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Dexamethasone-Binding Proteins in Cytosol and Nucleus of Rat Thymocytes

Purification of Three Receptor Proteins

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Dexamethasone-binding proteins from the cytosol and the nucleus of rat thymocytes were analyzed by ion-exchange chromatography on DEAE-cellulose. Three dexamethasone-binding proteins were revealed in cytosol, one in the flow-through (DE-1) and two (DE-2 and DE-3) eluting from the column with 0.13 M and 0.23 M NH_4Cl, respectively. In nuclear extracts only one receptor fraction, present in the flow-through, could be detected.

By a combination of affinity chromatography on Cl-Sepharose to which dexamethasone 21-methanesulfonate was linked through a disulfide bond and DEAE-cellulose chromatography, three receptor proteins were highly purified from cytosol, with molecular weights of 45000, 72000 and 90000 and one from nuclear extracts with molecular weight of 72000. Antibodies to the 45000-M_r and 90000-M_r proteins were elicited in rabbits. The antibodies to the 45000-M_r protein cross-react with the 90000-M_r protein. Similarly, the antibodies to the 90000-M_r protein cross-react with the 45000-M_r protein. Antibodies to either of the two proteins immunoprecipitate 60–70% of the dexamethasone-binding activity of rat thymus cytosol. Immunoaffinity chromatography of cytosol and nucleosol on columns of Sepharose linked to the IgG against either the 45000-M_r or the 90000-M_r protein leads to binding of these proteins on the columns but not of the 72000-M_r species. Two nuclear polypeptides with molecular weights of 36000 and 38000 remain attached to the immunoaffinity column; these polypeptides may represent degradation products of the cytoplasmic receptor upon entrance into the nucleus. Antibodies against two dexamethasone-binding proteins from rat liver cytosol immunoprecipitate the 45000-M_r and 90000-M_r cytosol receptors from rat thymus.

The presence of receptor proteins in thymocytes having high affinity to glucocorticosteroids has been amply demonstrated [1–5]. It is assumed that the interaction of the steroid with the receptor protein is the first step in the reaction chain ultimately leading to cellular degradation and lysis [6, 7]. As with other steroid hormones [8], a two-step mechanism has been proposed for the action of glucocorticoids in thymocytes and other lymph cells: a first step of binding of the hormone to the cytoplasmic receptor leading to its activation, followed by a second step of migration of the cytoplasmic receptor into the nucleus (translocation), culminating in binding to chromatin and in modulation of transcription. Experimental evidence in favour of the proposed scheme has accumulated during the last few years. However, definite proof for this scheme can only be given if the macromolecular binding components can be isolated from the various subcellular compartments and compared on a molecular basis.

In thymocytes, we have detected by DEAE-cellulose chromatography the presence of three dexamethasone-binding components in the cytosol and one in the nucleus (see below). In order to understand, at the molecular level, the relationship between the various cytoplasmic and nuclear binding proteins, in particular the possible modification of cytoplasmic receptor(s) during or after entrance into the nucleus, we purified these proteins by a combination of affinity and ion-exchange chromatography. In principle, these procedures had been successfully used by us for the purification of two dexamethasone-binding proteins from rat liver cytosol [9]. In addition, using antibodies elicited against purified thymocyte receptors, we employed the immunoaffinity technique both as a purification procedure and as a method for characterization of these proteins.

In our studies [^{3}H]dexamethasone was used as the labelled glucocorticoid in order to avoid interference by transcortin or transcortin-like proteins, which are known to be present in rat thymocytes [4] and which bind natural but not semisynthetic glucocorticoids.

MATERIALS AND METHODS

Chemicals

[1,2(n)-^{3}H]Dexamethasone (spec. act. 22–29 Ci/mmol, n indicates nominal position of labelling) was purchased from Amersham-Buchler (Braunschweig), DEAE-cellulose (DE-32 and DE-52) from Vetter (Heidelberg), Sephadex G-25 and Cl-Sepharose 4B from Pharmacia (Uppsala) and *Staphylococcus aureus* nuclease from Boehringer (Mannheim). All other chemicals were of analytical grade obtained commercially. Dexamethasone was a generous gift of Schering A.G. (Berlin/Bergkamen).

Animals

The thymus glands were derived from male Wistar rats weighing approximately 120 g, raised under standard conditions. For the immunization experiments albino rabbits weighing 4 kg were used.

Buffers

Buffer A consisted of 10 mM Tris/HCl, pH 7.5, 50 mM KCl, 5 mM 2-mercaptoethanol and 10% glycerol.

Buffer B had the same composition as buffer A except that the pH was raised to 8.5.

Buffer C consisted of 50 mM Tris/HCl, 10 mM $MgCl_2$, 25 mM KCl, 250 mM sucrose, pH 7.5.

Buffer E consisted of 10 mM Tris/HCl, pH 7.5 and 50 mM KCl.

Buffer F was composed of 10 mM phosphate, 140 mM NaCl and 0.01% NaN_3, pH 7.2.

Buffer G was composed of 10 mM Tris/HCl, pH 7.2, 10 mM phosphate and 70 mM NaCl.

Preparation of Cytosol

All steps were conducted at 0–4 °C. Thymus was homogenized in 2 vol. buffer A in a glass Potter–Elvejhem homogenizer fitted with a teflon pestle. The homogenate was submitted to centrifugation in a Sorvall RC2B centrifuge at 1000 rev./min for 15 min (SS-31 head) and the supernatant obtained subsequently centrifuged in a Beckman L5 ultracentrifuge for 2 h at 150000 × *g*. The supernatant was collected by careful aspiration and represented the cytosol.

Preparation of Nuclei and of Nucleosol

Nuclei were isolated in principle according to Chauveau et al. [10] using the modification of Beato et al. [11]. In short, thymi were homogenized in buffer C and passed through six layers of gauze. The homogenate was then centrifuged for 5 min at 2000 × *g* in a RC-5 Sorvall centrifuge. The pellet was suspended in 1.2 vol. buffer C, but in which sucrose was made 2.4 M and layered on 10 ml of the same solution. Further purification was accomplished according to [11]. To prepare the nucleosol, the purified nuclei were homogenized in buffer A with a loose-fitting pestle and incubated, after addition of 1 mM $CaCl_2$, with 5 units staphylococcal nuclease/A_{260} unit in the suspension, for 15 min at 25 °C. The suspension was subsequently centrifuged for 10 min at 8000 × *g* and the supernatant represented the 'nucleosol'.

Assay of [^{3}H]Dexamethasone Binding to Receptor Proteins

Cytosol or other fractions were incubated for 90 min at 0–4 °C in the presence of 0.05 μM [^{3}H]dexamethasone. In parallel incubations and in order to measure the specifically bound radioactivity, a 1000-fold excess of unlabelled steroid was added in addition to the labelled hormone. Free hormone was removed by the dextran-coated charcoal method [12] or by gel filtration on Sephadex G-25.

DEAE-cellulose and Phosphocellulose Chromatography

DEAE-cellulose (DE-32) and phosphocellulose (P-11) were pretreated as described in the Whatman manual. Equilibration of the DEAE-cellulose columns was in buffer B, whereas that of the phosphocellulose column in buffer A. The samples were applied to the column in the respective buffer. Elution in both cases was by linear 0.05–0.4 M NH_4Cl gradients in the respective buffer (see also [13]).

Preparation of the Affinity Gel [14]

Synthesis of Aminohexyl-Sepharose. Cl-Sepharose 4B was activated with CNBr as described by March et al. [15]. 100 ml CNBr-activated gel was placed in a conical flask containing 2 mmol hexamethylenediamine/ml gel and reacted overnight at 4 °C and pH 10 with continuous shaking. The gel was then extensively washed with water. The derivative contained approximately 10 μmol aminohexyl groups/ml Sepharose gel.

Synthesis of Bromoacetamidohexyl-Sepharose [15]. 10 mmol bromacetic acid and 12 mmol *N*-hydrosuccinimide were dissolved in 80 ml dioxan; 11 mmol dicyclohexylcarbodiimide were added and after 70 min dicyclohexylurea was removed by filtration. The solution was then added to a suspension of 200 ml aminohexyl-Sepharose at 0–4 °C. After 30 min the substituted Sepharose was washed with 5 l ice-cold 0.1 M NaCl.

Synthesis of Aminohexylcystaminium-Sepharose. 20 g bromacetamidohexyl-Sepharose were mixed with 0.1 M cystamin in 0.1 M Na_2CO_3 and left at room temperature for 5 days. After washing with 0.1 M $NaHCO_3$ and 2 l water (the gel contains 0.8–1 mmol amino groups) the gel was kept 24 h in 0.2 M aminoethanol at room temperature for the removal of the unreacted bromacetyl groups.

Conjugation of Dexamethasone 21-methanesulfonate with Aminohexylcystaminium-Sepharose. 100 ml of the Sepharose derivative were taken up in dioxan/water (2:1). 100 mg dexamethasone 21-methanesulfonate in dioxan was added to the Sepharose suspension and the pH raised to 9.5. The mixture was gently shaken for a week at room temperature and then washed on a sintered-glass funnel with 2 l dioxan, 5 l dioxane/water (1:1) and 5 l methanol/water (1:1). The Sepharose gel contained 86 nmol bound dexamethasone/g wet gel and was stored at 0–4 °C. In some cases the dexamethasone 21-methanesulfonate used was radioactively labelled. Using the same method as above, a gel containing 900 dis. [^{3}H]dexamethasone min^{-1} (g wet gel)$^{-1}$ was obtained (spec. act. of the [^{3}H]dexamethasone 21-methanesulfonate was 1 mCi/g). Before use the gel was equilibrated with buffer A without mercaptoethanol.

Affinity Chromatography of Nucleosol and Cytosol

Cytosol and nucleosol were derived from thymi of 200 animals. The cytosol and nucleosol, in buffer A, were subjected to gel filtration on Sephadex G-25 in buffer A without mercaptoethanol, to remove the mercaptoethanol. The preparations were passed slowly through 100-mm-long and 9-mm-wide column packed with 4–5 g affinity gel. The whole procedure lasted 12 h. The gels were then washed with 5 l buffer A without mercaptoethanol. The gels were then suspended in 15 ml in buffer A containing 100 mM 2-mercaptoethanol at 0–4 °C; 4 h later the gels were packed in columns and washed with buffer A without mercaptoethanol. The combined eluates containing the receptor activity were subjected to Sepharose G-25 filtration in buffer A to remove free hormone and excess mercaptoethanol.

Antibody Production

The method of Vaitukaitis et al. [16] was applied. The protein fractions were eluted from the preparative polyacryl-

amide gels (see below) with 1% sodium dodecylsulphate at 37 °C overnight. They were taken up in 1 ml of 0.9% NaCl and 1% sodium dodecylsulphate, boiled in a water bath at 100 °C and mixed with Freund's complete adjuvant (1:1). The rabbits received three injections of the proteins, a first one (2.5 µg), a second one after a week (5 µg) and the third after four weeks (5 µg); 7–8 days after the last injection blood was collected by cardiac puncture. Purification of the antibodies was performed as described by Livingston [17]. The purified IgG were lyophilized and stored as dry powder.

Immunological Procedures

Double-Diffusion Test according to Ouchterlony [18]. The precipitation bands were evident after 24–48 h at room temperature. The precipitates were stained with Coomassie brilliant blue after washing the agarose plates for 24 h with 0.14 M NaCl.

Immunoprecipitation of Cytosol Receptors with Antibody. Cytosol in buffer C was incubated with 0.05 µM [^{3}H]dexamethasone for 2 h and the un-bound hormone then removed by charcoal treatment [19]. 400-µl aliquots of the cytosol were incubated overnight at 0–4 °C with various concentrations of IgG (5–1000 µg) dissolved in buffer G. The samples were then centrifuged 10 min at 1000 rev./min in the Sorvall RC-2B centrifuge (HB-4 rotor) and the radioactivity in the supernatant and in the sediment measured. Controls were also performed in which the specific IgG was substituted with IgG prepared from preimmune serum.

Preparation of the Immunoaffinity Gel

CNBr-activated Sepharose 4B was suspended in 0.2 M sodium citrate, pH 6.5 and shaken for 48 h with IgG dissolved in the same buffer (see Pharmacia manual). The suspension was washed with water, citrate and 0.5 M NaCl/$NaHCO_3$. The unreacted active groups were blocked with 0.2 M 2-ethanolamine.

Immunoaffinity Chromatography

The columns containing the affinity gel were washed with buffer F. Cytosol or nucleosol prepared in buffer C was passed through Sephadex G-25 column equilibrated in buffer F. The macromolecular fraction was passed very slowly through the column at 0–4 °C. The duration of the procedure was 12 h. The column was then washed with buffer F containing 1% Triton X-100 until no protein could be detected in the eluate. The bound proteins were then eluted with 15–30 ml 3 M sodium thiocyanate.

Preparative Sodium Dodecylsulphate/Polyacrylamide Gel Electrophoresis

The protein fractions obtained after affinity and DEAE-cellulose chromatography were precipitated with 10% trichloroacetic acid and subjected to preparative gel electrophoresis according to the procedure of Laemmli [20] on 10% polyacrylamide gels. The protein bands were cut out and the proteins eluted from the gels by 1% sodium dodecylsulphate at 37 °C overnight under continuous shaking [21].

Protein Determination

Protein was determined according to Lowry et al. [22], using bovine serum albumin as standard.

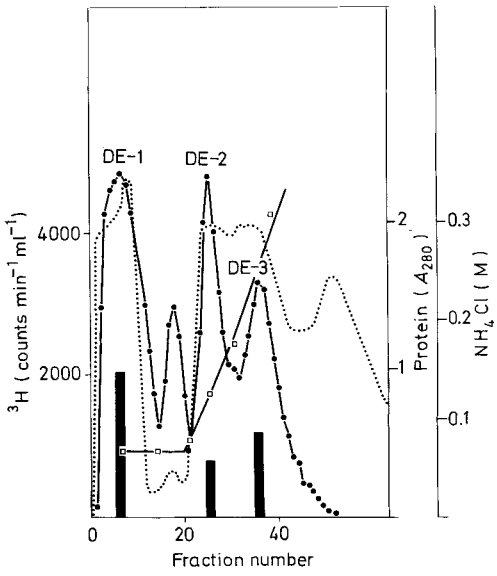

Fig. 1. *DEAE-cellulose chromatography of cytosol, preincubated with [^{3}H]dexamethasone.* Cytosol was incubated in the presence of 0.05 µM [^{3}H]dexamethasone at 0–4 °C for 90 min. Unbound hormone was removed by gel filtration on Sephadex G-25 equilibrated with buffer B. The volume of the DEAE-cellulose column was 20 ml. An 8-ml aliquot of the cytosol was submitted to chromatography at 0–4 °C. The column was eluted with a 50–400 mM NH_4Cl gradient (45 + 45 ml) in buffer B. Fractions of 2 ml were collected. (· · · · ·) Protein; (●——●) ^{3}H in fraction; (□——□) NH_4Cl concn. The solid bars represent the radioactivity remaining in the fractions after charcoal treatment

RESULTS

Ion-Exchange Chromatography of Cytosol Dexamethasone Receptor on DEAE-cellulose Columns

Maximal stability of glucocorticoid-binding activity is observed in a buffer consisting of 10 mM Tris/HCl, pH 7.5, 5 mM 2-mercaptoethanol, 50 mM KCl and 10% glycerol (buffer A, see also [13]). Cytosol therefore was prepared in this buffer, incubated in the presence of 0.05 µM dexamethasone for 90 min and then submitted to gel filtration on Sephadex G-25 equilibrated with buffer B. The gel filtration step serves both to change the pH of the cytosol from 7.5 to the desired 8.5 and also to remove unbound [^{3}H]dexamethasone. DEAE-cellulose chromatography has been performed at pH 8.5, as at pH 7.5 less than 20% of the hormone-receptor complex is retained on the ion-exchange column, compared to more than 60% retained on the column at pH 8.5.

The results of the chromatographic separation are depicted in Fig. 1. Four radioactive peaks are evident, one of which (DE-1) does not bind to the ion-exchange column under the conditions of the chromatography (70 mM Cl^-, 0–4 °C). The second radioactive peak represents free [^{3}H]dexamethasone, since the radioactivity is totally absorbed on charcoal (see also [13]), whereas the third (DE-2) and the fourth (DE-3) fraction bind to the column and elute with 0.13 M and 0.23 M salt, respectively.

Subsequent treatment of DE-1, DE-2 and DE-3 with charcoal or by gel filtration, demonstrated that only 29%, 15% and 30%, respectively, of the radioactivity is still bound to the receptors (Fig. 1 and Table 1). Attempts to recharge the receptor fractions with [^{3}H]dexamethasone (Table 1) demonstrated an almost complete loss of binding activity of

Table 1. *Binding capacity of receptor fractions after DEAE-cellulose chromatography of cytosol*
The peak radioactive fractions of the columns depicted in Fig. 1 were submitted to Sephadex G-25 gel filtration and subsequently incubated in the presence of 0.05 μM [^{3}H]dexamethasone in order to determine the dexamethasone-binding capacity of the fractions (see Materials and Methods for details of the assay). The radioactivity of the fractions after DEAE-cellulose chromatography was taken as 100%

Fraction	^{3}H remaining in fractions after		
	ion-exchange chromatography	gel filtration	renewed incubation with [^{3}H]dexamethasone
	%		
DE-1	100	29	55
DE-2	100	15	10
DE-3	100	30	15

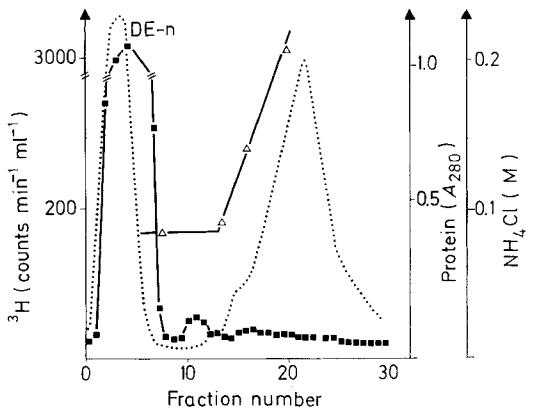

Fig. 2. *DEAE-cellulose chromatography of nucleosol dexamethasone-binding proteins.* Nucleosol was prepared by the nuclease digestion method (see Materials and Methods and legend to Fig. 7) and incubated with 0.05 μM [^{3}H]dexamethasone for 2 h at 0–4 °C. Conditions of chromatography as in legend to Fig. 1. (· · · · · ·) Protein; (■——■) ^{3}H in fraction; (△- - - - -△) NH_4Cl concn

receptors DE-2 and DE-3, whereas receptor DE-1 partially retains binding capacity.

The loss of the binding capacity of the fraction could be a consequence of the removal from the receptors during chromatography of components necessary for binding activity or to damage of the receptor protein during the fractionation procedure.

Ion-Exchange Chromatography of Nucleosol on DEAE-cellulose

Isolated thymocyte nuclei were treated with staphylococcal nuclease and the nucleosol obtained incubated at 0–4 °C with [^{3}H]dexamethasone; this treatment in more effective in releasing nuclear binding activity than 0.3 M KCl extraction (results not shown). The unbound labelled dexamethasone was removed by gel filtration. For the DEAE-cellulose chromatography the nucleosol was brought to pH 8.5 (see respective cytosol experiment).

The results of the DEAE-cellulose fractionation are depicted in Fig. 2. It is evident that, in contrast to the experiments with the cytosol, all of the dexamethasone-binding activity is found in the flow-through fractions (component DE-n). Subsequent charcoal treatment of the flow-through fraction demonstrated that at least 60% of the labelled dexamethasone is still protein-bound.

Purification of Dexamethasone-Binding Proteins by Affinity Chromatography

Starting material for the purification of the dexamethasone-binding proteins was rat thymus cytosol and nucleosol. To prepare the affinity material we have adapted the method of Sweet and Adair [23], in principle as used previously by us [9] for the purification of dexamethasone-binding proteins from rat liver cytosol. However, instead of using 11-deoxycorticosterone as the affinity ligand, we have now coupled dexamethasone, by way of a C-N bond, to the disulfide linker. The main steps of the preparation of the affinity material are described in Materials and Methods. Prior to use, the gel was washed with 50% methanol to remove non-covalently bound steroid which may be present due to hydrolysis of the gel during storage. The nucleosol and the cytosol were prepared from thymi of 200 animals. Purification was performed with 50 mg affinity gel/g thymus. The cytosol or nucleosol was slowly passed through the column in buffer A devoid of mercaptoethanol (see Materials and Methods), the whole period of the affinity chromatography lasting approximately 12 h. The attachment of the receptor proteins to the affinity column was evaluated by measuring binding activity in the cytosol and nucleosol before and after passage through the column. 68% of the cytosol receptor activity and 73% of that of the nucleosol was lost after passage through the column and, as shown below, remained attached to the affinity matrix. After passage of the fractions containing the receptor through the column the affinity gels were suspended in buffer A containing 100 mM mercaptoethanol. The combined eluates were then submitted to gel filtration on Sephadex G-25 equilibrated with buffer A to remove free dexamethasone and the excess mercaptoethanol. Due to the limited exchange of the unlabelled hormone from the hormone-receptor complex by [^{3}H]dexamethasone and in order to quantify the recovery of receptor activity, a parallel preparation was performed on a column in which dexamethasone was radioactively labelled. This was made possible by the synthesis of [^{3}H]dexamethasone 21-methanesulfonate (see Materials and Methods and [4]). We calculated that 42% and 57% of the binding activity of the cytosol and of the nucleosol, respectively, could be recovered from the affinity gel. The purification calculated was 8281-fold for the cytoplasmic and 11000-fold for the nuclear receptor (Table 2).

DEAE-cellulose Chromatography of the Receptor Fraction Purified by Affinity Chromatography

Preliminary characterization by sodium dodecylsulphate/polyacrylamide gel electrophoresis of the affinity-purified fractions revealed the presence of several protein species, in particular in the cytosol sample (see Fig. 3). We therefore proceeded to purify the receptor fractions further by DEAE-cellulose chromatography. DEAE-cellulose chromatography was performed at pH 8.5 (see also [13]). In some experiments the fractions to be submitted to chromatography stemmed from affinity columns in which dexamethasone was radioactively labelled, so that the hormone-receptor complex was also radioactively labelled. The separation on DEAE-cellulose columns of the receptor fraction purified by affinity chroma-

Table 2. *Purification by affinity chromatography of cytosol and nucleosol receptors for dexamethasone*

Fraction	Total protein	[^{3}H]Dexamethasone bound	Spec. act.	Purification
	mg	pmol (% original)	pmol/mg protein	-fold
Cytosol	1887	106 (100)	0.056	1
Purified receptor after affinity chromatography	0.1	46.8 (44)	463.7	8281
Nucleosol	1040	88 (100)	0.08	1
Purified receptor after affinity chromatography	0.062	52 (58)	880	11000

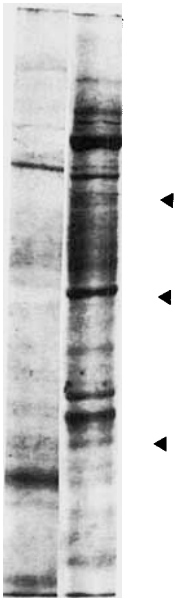

Fig. 3. *Sodium dodecylsulphate/polyacrylamide gel electrophoresis of the protein fraction recovered from the dexamethasone affinity column chromatography of cytosol and nucleosol.* 10% gels were used. 1 = cytosol fraction; 2 = nucleosol fraction. The arrows denote the positions of migration of marker proteins bovine serum albumin, ovalbumin and chymotrypsinogen

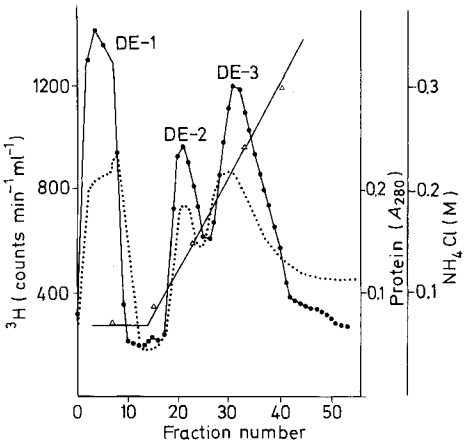

Fig. 4. *DEAE-cellulose chromatography of the receptor fraction obtained after affinity purification of the cytosol proteins on [^{3}H]dexamethasone-Sepharose columns.* The affinity purified fraction were submitted to Sephadex G-25 filtration and the macromolecular fraction was subsequently applied to a 15-ml column of DEAE-cellulose (see Materials and Methods and [13]). Elution was performed with a 50–400 mM NH_4Cl gradient (40 + 40 ml in buffer B). (· · · · · ·) Protein; (●——●) ^{3}H in fraction; (△——△) NH_4Cl concentration

tography of cytosol is shown in Fig. 4. Three fractions with binding activity can be detected, corresponding in their elution characteristics to DE-1, DE-2 and DE-3, which were also obtained with crude cytosol [13]. DEAE-cellulose chromatography of the receptor fraction purified by affinity chromatography of nucleosol reveals hormone-receptor complex only in the flow-through (results not shown), as also observed after chromatography of nucleosol [13].

Sodium Dodecylsulphate/Polyacrylamide Gel Electrophoresis of the Purified Fractions

The active fractions were pooled, treated with 10% trichloroacetic acid and the precipitated proteins submitted to electrophoresis in 10% polyacrylamide gels. As evident from Fig. 5A, fraction DE-1 contains mainly two polypeptides with M_r of 72000 and 45000, fraction DE-2 mainly one component with M_r 45000 and DE-3 mainly a protein with M_r 90000. Many minor protein species appear in the flow-through fraction of the column, whereas only a few minor protein species are present in fractions DE-2 and DE-3. The receptor fraction isolated from the nucleosol, DE-n, is mainly com-

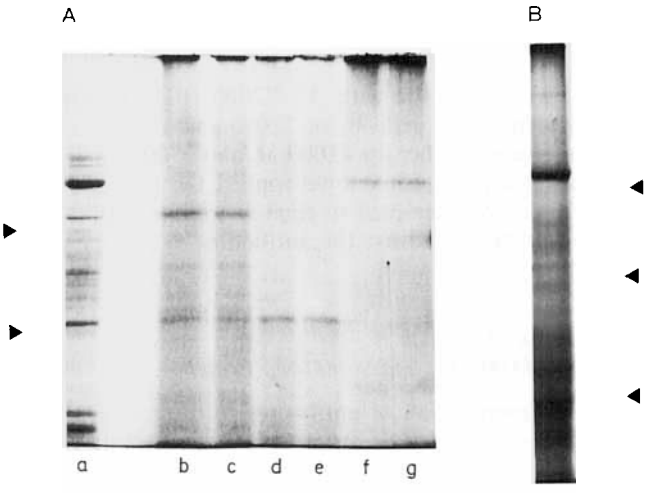

Fig. 5. *Sodium dodecylsulphate/polyacrylamide gel electrophoresis of the protein fractions recovered (A) from the DEAE-cellulose column chromatography of the affinity purified cytosol fraction depicted in Fig. 4 and (B) from the DEAE-cellulose chromatography of the affinity purified nuclear fraction.* 10% polyacrylamide gels were used. (A) Gel (a): affinity purified cytosol receptors (fraction submitted to DEAE-cellulose chromatography); (b) fractions 3, 4 and 5; (c) fractions 6 and 7; (d) fractions 19, 20 and 21; (e) fractions 22 and 23; (f) fractions 29, 30 and 31; (g) fractions 32, 33 and 34. (B) Flow-through fraction. The arrows denote the position of migration of marker proteins bovine serum albumin, ovalbumin and chymotrypsinogen

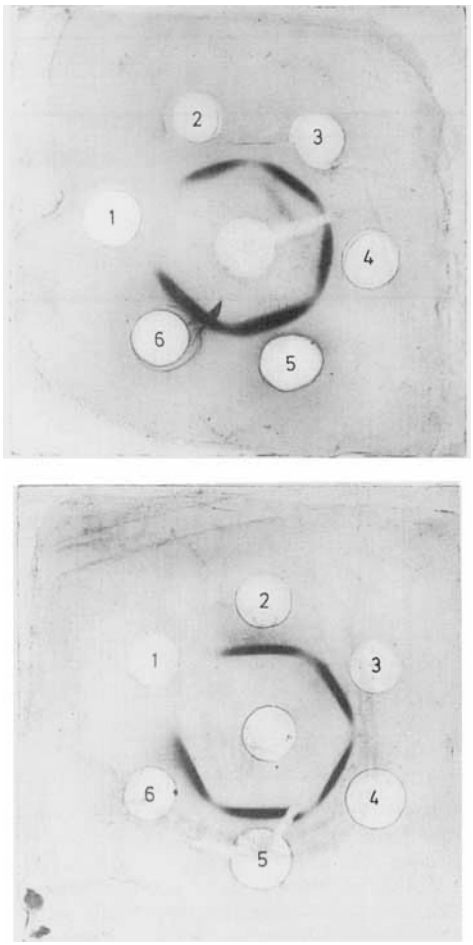

Fig. 6. *Ouchterlony double-diffusion tests.* (A) The middle well contains the 45000-M_r protein. 1 = preimmune IgG; 2, 5 = IgG to the 90000-M_r protein; 3, 4, 6 = IgG to the 45000-M_r protein. (B) The middle well contains the 90000-M_r protein. 1 = preimmune IgG; 2, 5 = IgG to the 90000-M_r protein; 3, 4, 6 = IgG to the 45000-M_r protein

posed of a polypeptide with M_r 72000 (Fig. 5B) and two smaller polypeptides with M_r of 22000 and 12000.

To ascertain whether the 45000-M_r and 90000-M_r proteins are indeed receptor proteins we applied the immunological approach, i.e. we attempted to elicit in rabbits antibodies to these two proteins and use the antibodies to precipitate the receptors.

Immunization of Rabbits against the 45000-M_r and 90000-M_r Proteins

For the preparation of antibodies to these two proteins preparative sodium dodecylsulphate/polyacrylamide electrophoresis [20] was applied and the respective proteins eluted from the gels with 1% sodium dodecylsulphate overnight at 37 °C (see Materials and Methods). The immunization procedure is described in Materials and Methods; the IgG fractions were obtained according to [7]. In Ouchterlony double-diffusion tests (Fig. 6) IgG against the 45000-M_r protein immunoprecipitates the 45000-M_r protein as well as the 90000-M_r protein. Similarly the antibody against the 90000-M_r protein precipitates both the 90000-M_r and the 45000-M_r proteins. No reaction with any of the two proteins is seen if the specific antibodies are substituted with IgG from preimmune serum (Fig. 6).

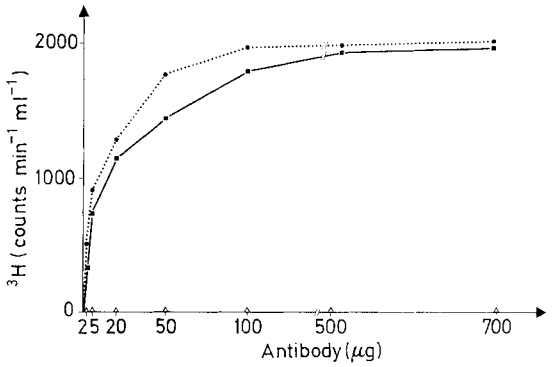

Fig. 7. *Immunoprecipitation of [^{3}H]dexamethasone receptor complexes from rat thymus cytosol.* Cytosol prepared in buffer G was incubated with 0.05 μM [^{3}H]dexamethasone for 120 min at 0–4 °C and subsequently treated with charcoal to remove unbound hormone. 400-μl aliquots were incubated with various concentrations of IgG to the 45000-M_r protein (■——■), to the 90000-M_r protein (● · · · · · · ●) and to IgG from preimmune serum (△——△) in buffer F for 16 h at 0–4 °C. The preparations were subsequently centrifuged at 1000 rev./min in the H4-B head of the Servall centrifuge for 10 min at 0–4 °C. The precipitates were submitted to liquid scintillation counting. The points are means of duplicate estimations

Immunoprecipitation of Cytoplasmic Dexamethasone Receptors with Antibodies to 45000-M_r and 90000-M_r Proteins

A strong indication that the two proteins represented the active binding components would be obtained if immunoprecipitation of these two proteins by their antibodies lead to impairment of the dexamethasone-binding activity of cytosol. We therefore incubated cytosol with [^{3}H]dexamethasone and then treated the cytosol with either of the two antibodies. Subsequently, the radioactivity in the precipitate was measured. As seen from Fig. 7, in both cases incubation with the specific IgG results in precipitation of receptor-bound [^{3}H]-dexamethasone (60–65% of total bound activity), whereas IgG from preimmune serum has no such effect.

In another experiment, cytosol charged with [^{3}H]dexamethasone and treated with specific IgG was submitted to sucrose gradient centrifugation. As controls, cytosol treated with buffer or with IgG from preimmune serum were run in parallel (Fig. 8A–D). It is evident that the interaction with the specific antibodies results in an aggregation of the hormone-receptor complexes, whereas the control assays show no or only minimal formation of aggregates.

Interaction of the antibodies to the two cytosol receptor proteins with nucleosol charged with [^{3}H]dexamethasone had no appreciable effect on the profile obtained after sucrose gradient centrifugation (results not shown) and it does not lead to appreciable precipitation of labelled material.

Immunoaffinity Chromatography of Cytosol and Nucleosol

Immunoaffinity chromatography was performed (see Materials and Methods) on two columns, one coated with antibodies against the 45000-M_r protein and the other against the 90000-M_r protein. The proteins eluted from the immunoaffinity column with 3 M sodium thiocyanate were submitted to sodium dodecylsulphate/polyacrylamide gel electrophoresis. The results are shown in Fig. 9. In the eluates of both columns to which cytosol was applied we could detect mainly the 45000-M_r protein and small amounts of the 90000-M_r

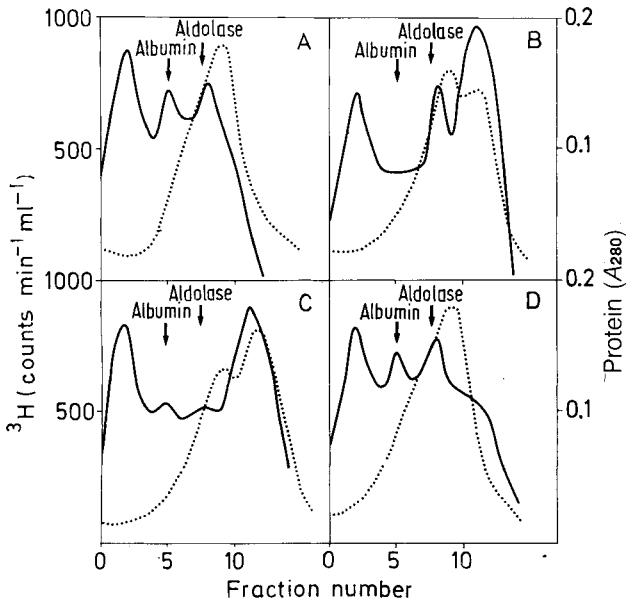

Fig. 8. *Influence of antibodies to receptor proteins on the sedimentation behavior of cytosol receptors.* Cytosol incubated with 0.05 μM [^{3}H]-dexamethasone for 90 min at 0–4 °C in buffer G and subsequently treated with charcoal to remove unbound hormone was incubated in buffer F without additions (A), or in the presence of IgG to either the 45000-M_r protein (B), to IgG to the 90000-M_r protein (C) or to pre-immune IgG (D) for 12 h at 0–4 °C. The preparations were then submitted to sucrose gradient centrifugation in 5–20% linear sucrose gradients in an SW41 Beckman head at 39000 rev./min for 19 h. (——) ^{3}H in fraction; (· · · · · ·) protein. The arrows denote the migration of marker proteins

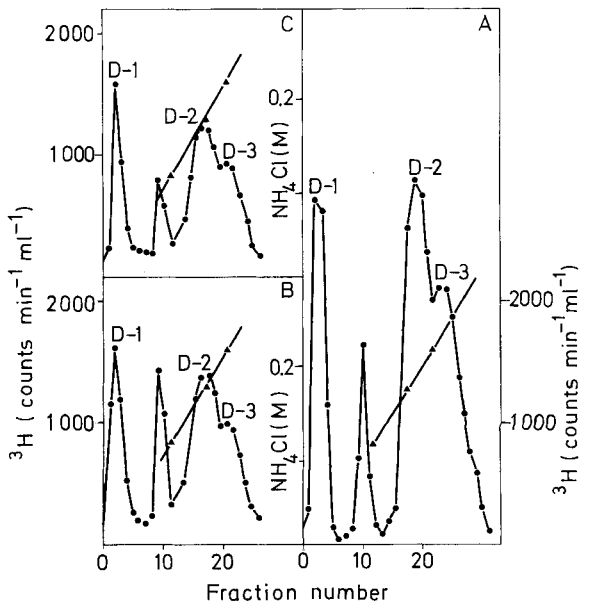

Fig. 10. *DEAE-cellulose chromatography of receptors from thymus cytosol previously submitted to immunoaffinity chromatography.* Rat thymus cytosol was incubated at 0–4 °C for 90 min with 0.05 μM [^{3}H]dexamethasone and subsequently submitted to Sephadex G-25 filtration in buffer G. The macromolecular fraction was passed through Sepharose 4B columns coated with (A) preimmune IgG, (B) IgG to the 45000-M_r rat liver cytosol receptor, (C) IgG to the 90000-M_r rat liver cytosol receptor. The flow-through fractions were then submitted to DEAE-cellulose chromatography (see legend to Fig. 3 and [13]). (●) ^{3}H; (▲) NH_4Cl concentration

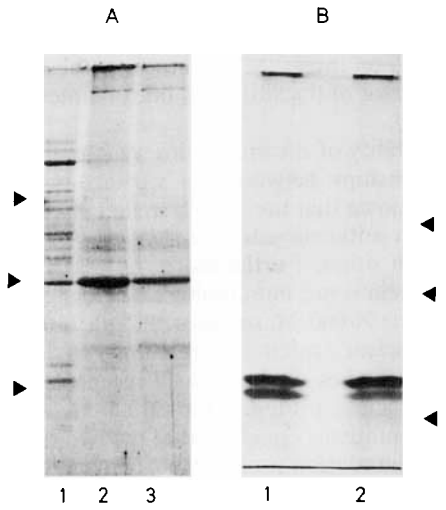

Fig. 9. *Sodium dodecylsulphate/polyacrylamide gel electrophoresis of (A) cytosol proteins and (B) nucleosol proteins bound to the immunoaffinity columns.* Cytosol and nucleosol were submitted to immunoaffinity chromatography on Sepharose 4B linked to IgG either to the 45000-M_r protein or to the 90000-M_r protein. The proteins eluted from the column with 15 ml of 3 M sodium thiocyanate were precipitated with 10% trichloroacetic acid, washed with ethanol and submitted to electrophoresis. 10% polyacrylamide gels were used. (A) 1 = cytosol receptor fraction purified on dexamethasone affinity column; 2 = cytosol proteins eluted from anti-(90000-M_r protein) IgG immunoaffinity column; 3 = cytosol proteins eluted from anti-(45000-M_r protein) IgG immunoaffinity column. (B) 1 = nucleosol proteins eluted from anti-(90000-M_r protein) IgG immunoaffinity column; 2 = nucleosol proteins eluted from the anti-(45000-M_r protein) IgG immunoaffinity column. The arrows denote the position of migration of marker proteins bovine serum albumin, ovalbumin and chymotrypsinogen

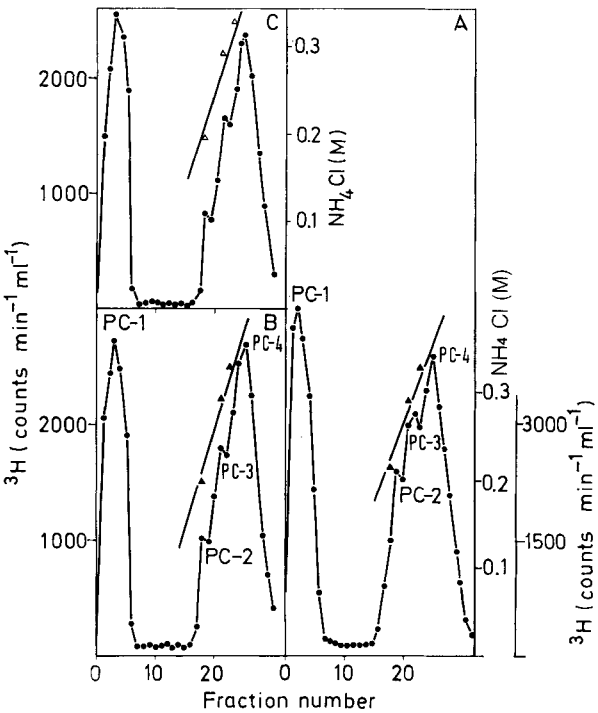

Fig. 11. *Phosphocellulose chromatography of cytosol receptors from thymus previously submitted to immunoaffinity chromatography.* The conditions of labelling of the cytosol with [^{3}H]dexamethasone and of the immunoaffinity chromatography is as described in legend to Fig. 10. The conditions of phosphocellulose chromatography are as described in Materials and Methods and in [13]. (●) ^{3}H; (▲) NH_4Cl concentration

protein, corroborating the results of the Ouchterlony double-diffusion test on the cross-reactivity of these proteins. Some other proteins bands were also observed which have not yet been identified (Fig. 9A).

Immunoaffinity chromatography of the nucleosol fraction on either of the two columns (Fig. 9B) resulted in the adsorption of two proteins with M_r 36000 and 38000. The 72000-M_r protein, which represents the main protein component obtained after affinity chromatography of the nucleosol, could not be detected on the immunoaffinity column.

DEAE-cellulose and Phosphocellulose Chromatography of Thymus Cytosol after Treatment with Antibodies to the 45000-M_r and 90000-M_r Proteins from Rat Liver Cytosol

In preliminary experiments with the Ouchterlony double-diffusion technique we detected cross-reactivity of antibodies to rat liver cytosol receptors [9] towards thymus cytosol receptors. To document this intertissue reactivity further, we submitted thymus cytosol prelabelled with [^{3}H]dexamethasone to immunoaffinity chromatography on Sepharose 4B columns coated either with IgG to the 45000-M_r or the 90000-M_r liver cytosol receptor [9] or with IgG from preimmune serum. The flow-through fractions were then submitted to either DEAE-cellulose or phosphocellulose chromatography as described in Materials and Methods and [13]. As mentioned above, DEAE-cellulose chromatography of cytosol reveals the presence of three binding fractions whereas phosphocellulose chromatography shows four binding components named PC-1 to PC-4 (unpublished results). The results shown in Fig. 10 and 11 demonstrate that the reaction with either of the specific IgG results in a decrease of total binding activity, but not of selective decrease in the activity of any of the various receptor peaks separated by the ion-exchange chromatography. Similar results have been obtained if thymus receptor antibodies are used instead of liver IgG.

DISCUSSION

The detection in the cytosol of rat thymocytes of multiple decamethasone-binding activities and the large capacity of the nucleus of the thymocyte to bind dexamethasone [4] raised a series of questions concerning the interrelationship between the various cytosol binding proteins, as well as between cytosol and nuclear binding components. Particularly interesting is the question of whether the cytosol receptor(s) undergoes molecular changes during its probable translocation from the cytoplasm into the nucleus. In order to answer these questions the various macromolecules involved had to be purified. In a previous publication [9] we described the extensive purification of two dexamethasone-binding proteins from rat liver cytosol, using as the main purification step affinity chromatography on Sepharose to which 11-deoxycorticosterone was linked through a disulfide bond [23]. In that work the steroid ligand was bound to the aminohexylcystaminium-Sepharose by way of a C-N bond, instead of the easily hydrolysable ester bond, initially introduced by Sweet and Adair [23]. In our present work we have substituted 11-deoxycorticosterone by dexamethasone, as in preliminary experiments we had observed a much higher purification of the receptors using dexamethasone as the steroid ligand [24]. The presence of the disulfide linker between the steroid ligand and the affinity matrix [23] makes it very easy to recover the receptors bound to the affinity column by cleavage of the disulfide bond with mercaptoethanol or other thiol reagents. Using this procedure we could obtain a many-thousand-fold purification of both cytosol and nucleosol binding components, which, with an additional DEAE-cellulose chromatography step, yielded extensively purified dexamethasone receptor fractions. In previous work with rat liver cytosol [13] and in the present work with thymus cytosol, chromatography on DEAE-cellulose yielded three major binding fractions: DE-1, present in the flow-through of the column and DE-2 and DE-3 eluting with approximately 0.13 M and 0.23 M salt, respectively. The nuclear extract yielded one single binding component, present in the flow-through (DE-n). In accordance with these results, the affinity-purified fractions from cytosol yielded three binding activities after DEAE-cellulose chromatography (DE-1, DE-2 and DE-3), whereas those from nucleosol yielded one binding component, DE-n.

Analysis of these components by sodium dodecylsulphate/polyacrylamide gel electrophoresis revealed that DE-1 contains mainly two proteins (with M_r of 45000 and 72000), DE-2 one major band (M_r 45000), DE-3 also one major protein component (M_r 90000), whereas in DE-n the major protein component is the 72000-M_r one and two smaller polypeptides (M_r 22000 and 12000).

Using the technique of Vaitukaitis et al. [16], it was possible to elicit antibodies to the 45000-M_r and 90000-M_r species. Applying immunoprecipitation technique we could demonstrate that these two proteins represent the major dexamethasone-binding components of the cytosol. Similar results had been obtained by us previously [9] with rat liver cytosol, where two major dexamethasone-binder proteins of M_r of 45000 and 90000 had been demonstrated. Recently Gustafsson et al. [25] have purified a 89000-M_r receptor from rat liver cytosol and suggest that the 45000-M_r species could represent a degradation product of the 90000-M_r molecule. Govindan [26], on the other hand regards the 45000-M_r protein as a precursor of the 90000-M_r one (a dimer of the 45000-M_r protein [9]).

The availability of the antibodies was important in establishing relationships between the various binding components. It was shown that the 45000-M_r and 90000-M_r proteins share common antigenic sites, as they cross-react with antibodies to each other. Furthermore, it was shown that the 72000-M_r protein is not immunologically related to either the 45000-M_r or the 90000-M_r receptor. As the 72000-M_r protein is the major nuclear protein having affinity to dexamethasone and as the antibodies to the cytosol receptors interact with two smaller nuclear proteins (the 36000-M_r and 38000-M_r proteins) we tentatively conclude that the two smaller nuclear proteins are degradation products of the translocated cytosol receptor. Climent et al. [27] have partially purified from rat liver cytosol a glucocorticoid receptor with a molecular weight of 33500. In rat liver nuclei, however, only the 90000-M_r receptor could be detected [26]. Thus the presence of the 72000-M_r receptor in thymus nuclei could either reflect high proteolytic activity of the thymus extracts or an intranuclear fate of the translocated thymus cytosol receptor different than that of the liver receptor. Experiments are now in progress to elicit antibodies against the 72000-M_r receptor, to be used in exploring the possible relationship of this protein to the other receptor species.

On the basis of the sedimentation coefficient of the nuclear receptor (7S, see [28]) we must assume that the cytosol receptor upon entry into the nucleus forms larger salt-stable structures, either by associating with nuclear proteins or with further receptor molecules. As these larger complexes do not

precipitate with the antibodies to the cytosol receptors, we conclude that the antigenic sites are either buried and not accessible for reaction or that they have been cleaved by proteolysis as part of the intranuclear modification of the receptor. The two smaller proteins which bind to the immunoaffinity column could represent the cleaved part of the receptor molecule, representing the antigenic sites reacting with the respective antibodies.

We have recently introduced an immunocytochemical test [29], based on the availability of antibodies to the two thymus cytosol receptors, for the detection of glucocorticoid receptors in various cell types, such as rat and mouse thymocytes, human peripheral lymphocytes, HeLa cells [29] and human mammary tumors [30]. Using phytohemagglutinin-activated lymphocytes from human peripheral blood [29] and incubating these cells with dexamethasone, we observed that the immunofluorescence staining of the cytoplasm decreased during the incubation with concomittant appearance of immunofluorescence in the nucleus. On the basis of our present experiments, we could ascribe the appearance of immunofluorescence in the nucleus to the 36000-M_r and 38000-M_r species. Further studies are necessary to establish whether these nuclear proteins can be regarded as products of a physiologically significant cleavage mechanism, irreversibly modifying the translocated receptor, or simply as degradation products of unspecific nuclear proteases.

A similar immunological technique has been applied by Govindan [31] to demonstrate translocation of glucocorticoid receptors from the cytoplasm into the nucleus of HTC cells. In this case no proteolytic cleavage of the receptor in the nucleus has been observed [26].

The interaction of antibodies elicited against rat liver cytoplasmic receptors [9] with thymus cytosol receptors, observed in the present study, demonstrate tissue cross-reactivity, which is further underlined by the interaction of the antibodies against rat thymus cytosol receptors with cells from other species, such as humans, mice and a variety of cells in culture [29, 30].

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