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Maximizing the Purification of the Activated Glucocorticoid Receptor by DNA–Cellulose Chromatography

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With heat treatment (20°C for 30min), the glucocorticoid–receptor complex becomes 'activated' and undergoes an increase in affinity for DNA. A two-stage procedure was used to separate sequentially the rat liver glucocorticoid–receptor complex from proteins with high and low affinity for DNA. DNA–cellulose column chromatography of unheated cytosol resulted in the retention of DNA-binding proteins, but not the unactivated receptor complex. Heat treatment of the column eluate resulted in increased affinity of the receptor complex to DNA, and chromatography on DNA–cellulose then yielded receptor complex free from proteins with low affinity for DNA. Removal of DNA-binding proteins during the first chromatographic step was critically dependent on ionic conditions and the ratio of cytosol chromatographed to DNA–cellulose. A purification of 11000-fold (85% yield) was achieved by this procedure. The partially purified receptor complex was taken up by rat liver nuclei.

Glucocorticoids, like other steroid hormones, bind to protein receptors in the cytosol of target cells (Wira & Munck, 1970). The glucocorticoid–receptor complex then enters the nucleus (Rousseau *et al.*, 1973). Current evidence suggests that the receptor complex acts in the nucleus to mediate the effects of glucocorticoids on gene transcription (Higgins *et al.*, 1973; Yamamoto & Alberts, 1974). Despite the importance of the glucocorticoid–receptor complex as a putative 'regulator' of eukaryotic gene transcription, most of the knowledge of its properties has come from crude systems. The glucocorticoid–receptor complex has proved very difficult to purify by conventional techniques because of its low concentration and instability. Failla *et al.* (1975) have covalently linked a corticosterone derivative to agarose and have used this as an affinity ligand for partially purifying the glucocorticoid receptor from hepatoma (HTC) cells. Another potentially useful class of ligands for affinity chromatography was suggested by observations that the glucocorticoid receptor bound to DNA (Milgrom *et al.*, 1973; Rousseau *et al.*, 1975) and to other polyanions (Milgrom *et al.*, 1973). Using DNA immobilized on cellulose (Alberts & Herrick, 1971), we have developed a rapid two-stage procedure for highly purifying the glucocorticoid–receptor complex from rat liver.

Experimental

Male Sprague–Dawley rats, weighing 175–200g, were adrenalectomized and maintained on food *ad libitum* and drinking water supplemented with 0.3% NaCl and 1% dextrose. They were killed by decapitation 7–14 days after adrenalectomy. Livers were perfused via the portal vein with 10ml of ice-cold 0.9% NaCl, minced, and homogenized at 200rev./min with a loosely fitting Teflon/glass homogenizer in 2vol. of 10mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] buffer, pH7.6, containing 10% (v/v) glycerol, 1mM-EDTA, 1mM-dithiothreitol (hereafter called Hepes/glycerol buffer), and NaCl at concentrations indicated in the text. The homogenate was centrifuged at 5000*g* for 10min. The supernatant was then centrifuged (105000*g* for 1h) to obtain a cytosol fraction that contained the glucocorticoid receptor. [^{3}H]Triamcinolone acetonide (16Ci/mmol, from New England Nuclear Corp., Boston, MA, U.S.A.) was added to form the glucocorticoid–receptor complex.

DNA–cellulose was prepared (Alberts & Herrick, 1971) by using native calf thymus DNA (P–L Laboratories, Milwaukee, WI, U.S.A.); 0.7mg of DNA was bound/packed ml of DNA-cellulose. Before use columns containing DNA–cellulose were washed extensively with Hepes/glycerol buffer containing 450mM-NaCl and then were equilibrated with this buffer containing the NaCl concentration indicated in the text. Nuclei from livers of adrenalectomized rats were isolated as previously described (Zieve, 1972).

DNA was assayed by the method of Kisane & Robins (1958). Protein was measured by a fluorescamine assay (Udenfriend *et al.*, 1972); crystalline bovine albumin was used as a standard. ^{3}H radioactivity was counted by dissolving the sample in 10ml of Hydromix liquid-scintillation fluid (York-

town Research Laboratory, Hackensack, NJ, U.S.A.). Samples were counted for radioactivity at 30% effiency in a Packard Tri-Carb liquid-scintillation counter (model 544).

Polyacrylamide gels (7.5% acrylamide, 0.02% *NN'*-methylenebisacrylamide) containing 0.1% sodium dodecyl sulphate were prepared as described by Weber & Osborn (1969), except that the gel-phase buffer contained 75mM-Tris/HCl, pH9.0 at 20°C, and 0.1% sodium dodecyl sulphate, and the running buffer contained 50mM-Tris/200mM-glycine, pH8.3 at 20°C, and 0.1% sodium dodecyl sulphate. Samples (25μl) were heated for 3min at 100°C in the presence of 1% sodium dodecyl sulphate and 1% 2-mercaptoethanol, cooled to 20°C and layered directly on the gels (0.6cm×10cm). Electrophoresis was carried out at 2mA/tube. Gels were fixed in 50% (v/v) propan-2-ol/10% (w/v) trichloroacetic acid for 18h and then stained with Coomassie Brilliant Blue G (Reisner *et al.*, 1975). Molecular-weight standards (β-galactosidase, 130000; ovalbumin, 43000; bovine albumin, 66000; cytochrome *c*, 12000) were all from Schwartz/Mann, Orangeburg, NY, U.S.A.)

Results

DNA–cellulose chromatography has been used to characterize or partially purify several steroid-hormone receptors (Clemens & Kleinsmith, 1972; Mainwaring & Irving, 1973; Eisen & Glinsmann, 1975). In general, these procedures involved the binding of the activated form of the steroid–receptor complex to DNA-cellulose at low ionic strength, and its elution at higher ionic strength. However, other proteins in cytosol also were bound to DNA–cellulose, and these proteins severely limited the purification of the steroid–receptor complex.

The observation that the glucocorticoid–receptor complex is found in two states with markedly different affinities for DNA suggested the following strategy for separating it from cytosol DNA-binding proteins: DNA–cellulose chromatography of unheated cytosol should result in the retention of DNA-binding proteins, but not the unactivated form of the glucocorticoid–receptor complex. Heat treatment should result in the binding of the glucocorticoid–receptor complex to DNA; chromatography on DNA-cellulose then should yield receptor complex free from DNA-binding protein. We and other investigators have used this basic strategy for partially purifying the glucocorticoid–receptor complex; however, purifications achieved differed substantially, from 100-fold (Simons *et al.*, 1976) to 1800-fold (Eisen & Glinsmann, 1976). This suggested that systematic evaluation of the variables involved might explain some of these differences and might lead to substantial improvement in the selectivity of the procedure.

The principal contaminants of the steroid–receptor complex after the second chromatographic step were expected to be DNA-binding proteins that had not been removed during the first chromatographic step. Ionic conditions affecting the binding of the receptor complex and other proteins to DNA–cellulose were examined, since differential sensitivity to ionic strength offered one way of separating the receptor complex from these proteins. In the experiment shown in Fig. 1, heat-treated cytosol was

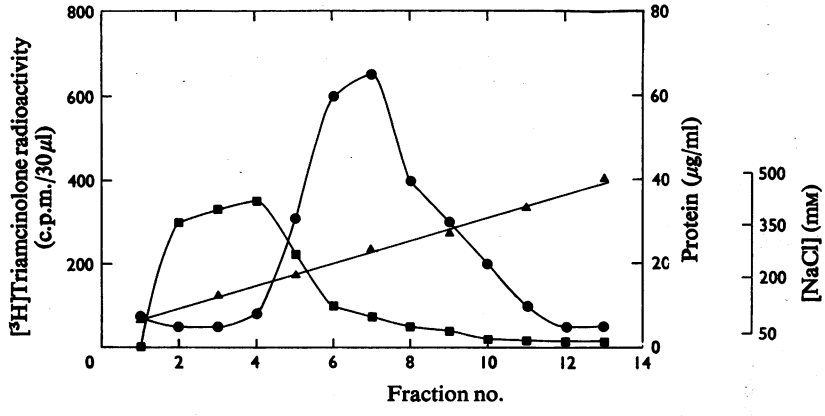

Fig. 1. *Elution of [^{3}H]triamcinolone–receptor complex from DNA–cellulose*

Cytosol was prepared in Hepes/glycerol buffer containing 50mM-NaCl. [^{3}H]Triamcinolone (60nM) was added; after 1h at 4°C, the cytosol was heated at 20°C for 30min, cooled and applied to DNA–cellulose (1.8cm×16cm, 40ml bed volume). The column was washed with 100ml of Hepes/glycerol buffer containing 50mM-NaCl and then eluted with a linear NaCl gradient (50–500mM-NaCl in Hepes/glycerol buffer). Protein (■), ^{3}H (●) and NaCl concentration (▲) were measured in the gradient fractions (3ml).

Table 1. *Protein binding to DNA–cellulose*

For step 1, cytosol preparations were made by using the Hepes/glycerol buffer containing 50mM- (*a*) or 100mM- (*b*) NaCl. The cytosol preparations (10ml containing 300mg of protein) were passed through 20ml DNA–cellulose columns. The columns were washed with 40ml of the initial buffers, and protein bound was eluted with Hepes/glycerol buffer containing 450mM-NaCl. For step 2, flow-through fractions containing cytosol protein that did not bind to step-1 columns were individually applied to 5ml DNA–cellulose columns. These columns were washed with 20ml of the initial buffer, and protein bound was eluted with Hepes/glycerol buffer containing 450mM-NaCl.

		Protein bound (μg)			
	[NaCl] (mM)	Step 1	Step 2		
Fraction ...			1	2	3
(*a*)	50	450	40	65	80
(*b*)	100	40	4	6	10

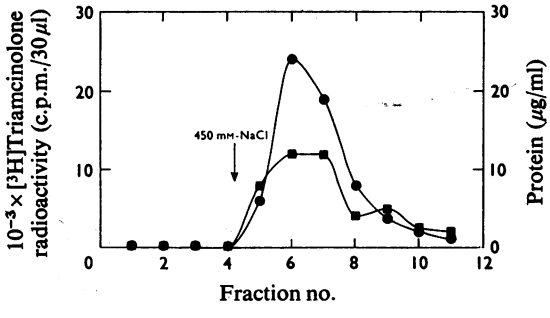

Fig. 2. *Two-stage DNA–cellulose chromatography of [³H]triamcinolone–receptor complex*

Cytosol was prepared with Hepes/glycerol buffer containing 100mM-NaCl; [³H]triamcinolone (60nM) was added, before the high-speed centrifugation, to form the glucocorticoid–receptor complex. Cytosol (25ml) was passed through a DNA–cellulose column (2.6cm×25cm; 150ml bed volume) equilibrated with Hepes/glycerol buffer containing 100mM-NaCl. [To elute DNA-binding proteins from the first DNA–cellulose column, this column was washed with 300ml of Hepes/glycerol buffer (containing 100mM-NaCl) and then eluted with buffer containing 450mM-NaCl.] Eluate fractions were pooled, heated at 20°C for 30min, and then cooled to 4°C before application to a second DNA–cellulose column (1.3cm×12cm; 30ml bed volume). The column was washed with 100 ml of Hepes/glycerol buffer containing 100mM-NaCl, and the bound receptor complex was eluted with Hepes/glycerol buffer containing 450mM-NaCl. ³H (●) and protein (■) were measured. Only fractions (1.3ml) from the 450mM-NaCl elution of the second DNA–cellulose column are shown in the Figure.

applied to a DNA–cellulose column. The column was washed extensively with Hepes/glycerol buffer containing 50mM-NaCl and then eluted with a linear NaCl gradient in the same buffer. The glucocorticoid–receptor complex was eluted in a broad peak between 125mM- and 450mM-NaCl, whereas most of the DNA-binding proteins were eluted at lower ionic strength. This suggested that the use of NaCl concentrations close to 125mM would limit the binding of these proteins to DNA, and would substantially improve purification. To test this idea directly and to define other parameters that might affect the removal of DNA-binding proteins, two-step DNA–cellulose chromatographic procedures were carried out under different ionic conditions and the protein bound to the columns was measured (Table 1). NaCl concentrations greater than 100mM were found to inhibit binding of the receptor complex to DNA–cellulose and were therefore not used. However, a striking difference in protein bound to DNA–cellulose was noted at the ionic strengths studied (50mM- and 100mM-NaCl). Furthermore, the first-stage column eluates containing cytosol were divided and applied individually to second-stage columns. Protein bound to the columns was less in the initial fractions and increased substantially in the later fractions. This observation suggested that DNA–cellulose has a limited capacity to clear DNA-binding proteins from cytosol, and that very low cytosol loads offered the best condition for maximal removal of these proteins.

Although DNA–cellulose columns are relatively inefficient at clearing cytosol of DNA-binding proteins, only a small proportion of cytosol protein binds to DNA–cellulose, and thus a remarkably high degree of purification of the receptor complex can be achieved. Using conditions defined by the above data, we were able to purify the activated [³H]triamcinolone receptor 11000-fold with a yield of 85% (Fig. 2, Table 2). Polyacrylamide gels (containing 0.1% sodium dodecyl sulphate) indicate the heterogeneous nature of the cytosol DNA-binding proteins (Fig. 3), and are shown primarily to document the effective clearance of DNA-binding proteins by the first-stage column. [³H]Triamcinolone dissociates from the receptor under denaturing conditions, hence we cannot locate the glucocorticoid receptor on these gels.

The purified receptor complex had a sedimentation coefficient of 4S on glycerol gradients containing 450mM-NaCl (Fig. 4). The receptor complex was apparently denatured by gel-permeation chromatography on agarose or on polyacrylamide gels that were expected to include the receptor complex (Sephadex G-100 or Bio-Gel P-60). Failla *et al.* (1975) also noted denaturation of partially purified receptor complex on gel filtration. Therefore we were unable to calculate the Stokes radius of the receptor complex.

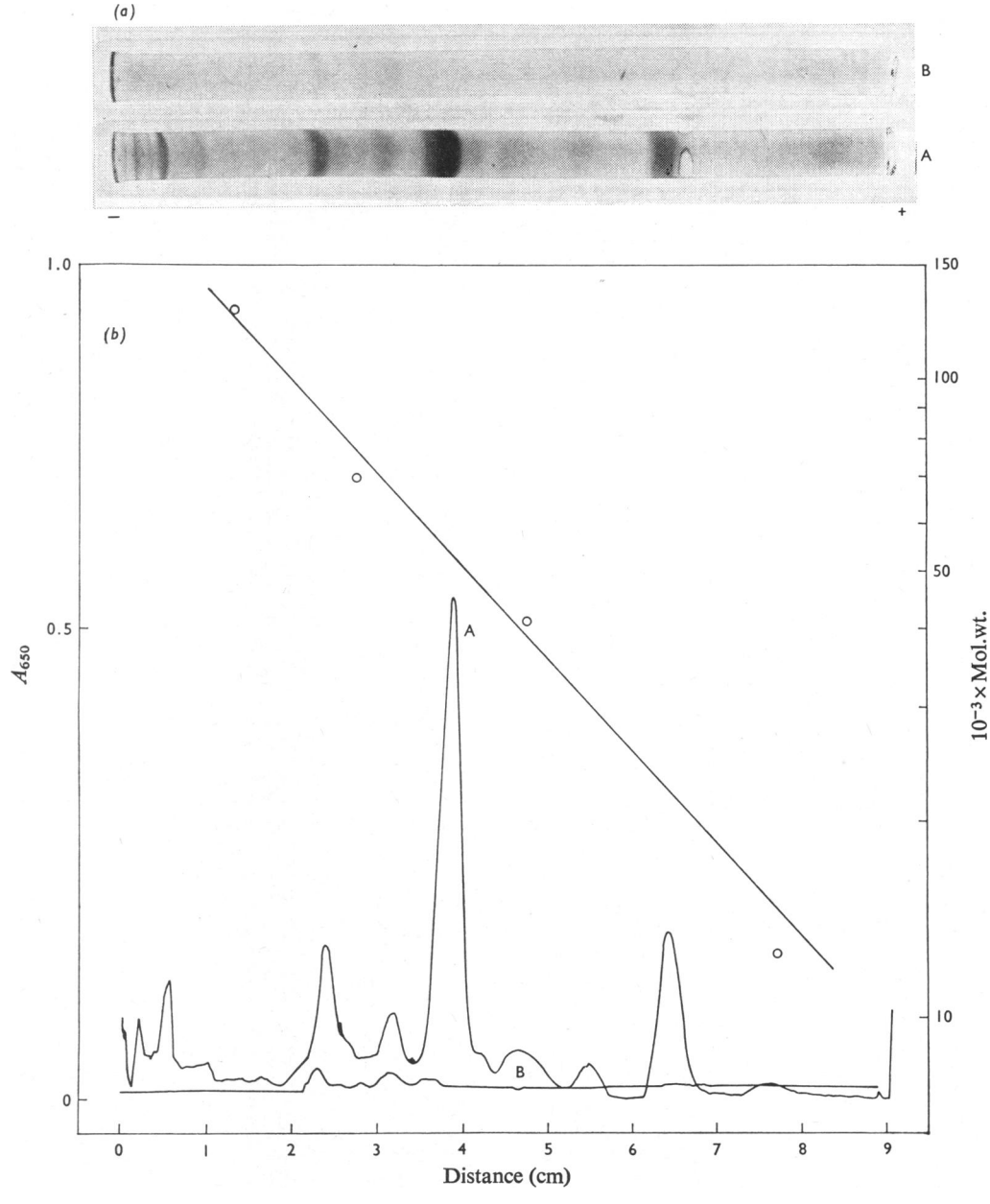

Fig. 3. *Polyacrylamide-gel electrophoresis in the presence of* 0.1% *sodium dodecyl sulphate*

The 450mM-NaCl eluates of the columns described in Fig. 2 were concentrated 5-fold by using CF-25 Centriflo cones (Amicon). Samples (25 μl) were denatured and electrophoresed as described in the Experimental section: (*a*) A, DNA-binding proteins from the first DNA–cellulose step (23 μg of protein applied to gel); B, partially purified receptor from the second DNA–cellulose step (1.75 μg of protein applied to gel). Spectrophotometric scans of these gels are also shown (*b*). Molecular-weight calibration is based on spectrophotometric scan of gel containing standards (○) given in the Experimental section.

Table 2. *Purification of [^{3}H]triamcinolone–receptor complex*

[^{3}H]Triamcinolone bound to macromolecules was determined by gel filtration on columns of Sephadex G-25; non-specific binding of [^{3}H]triamcinolone in crude cytosol was measured as previously described (Eisen & Glinsmann, 1976) and was subtracted from total binding.

	Volume (ml)	Protein (mg)	[^{3}H]Triamcinolone (pmol)	Specific activity (pmol/mg)
(*a*) Cytosol	25	750	490	0.58
(*b*) 450mM-NaCl eluate	7.8	0.060	390	6500

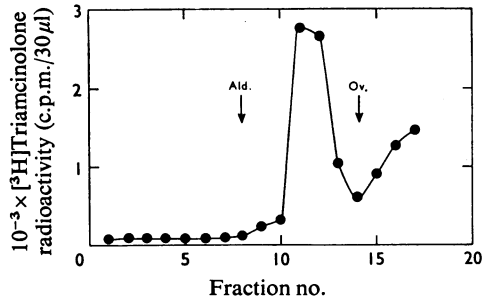

Fig. 4. *Velocity sedimentation of purified receptor in 15–30% glycerol gradient*

Purified [^{3}H]triamcinolone–receptor complex (100μl) was applied to a 4ml gradient [15–30% (v/v) glycerol in Hepes buffer containing 450mM-NaCl] and centrifuged for 16h at 55000rev./min in a Beckman SW-60 rotor. Fractions (0.2ml) were collected and counted for ^{3}H radioactivity. As standards, 50μg of ovalbumin (Ov., 3.5S) and 50μg of aldolase (Ald., 8S) in 100μl were centrifuged in a parallel gradient.

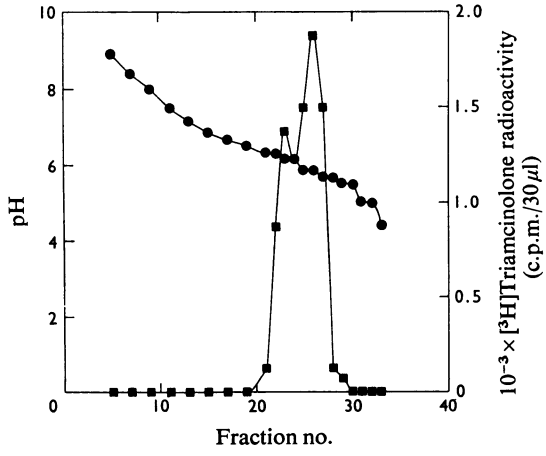

Fig. 5. *Isoelectric focusing of the partially purified [^{3}H]-triamcinolone–receptor complex*

Isoelectric focusing was done in small glass columns by the method of Godson (1970); focusing was carried out in a 10–50% (v/v) glycerol gradient (10ml) containing 1% Ampholines (pH5–8) and 1mM-dithiothreitol. Desalted [^{3}H]triamcinolone–receptor complex was added to make the 10% glycerol solution for the gradient. Focusing was carried out for 16h at 400V. Fractions (0.4ml) were collected; pH (●) and ^{3}H content (■) were measured in each fraction.

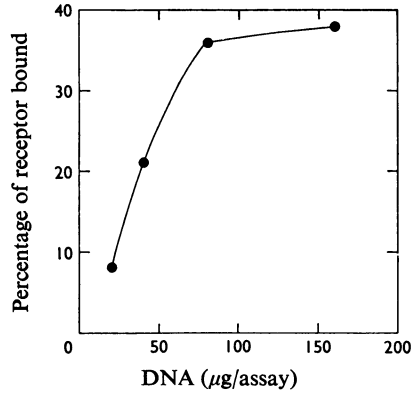

Fig. 6. *Nuclear binding of [^{3}H]triamcinolone–receptor complex*

Purified [^{3}H]triamcinolone–receptor complex (final concentration 4nM) and various concentrations of isolated rat liver nuclei were incubated at 4°C for 60min in 10mM-Hepes (pH7.6)/20% glycerol/3mM-$MgCl_2$/1mM-dithiothreitol/90mM-NaCl. Nuclei were separated by centrifugation (2min in a Beckman microfuge, model 152), washed three times with the above buffer, suspended in water and then sonicated. ^{3}H was determined. Values represent averages of duplicate assays. Nuclear concentration is expressed in terms of DNA content (Kisane & Robins, 1958).

However, the receptor complex was quantitatively excluded from Sephadex G-25, and we have used gel filtration on small Sephadex G-25 columns to measure macromolecular binding of [^{3}H]triamcinolone (Eisen & Glinsmann, 1976). The receptor complex has pI5.9–6.1 (Fig. 5), which is similar to the value reported by other investigators (Koblinsky *et al.*, 1973) for the activated glucocorticoid–receptor complex from rat liver.

The only established property of the glucocorticoid receptor complex that correlates with function is its ability to undergo nuclear uptake, and the receptor complex purified by this method is taken up by isolated liver nuclei (Fig. 6). The maximum proportion of receptor taken up by nuclei was determined by using an 'excess nuclei' assay in which

Table 3. *Binding of purified [^{3}H]triamcinolone–receptor complex to DNA–cellulose and nuclei*

In (*a*), purified receptor complex (500 μl) was diluted 1:5 with Hepes/glycerol buffer to make the NaCl concentration 90mM. The sample was applied to a 10ml DNA–cellulose column; the column was washed with 40ml of Hepes/glycerol buffer containing 100mM-NaCl and then eluted with the buffer containing 450mM-NaCl. Nuclear binding of the diluted receptor preparation was determined as described in Fig. 6. Tabulated values represent percentages of total ^{3}H bound to DNA–cellulose or nuclei. In (*b*), fractions from DNA–cellulose chromatography of the purified receptor were analysed for nuclear binding (see Fig. 6).

	[^{3}H]Triamcinolone bound (%)	
	DNA–cellulose	Nuclei
(*a*) Purified receptor	45	40
(*b*) Fractions from DNA–cellulose chromatography of purified receptor		
(1) 100mM-NaCl wash		0
(2) 450mM-NaCl eluate		47

the nuclear concentration is increased until the fraction of receptor bound reaches a maximum value. The fraction of receptor bound was 40–50% immediately after purification and it decreased to 25% with storage at 4°C for 48h. The proportion of receptor capable of binding to nuclei was not increased by repeating heat treatment (20°C for 30 min). The proportion of receptor that was able to re-bind to DNA–cellulose was proportionally decreased (Table 3). Purified receptor that was bound to DNA–cellulose also underwent nuclear uptake (Table 3), whereas receptor that did not bind to DNA–cellulose did not subsequently bind to nuclei.

Discussion

Biochemical studies of the function of the glucocorticoid receptor will require precise definition of its components and purification of these components to homogeneity. Rapid, highly selective, purification methods are important because of the lability of the receptor (Schaumberg, 1972; Bell & Munck, 1973). Two-stage procedures based on the change in affinity to DNA (Simons *et al.*, 1976) or phosphocellulose (Colman & Feigelson, 1976; Climent *et al.*, 1976; Atger & Milgrom, 1976) have been described for partially purifying the glucocorticoid–receptor complex. By defining optimal ionic conditions and determining the clearance properties of DNA–cellulose columns, we have significantly increased the selectivity of this procedure. Since an 'activation' process is a common property of steroid-hormone receptors, these straightforward observations may be extended to the purification of other steroid-hormone receptors.

If a mol.wt. of 60000 (Koblinsky *et al.*, 1973) and a single steroid-binding site per molecule are assumed, a specific activity of 16nmol of [^{3}H]triamcinolone bound/mg of protein would represent homogeneous receptor. The present procedure results in a specific activity of 6nmol/mg and may represent more than 30% pure receptor. This represents the highest purification reported for the glucocorticoid receptor.

We have noted that nearly 50% of the receptor complexes eluted from the second DNA–cellulose column do not bind to nuclei and do not bind to a third DNA–cellulose column (Table 3). These complexes may represent receptor subunits with no intrinsic DNA-binding function or receptor subunits with inactivated DNA-binding sites. Young *et al.* (1975) have shown that the DNA-binding site on the glucocorticoid–receptor complex can be inactivated by iodoacetamide or *N*-ethylmaleimide. If occupied by a glucocorticoid, the steroid-binding site is not affected by these agents. Thus it is possible to inactivate the DNA-binding site without inactivating the steroid-binding site of the receptor complex, and such inactivation may have occurred during purification of the receptor complex.

Ideally, one would like to use both the steroid- and DNA-binding sites for affinity chromatography. Failla *et al.* (1975) have made significant progress in partially purifying the glucocorticoid receptor on the basis of its affinity for corticosterone. The receptor complex remains in its unactivated state after that procedure and could be subsequently purified by the two-stage procedure described in the present paper.

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