to the other forms of kidney receptor for fluorinated glucocorticoids. We have not found evidence for a distinct class of [^{3}H]-corticosterone receptors (type III) by these methods.

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Effects of Pyrophosphate, Triphosphate, and Potassium Chloride on Adenylate Deaminase from Rat Muscle†

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ABSTRACT: Inorganic pyrophosphate and triphosphate inhibit adenylate deaminase from rat skeletal muscle with K_I values of 10 and 1.5 μM, respectively, in the presence of 150 mM KCl at pH 7. They act by reducing the apparent affinity of the enzyme for AMP, with relatively small effects on V_{max}. The inhibitions are diminished by H^+, the K_I values increasing two- to threefold in going from pH 7.0 to 6.2, and are relieved by ADP. These properties are similar to the inhibitions produced by GTP and ATP, indicating that pyrophosphate and triphosphate act like analogues of the nucleoside triphosphates. Neither of these inhibitors shows relief of inhibition at high concentrations as do ATP and GTP. These results suggest that nucleotides interact with the inhibitor site of the enzyme primarily through their phosphate moieties and with the activator site primarily through their nucleoside moieties. As the concentration of KCl is increased from 25 to 300 mM, the apparent affinities of the enzyme for ATP, GTP, orthophosphate, pyrophosphate, and triphosphate are decreased 8-100-fold. The cooperativity of the inhibitions is increased, with the Hill coefficient rising from 1.0 to 1.3-1.8, and the maximum inhibition approaches 100%. Maximum activation by ADP is reduced from 1800% at 25 mM KCl to 80% at 200 mM KCl. Experiments with $(CH_3)_4NCl$ indicate that activation of the enzyme by KCl involves both specific K^+ effects and ionic strength effects.

Adenylate deaminase from muscle is regulated by nucleoside di- and triphosphates. ATP and GTP are inhibitors; ADP and GDP are activators (Lyubimova & Matlina, 1954; Ronca et al., 1968; Smiley & Suelter, 1967). Moreover, at high concentrations ATP and GTP reverse their own inhibition (Ashby & Frieden, 1978; Wheeler & Lowenstein, 1979b). Inorganic pyrophosphate and triphosphate also inhibit the enzyme from muscle (Nikiforuk & Colowick, 1956; Wheeler & Lowenstein, 1979a) and other tissues (Setlow & Lowenstein, 1968; Yun & Suelter, 1978). We have studied their effects on enzyme activity in order to clarify certain aspects of the nucleoside di- and triphosphate specificity of the enzyme.

The regulatory properties of adenylate deaminase are strongly dependent on the concentration and composition of added salt (Coffee & Solano, 1977; Ronca et al., 1972; Ronca-Testoni et al., 1970; Sammons et al., 1970). Previous investigators used 100-150 mM KCl as an approximation to intracellular conditions. We have examined the effects of H^+, ADP, and inhibitors on the activity of the enzyme at different KCl concentrations in order to relate such studies to conditions in vivo and to better understand the regulatory properties of the enzyme.

Experimental Procedures

The purification of adenylate deaminase, assay procedures, and treatment of data were described previously (Wheeler & Lowenstein, 1979b). Sodium pyrophosphate was obtained from Fisher, sodium tripolyphosphate from Howe and French, and tetramethylammonium chloride from Eastman.

Results

Inhibition by Pyrophosphate and Triphosphate. Adenylate deaminase activity in the presence of 20 μM AMP and 150 mM KCl, pH 7.0, is inhibited 58 and 92% by 12 and 60 μM inorganic pyrophosphate, respectively. Under the same conditions it is inhibited 61 and 94% by 2 and 10 μM inorganic triphosphate, respectively. The inhibition is cooperative (n = 1.1-1.6) and becomes weaker as the pH is decreased from 7.0 to 6.2, the K_I increasing from 10 to 33 μM in the case of pyrophosphate and from 1.5 to 2.7 μM in the case of triphosphate. (K_I is defined as the concentration of inhibitor required for half-maximal inhibition.) ADP relieves the inhibition produced by both substances. For example, about 100 μM ADP completely reverses the inhibition produced by 100 μM pyrophosphate or 10 μM triphosphate. The effects of H^+ and ADP on the inhibition by pyro- and triphosphate are similar to those observed for the inhibitions caused by GTP,

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