Chromatographic Separation on Phosphocellulose of Activated and Nonactivated Forms of Steroid–Receptor Complex. Purification of the Activated Complex†

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ABSTRACT: Steroid–receptor complexes formed at low temperature and ionic strength are unable to bind to target cell nuclei. After a temporary exposure to high ionic strength and/or temperature they become activated (i.e., able to bind to nuclei). However, there exists an equilibrium between activated and nonactivated complexes; thus, mixtures of both populations are obtained. In this paper it is shown that activated [^{3}H]triamcinolone acetonide–rat liver receptor complexes bind strongly to phosphocellulose, whereas nonactivated complexes do not. Thus, it is chromatographically possible to isolate these two populations of complexes. The experimental conditions of the separation have been established. The most important feature is that upon prolonged exposure to phosphocellulose, nonactivated complexes become progressively activated. The separation on phosphocellulose has at least three potential applications. A first application is the possibility of measuring the concentration of activated complexes in incubates. However, when activated complexes were titrated with rat liver nuclei in excess or assayed through binding to phosphocellulose, slightly different results were obtained. This discrepancy was due on one hand to the difficulty of obtaining binding of all the activated complexes and on the other hand to the secondary activation of some of the complexes during their exposure to phosphocellulose. A second application was the possibility of obtaining a homogeneous population of activated complexes. This was actually achieved, since the complexes eluted from phosphocellulose were demonstrated to be 90–100% activated. The use of such homogeneous preparations simplifies considerably studies on binding of steroid–receptor complexes to nuclear acceptors (nuclei, chromatin, DNA). A third application is the use of phosphocellulose for the purification of receptor. Cytosol containing nonactivated complexes was filtered through phosphocellulose; the complexes present in the breakthrough of the column were then activated and bound to phosphocellulose in a second chromatography. Advantage was also taken of the "amphoteric" behavior of the receptor that binds to both anionic (phosphocellulose) and cationic (diethylaminoethylcellulose) resins. Purification (940-fold) with 24% yield could be obtained in preparations taking less than 2 days. The partially purified receptor was a heavy aggregate (> 12 S) that could be dissociated into 4S subunits by exposure to 0.3 M KCl. It has kept its property of interacting with nuclear acceptor. Preliminary experiments have shown that this technique could be of general application for steroid hormone receptors: activation enhanced binding to phosphocellulose of progesterone, aldosterone, and estradiol receptors.

After it has bound steroid, receptor protein acquires a high affinity for nuclei (Brecher et al., 1970; DeSombre et al., 1972; Gschwendt and Hamilton, 1972; Jensen et al., 1972; Buller et al., 1975; Kalimi et al., 1975). This process, called activation, is exceedingly slow at low temperature and ionic strength (Milgrom et al., 1973). Thus, it is possible to obtain incubates with a majority of nonactivated complexes (if these have been kept at low ionic strength and temperature) and also incubates containing mixtures of activated and nonactivated complexes (if the complexes have been temporarily submitted to high ionic strength or temperature). In order to study the process of activation and also the binding of activated complexes to acceptors (nuclei, chromatin, DNA), it could be of great importance on the one hand to separate activated from nonactivated complexes and on the other hand to quantitate the proportion of activated complexes in complex mixtures. Activation may also be used as a tool to purify receptor–steroid complexes.

We have previously shown (Milgrom et al., 1973) that upon activation steroid–receptor complexes acquire a high affinity not only for nuclei but also for various polyanions including, for instance, carboxymethyl-Sephadex and sulfopropyl-Sephadex. It thus appeared possible to use such anionic gels to chromatographically separate activated from nonactivated complexes. A preliminary work having shown that the best separation was obtained with phosphocellulose, we have used this gel in all the following experiments. A similar observation has recently been reported by Colman and Feigelson (1975).

Material and Methods

Steroids. [1,2,4-^{3}H]Triamcinolone acetonide[1] (4 or 16 Ci/mmol) was obtained from Schwarz/Mann (Orangeburg, N.Y.). Its purity was checked by chromatography on thin-layer silica gel in the benzene/acetone (4:1) system. Nonradioactive triamcinolone acetonide was from Sigma Chemical Co. (St Louis Mo.).

Buffers. Tris-HCl[2] 0.01 M, pH 7.4, β-mercaptoethanol 1 mM buffer was used (Tris buffer). In most experiments EDTA 1.5 mM was added (Tris–EDTA buffer). In experiments on

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[1] Triamcinolone: 9α-fluoro-11β,16α,17,21-tetrahydroxypregna-1,4-diene-3,20-dione.

[2] Abbreviations used are: DEAE, diethylaminoethyl; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; EDTA, (ethylenedinitrilo)tetraacetic acid.

nuclei, either sucrose 0.25 M (Tris–sucrose buffer), sucrose 0.25 M and EDTA 1.5 mM (Tris–EDTA–sucrose buffer), or sucrose 0.25 M Mg^{2+} 3 mM (Tris–sucrose–Mg buffer) was added to the Tris buffer. Potassium phosphate, pH 7.4 (potassium buffer), was used at various molarities (0.005–0.5 M).

Phosphocellulose (P11 Whatman Biochemicals Ltd) was treated before use as described by Burgess (1969). Hydroxylapatite (Bio-Rad Laboratories) was suspended in the buffer and used without further treatment.

Animals. Male Wistar rats (250 g) were adrenalectomized 5–10 days before use. They were killed by decapitation. The absence of adrenal glands was checked, and liver was excised and perfused with cold saline. Periodically, samples of blood were taken to assay corticosterone by competitive protein binding in order to check the absence of adrenal secretion.

Preparation of cytosol and incubation with hormone were performed at 0 °C. The tissue was homogenized (0.66 ml of Tris–EDTA buffer/g wet weight) with a Teflon–glass Potter Elvehjem apparatus. The homogenate was centrifuged for 90 min at 105 000*g*. The steroid dissolved in benzene was introduced into a Packard glass counting vial, evaporated under an air stream, and the supernatant (cytosol) of the 105 000*g* centrifugation was added. Usually a concentration of 20 nM [^{3}H]triamcinolone acetonide was used. This concentration saturates the receptor and equilibrium is attained in less than 120 min of incubation at 0 °C. A parallel incubation in the presence of 100 μM unlabeled triamcinolone acetonide was performed. It was used for the estimation of the nonspecific nonsaturable binding (Milgrom et al., 1973).

Preparation of Nuclei. Nuclei were prepared by the method of Chauveau (1956) as previously described (Milgrom et al., 1973). Mg^{2+}, which is present in the buffer used in this technique, decreases the binding of the receptor to nuclei (Atger and Milgrom, unpublished observations). For this reason the nuclei were washed twice in Tris–EDTA buffer before their use for the titration of the activated form of glucocorticoid–receptor complexes.

Incubation of Cytosol with Nuclei. Cytosol was incubated with nuclei (DNA concentration and volume of incubation are given for each experiment) for 30 min at 0 °C. This time has been found to be sufficient to attain equilibrium at the high concentration of nuclei used here. The pellet obtained by a 3000*g*, 15 min centrifugation was washed twice with 6 ml of Tris–EDTA buffer. It was counted for radioactivity. A correction was made for the nonspecific, nonsaturable binding using the cytosol incubated with excess unlabeled hormone. The nonspecific binding ranged from 1 to 5% of total binding in these experimental conditions.

Assay of Steroid–Receptor Complexes in the Cytosol or in the Soluble Fraction Obtained after Incubation with the Nuclei. Aliquots (50–200 μl of the solution were chromatographed on small columns of hydroxylapatite and washed with 50 ml of buffer. The gel was counted for radioactivity. This technique was adapted from the assay described by Erdos et al. (1970) for estrogen receptors. It was verified that steroid did not dissociate from receptor during the washing procedure. It was also shown that the assay was linear and that nonreceptor proteins of the cytosol did not interfere.

Counting of radioactivity and protein and DNA assays were performed as previously described (Milgrom et al., 1973).

Results

Affinity for Phosphocellulose of Activated and Nonactivated [^{3}H]Triamcinolone Acetonide–Receptor Complexes. Cytosol was incubated with [^{3}H]triamcinolone acetonide, activated by heating (15 min at 25 °C), and chromatographed on phosphocellulose. After washing with a buffer containing 0.15 M KCl, buffers containing 0.4 and 2 M KCl were applied successively. It may be seen that a peak of radioactivity was eluted at 0.4 M KCl (Figure 1B, line a). It had the characteristics of receptor–steroid complexes, since it was saturable (displaced by excess unlabeled hormone) (Figure 1B, line b) and abolished by incubation with *p*-hydroxymercuribenzoate (not shown). A similar result was observed when activation was obtained by a transitory rise in ionic strength (Figure 1C).

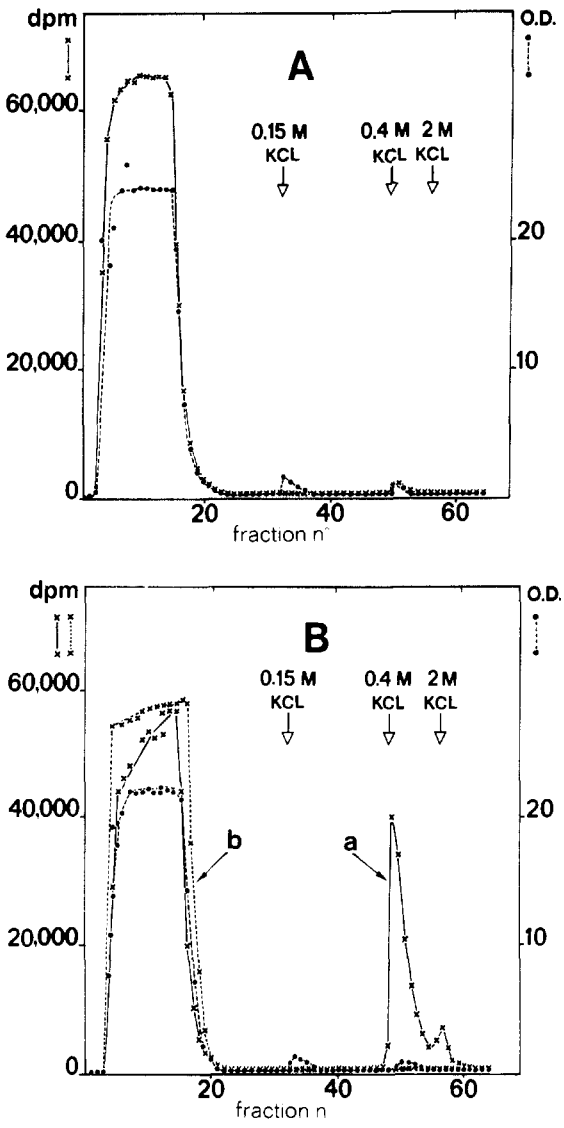
On the contrary, when the complexes have been kept at low temperature and ionic strength and thus were nonactivated, no radioactivity was retained on phosphocellulose (Figure 1A).

Optimal Conditions for the Retention of Activated Complexes on Phosphocellulose. If chromatography on phosphocellulose was to be used for the separation of nonactivated and activated complexes it had to be checked that the latter were firmly bound. Activated complexes did not "leak" from the gel even when 96 column volumes of washing buffer were applied. Five column volumes were sufficient to wash out more than 97% of non-receptor-bound hormone.

The capacity of the resin was high, since no limitation of binding was observed when up to 4.2 pmol of complexes werefixed and 40 mg of protein were chromatographed on 1 ml of gel.

The effect of divalent cations was of interest, since studies on binding of receptor–steroid complexes to nuclei or chromatin are often performed in their presence. It was found that Mg^{2+}, Ca^{2+}, and EDTA at 1–5 mM concentrations only very slightly enhanced the binding of activated complexes to phosphocellulose.

Activation of Glucocorticoid–Receptor Complexes upon Prolonged Exposure to Phosphocellulose. Binding to Nuclei of Complexes Eluted from the Resin. The experiments described above have shown that activated glucocorticoid–receptor complexes are firmly bound to phosphocellulose. However, the question of how fast this interaction took place and if a rapid filtration through the gel was sufficient to obtain maximal binding could be raised. In order to check this, cytosol containing activated receptor–steroid complexes was introduced into the gel and kept in contact with phosphocellulose for various periods of time ranging from a few seconds to 120 min. As shown in Figure 2 line a, it was observed that the concentration of complexes retained on the gel increased quickly during the first 20 min and then slowly until about 75% of the steroid–receptor complexes were bound. This result was difficult to reconcile with the previously demonstrated fact that under these precise experimental conditions only 50–60% of the complexes contained in the cytosol were activated. The explanation of this discrepancy was obtained when cytosol containing nonactivated complexes was exposed to a prolonged contact with phosphocellulose (Figure 2 line b). It was observed that the amount of receptor–steroid complexes raised progressively and after 2 h attained nearly the level of that obtained with activated steroid–receptor complexes. Two explanations of these experimental data were possible: either complexes under both activated and nonactivated forms were able to bind to phosphocellulose but with different rates of association or, initially nonactivated complexes were progressively activated during their contact with phosphocellulose. In order to discriminate between these hypotheses, it was necessary to recover the complexes bound to phosphocellulose after a prolonged exposure to the gel and to check by titration



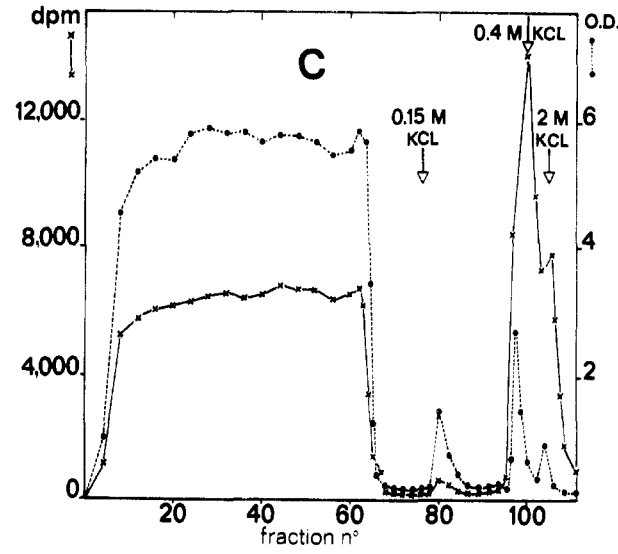


FIGURE 1: Binding to phosphocellulose of [^{3}H]triamcinolone acetonide–receptor complexes. (A) Nonactivated complexes; (B) complexes activated by heating; (a) cytosol incubated with [^{3}H]triamcinolone acetonide, 20 nM, (b) cytosol incubated with [^{3}H]triamcinolone acetonide 20 nM and unlabeled triamcinolone acetonide 10 μM; (C) complexes activated by ionic strength. Cytosol was incubated with either 20 nM [^{3}H]trimacinolone acetonide or 20 nM [^{3}H]triamcinolone acetonide and 10 μM unlabeled triamcinolone acetonide (120 min at 0 °C). Eight-milliliter aliquots were used for each experiment. In experiment A, the incubate was chromatographed without any further treatment. In experiment B, cytosol was heated (15 min at 25 °C) and cooled (15 min at 0 °C) before chromatography. In experiment C, Tris–EDTA buffer containing 3 M KCl was added in order to obtain a concentration of 0.3 M KCl. After 120 min exposure to this ionic strength (known to activate the complexes (Milgrom et al., 1973), the incubate was diluted fivefold with Tris–EDTA buffer in order to bring back the ionic strength to 0.06 M KCl. It was checked that cytosol diluted [illegible]ld in the same conditions, but not subjected to a rise in ionic str[illegible] gave the same result as in the Figure 1A. Chromatography was performed on a column of phosphocellulose (2 ml, 0.8-cm diameter). After application of the sample, the gel was washed with 12 ml of Tris–EDTA buffer, 12 ml of Tris–EDTA–KCl, 0.15 M, buffer, and eluted successively with 6 ml of Tris–EDTA–KCl, 0.4 M, and 6 ml of Tris–EDTA–KCl, 2 M, buffers. Fractions (0.75 ml) were collected of which 0.1 ml was counted for radioactivity.

with nuclei in excess what proportion of them was activated. When such an experiment was performed it was found that 93% of the eluted complexes could be bound by nuclei and, thus, may be considered as activated (Figure 3). The missing 7% could be accounted for by losses during washing of the nuclear pellet, since no remaining complexes were detected in the soluble fraction after incubation with nuclei. Over 90% of the eluted complexes could also be rebound to phosphocellulose during a rapid chromatography.

This experiment showed that prolonged exposure to phosphocellulose results in progressive activation of initially nonactivated complexes and also that the totality of complexes eluted from phosphocellulose are under the activated state.

Comparison of the Binding of Glucocorticoid Receptor Complexes to Phosphocellulose and to Rat Liver Nuclei. The differential binding of activated and nonactivated glucocorticoid–receptor complexes to phosphocellulose could be used to achieve various aims. The first application was the possibility of isolating a homogeneous population of activated complexes from incubates containing mixtures of activated and nonactivated complexes. As shown in Figure 3, this can actually be achieved.

A second possible application was the use of small columns of phosphocellulose in order to measure the concentration of activated complexes. The results of such measurements were compared to a titration of activated complexes by rat liver nuclei in excess (Figure 4). In heat-activated cytosol, the concentration of activated complexes measured by titration with nuclei was 244 fmol/incubation; the assay using phosphocellulose binding gave a value of only 203 fmol. On the contrary, titration of nonactivated cytosol by nuclei gave a value of 45 fmol, whereas phosphocellulose binding gave 68 fmol. Thus, in a rapid chromatography not all the activated complexes are retained by phosphocellulose and, conversely, some initially nonactivated complexes are activated during the chromatography. These experiments that repeatedly gave the same results show that according to the conditions, an assay using phosphocellulose would lead to an overestimation or an underestimation of the concentration of activated complexes. This assay can thus be used only when comparative measurements should be made but not when accurate values of the concentration of activated complexes are needed. In the latter case titration with nuclei should be preferred (Atger and Milgrom, 1976).

Purification of [^{3}H]Triamcinolone Acetonide–Receptor Complexes. As the receptor is a very labile protein, it was necessary to set up a purification procedure where the various steps could be performed as rapidly as possible. In order to

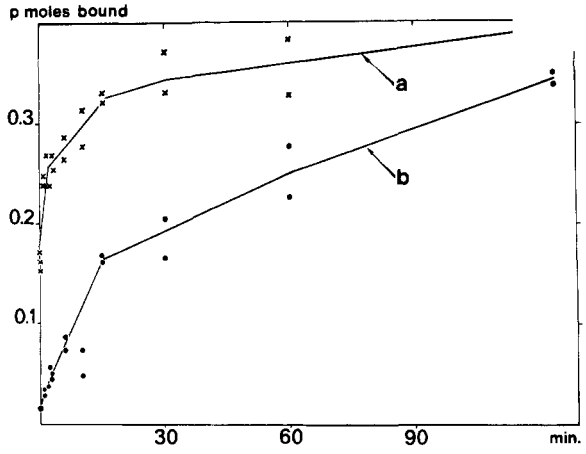


FIGURE 2: Activation of glucocorticoid-receptor complexes during prolonged exposure to phosphocellulose at 0 °C. (a) Initially activated complexes; (b) initially nonactivated complexes. Cytosol was incubated 2 h at 0 °C with 20 nM [^{3}H]triamcinolone acetonide. One part was activated (15 min at 25 °C) and cooled (15 min at 0 °C), another part was kept at 0 °C. Fifty-microliter aliquots of activated and nonactivated cytosol were loaded onto small phosphocellulose columns (2 ml, diameter 1.3 cm). After the penetration of the incubate into the gel, 200 μl of buffer were deposed and allowed to penetrate into the gel. The chromatography was then stopped for various periods of time. It was resumed by a wash with 25 ml of buffer. The gel was dried and counted for radioactivity (Erdos et al., 1970). Correction was made for the nonspecific, nonsaturable binding by parallel incubations in the presence of 10 μM unlabeled triamcinolone acetonide.

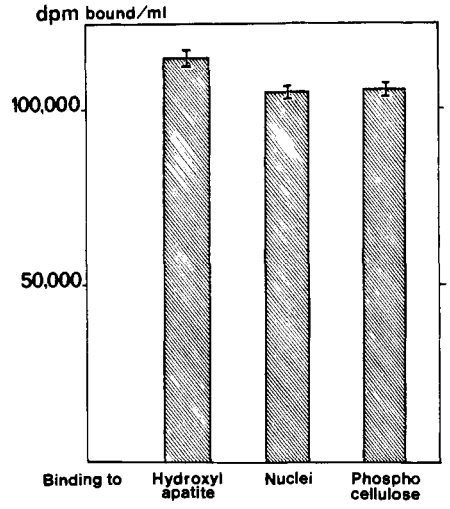


FIGURE 3: Binding to nuclei of glucocorticoid-receptor complexes eluted from phosphocellulose. Fifteen milliliters of rat liver cytosol was incubated 2 h at 0 °C with 20 nM [^{3}H]triamcinolone acetonide, activated (15 min at 25 °C) and cooled (15 min at 0 °C). The incubate was layered on a column of phosphocellulose (2.5 × 10 cm), and allowed to enter the gel. Five milliliters of Tris-EDTA buffer was then introduced into the gel and the chromatography was stopped. Three hours later the gel was washed with 60 ml of Tris-EDTA and 100 ml of Tris-EDTA-KCl, 0.15 M, buffer. Elution was performed with Tris-EDTA- KCl, 0.4 M, buffer. The fractions containing the receptor were pooled and desalted on Sephadex G-25. Aliquots were taken for the determination of total concentration of complexes (by binding to hydroxylapatite), of concentration of activated complexes (by binding to nuclei in excess) (2 mg of DNA/ml), and of concentration of complexes binding to small phosphocellulose columns (2 ml) during a rapid chromatography (see Figure 2). Results are given as the mean ± standard error of the mean (six determinations).

achieve this, preliminary experiments were undertaken where the behavior of steroid-receptor complexes, when chromatographed through DEAE-cellulose, hydroxylapatite, and phosphocellulose, was examined. From these experiments, washing and elution conditions were deduced that were "blindly" applied during the actual purification. No fractions were collected and only the eluates where the receptor was known to be present were kept. The total procedure could then be achieved in less than 2 days.

(*1*) *Purification Procedure* (*Table I*). The cytosol containing nonactivated complexes was filtered through a column of phosphocellulose. The filtrate was directly applied to a column of DEAE-cellulose. After washing with 0.06 M KCl, the receptor was eluted with 0.2 M KCl.

Precipitation with ammonium sulfate (33% saturation) was followed by resuspension in 5 mM phosphate buffer and direct application to hydroxylapatite (ammonium sulfate at 5% saturation does not interfere with hydroxylapatite chromatography). After washing with 0.125 M phosphate, the receptor was eluted with 0.3 M phosphate.

Dialysis was then performed over 10 h against Tris-EDTA buffer and the receptor was applied to a second phosphocellulose column. After washing with 0.2 M KCl, it was eluted with 0.4 M KCl. The receptor solution was desalted on Sephadex G-25 and lyophilized. The receptor was then purified 940-fold, with a yield of 23.7%. Its specific activity was 373 pmol of bound hormone/mg of protein. In various preparations very similar results were obtained. However, even if the specific activity of the initial cytosol varied between 0.25 and 0.75 pmol of receptor-bound hormone/mg of protein, the specific activity at the end of the purification was always of the same order as that reported in Table I.

(*2*) *Characteristics of the Partially Purified Receptor.* When the partially purified receptor was centrifuged in low ionic strength buffer it sedimented (Figure 5A) as a very large macromolecule (over 12 S), since all the radioactivity was found at the bottom of the tube. In the presence of 0.3 M KCl, 70% of the radioactivity sedimented as a sharp 4S peak, while some aggregates were still present on the bottom of the tube.

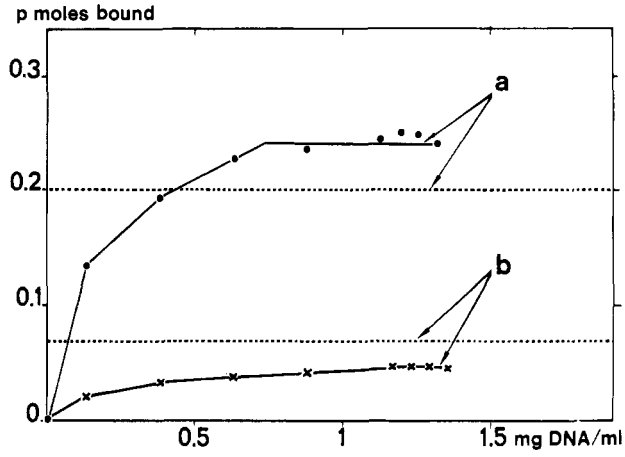


FIGURE 4: Compared binding of glucocorticoid-receptor complexes to nuclei and phosphocellulose. (a) Activated complexes; (b) nonactivated complexes (full line, binding to nuclei; dashed line, binding to phosphocellulose). Rat liver cytosol was prepared in Tris-EDTA-sucrose 0.25 M, buffer. It was incubated 2 h at 0 °C with 20 nM [^{3}H]triamcinolone acetonide. One part was activated (15 min at 25 °C) and cooled (15 min at 0 °C), another part was kept at 0 °C. Aliquots (50 μl were either rapidly chromatographed on small phosphocellulose columns (see Figure 2) or incubated 30 min at 0 °C with increasing concentrations of nuclei (total volume of incubation 1.1 ml) in Tris-EDTA-sucrose, 0.25 M buffer Nuclei were washed twice with 6 ml of buffer and the pellet was counted Corrections were made for the nonsaturable, nonspecific binding (see Figure 2).

TABLE I: Purification of [^{3}H]Triamcinolone Acetonide-Receptor Complex.[a]

Column	Vol (ml)	Protein[b] (mg)	Receptor (pmol of bound hormone)	Sp act (pmol/mg of protein)	(Yield %)	Purification (fold)
Cytosol	80	2774	1101	0.397		
First phosphocellulose	155.8	2370	1008	0.425	91.6	1.1
DEAE-cellulose	81	357	743	2.08	67.5	5.2
Ammonium sulfate	16	108	707	6.55	64.2	16.5
Hydroxylapatite	42	12.7	439	34.6	39.9	87.2
Second phosphocellulose	17	0.70	261	373	23.7	940

[a] 14 rats were used (190 g of liver). Cytosol was incubated (90 min at 0 °C) with 50 nM [^{3}H]triamcinolone acetonide (4 Ci/mmol). It was filtered through a phosphocellulose column (5 × 5 cm, flow 2.5 ml/min). The filtrate was applied to a DEAE-cellulose column (5 × 2.5 cm, flow 6 ml/min). The resin was washed with 60 ml of Tris-EDTA buffer and 140 ml of Tris-EDTA-KCl 0.06 M. The receptor was eluted with Tris-EDTA-KCl 0.2 M. Ammonium sulfate precipitation (33% saturation) was performed over 1 h. The precipitate was resuspended in 5 mM phosphate buffer, the nondissolved particles were eliminated by centrifugation (6000g for 15 min), and the supernatant was applied to a hydroxylapatite column (1.5 × 5.7 cm, flow 1.5 ml/min). The gel was washed with 10 ml of 0.05 M phosphate and 25 ml of 0.125 M phosphate. The receptor was eluted with 0.25 M phosphate. It was dialyzed 10 h against 2 l. of Tris-EDTA-buffer containing 1 nM [^{3}H]triamcinolone acetonide and applied to a phosphocellulose column (0.8 × 4 cm, flow 0.5 ml/min). The column was washed successively with Tris-EDTA buffer (10 ml), Tris-EDTA-KCl, 0.15 M (10 ml), Tris-EDTA-KCl, 0.2 M (4 ml), and eluted with Tris-EDTA-KCl, 0.4 M. The receptor was desalted on Sephadex G-25 and lyophilized. Temperature was strictly controlled at 0-3 °C during all procedures. [b] Optical density at 280 nm ($E_{1\ mg/ml}^{1\ cm} = 1$).

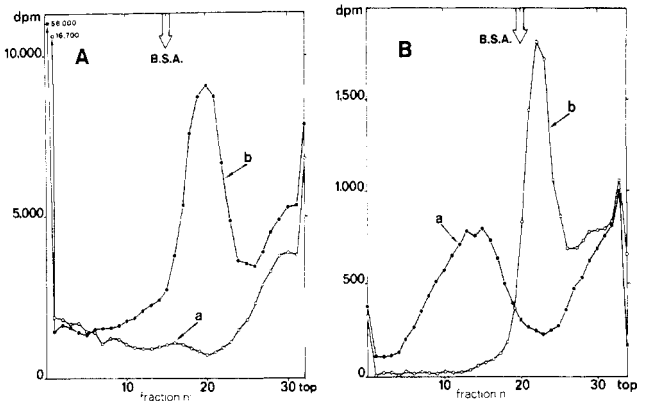


FIGURE 5: Glycerol gradient sedimentation of [^{3}H]triamcinolone acetonide-receptor complexes purified (A) or in crude cytosol (B). (a) In low ionic strength; (b) in 0.3 M KCl. (A) Partially purified and lyophilized receptor-steroid complexes (see Table I) were redissolved in Tris-EDTA buffer. The solution was clarified by centrifuging 30 min at 6000g. Aliquots (0.2 ml) (containing 47.9 pmol of steroid-receptor complex) were then layered onto 10-30% glycerol gradients in either Tris-EDTA or Tris-EDTA-KCl, 0.3 M, buffers. Centrifugation was at 64 000 rpm for 11 h in a SW 65 rotor. Specific activity of [^{3}H]triamcinolone acetonide was 4 Ci/mmol. (B) Liver cytosol prepared in Tris-EDTA buffer was incubated 2 h at 0 °C with 2 nM [^{3}H]triamcinolone acetonide (specific activity 16 Ci/mmol). Aliquots (0.2 ml) were then layered on 10-30% glycerol gradients in either Tris-EDTA or Tris-EDTA-KCl, 0.3 M, buffer. Centrifugation was at 45 000 rpm for 18 h in a SW 50.1 rotor.

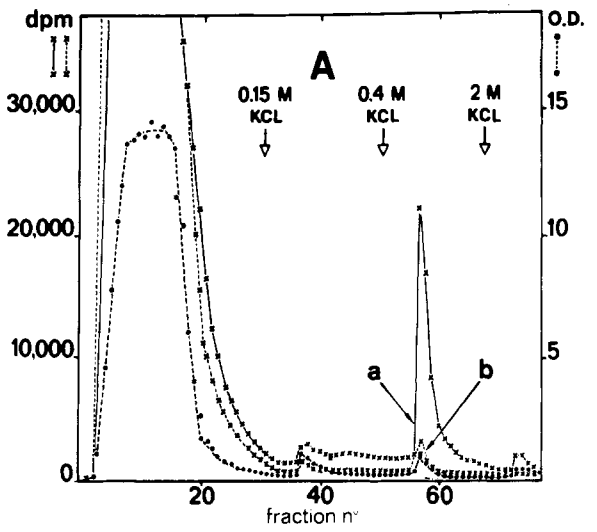
For comparison, a similar experiment performed with the receptor present in the crude cytosol (Figure 5B) showed it to sediment in low ionic strength as a wide peak at 7S, whereas in 0.3 M KCl a sharp 4S peak was observed. Thus, at high ionic strength purified and nonpurified receptor behaved similarly, whereas at low ionic strength purified receptor had a more marked tendency to aggregation than did the nonpurified receptor.

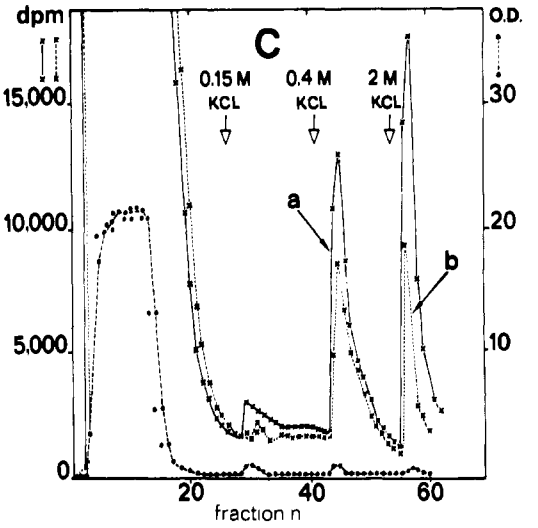
Chromatography on Sephadex G-200 confirmed that the partially purified receptor was a large aggregate (excluded from the gel) at low ionic strength and could be dissociated (included in the gel) at 0.3 M KCl (not shown). When titrated with nuclei, 95% of the purified [^{3}H]triamcinolone acetonide-receptor complexes were found to be under the activated form.

Binding to Phosphocellulose of Activated and Nonactivated Forms of Progesterone-, Aldosterone-, and Estradiol-Receptor Complexes (Figure 6). When activated and nonactivated forms of progesterone- (Figure 6A) and aldosterone- (Figure 6B) receptor complexes were chromatographed on phosphocellulose, a situation very similar to that observed with glucocorticoid receptor was seen. Only activated complexes were retained on phosphocellulose. In the case of the estrogen receptor (Figure 6C) a markedly smaller difference in affinity was observed, since the binding of the nonactivated complexes was about half that of activated complexes. It may be noticed that some slight variations in the ionic strength necessary to elute the complexes were also observed, aldosterone receptor being eluted at lower and estradiol receptor at higher ionic strength than glucocorticoid receptors.

Discussion

Activated steroid-receptor complexes are able to bind not only to nuclei, chromatin, or DNA, but also to synthetic polyanions, among them phosphocellulose. To explain this finding, we have proposed the hypothesis that activation consists of the stabilization by hormone of a conformation of receptor having a region of positive charges at its surface (Milgrom et al., 1973). At low temperature and ionic strength the rates of intertransformation of activated and nonactivated forms of receptor-steroid complexes are exceedingly slow. Thus, it is possible to keep complexes either in the nonactivated or activated state for long periods of time. The preparation of solutions containing only or mainly nonactivated complexes is easily achieved by incubating the cytosol with hormone at low temperature and ionic strength. On the contrary, preparation of solutions containing only (or mainly) activated complexes is impossible without chromatographic separation, since an equilibrium is obtained where 40% of the complexes are not activated even in the most favorable situation (Atger and Milgrom, 1976). Using phosphocellulose chromatography it is possible to obtain homogeneous populations of activated complexes. This simplifies the situation when interaction of





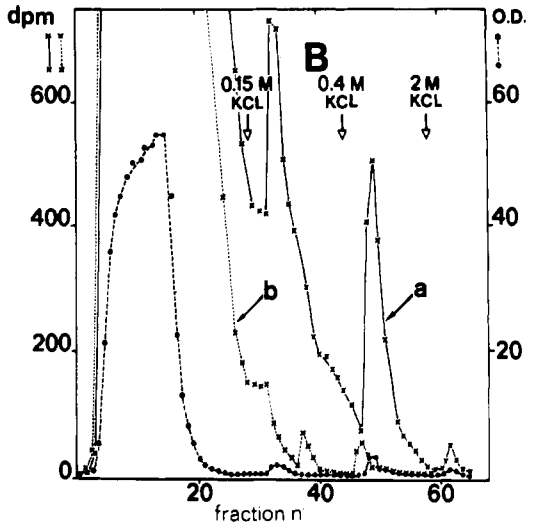


FIGURE 6: Binding to phosphocellulose of progesterone (A)-, aldosterone (B)-, and estradiol (C)-receptor complexes. (a) Activated steroid-receptor complexes; (b) nonactivated steroid-receptor complexes. (A) Three-hundred-gram female Hartley guinea pigs were ovariectomized and primed 1 week later with estradiol (5 μg in 0.5 ml of sesame oil during 3 days). Uteri were removed and homogenized (3.7 ml of Tris-EDTA buffer/g wet weight). Cytosol was prepared by a 105 000g, 90-min centrifugation and incubated 2 h at 0 °C with 20 nM [^{3}H]progesterone (specific activity 47 Ci/mmol). (B) Two hundred-gram male Wistar rats were adrenalectomized 6 days before the experiment. Kidneys were perfused with saline and homogenized (0.5 ml of Tris-EDTA buffer/g). Cytosol was obtained and incubated with 20 nM [^{3}H]aldosterone (46 Ci/mmol) as described above. (C) Calf uteri were obtained from a slaughterhouse and homogenized (1.4 ml of Tris-EDTA buffer/g). Cytosol was obtained and incubated with [^{3}H]estradiol (85 Ci/mmol) as described above. In all cases, complexes were either activated (15 min at 25 °C) and cooled (15 min at 0 °C) or kept at 0 °C. Eight milliliters of cytosol was then chromatographed at 0 °C through a phosphocellulose column (2 ml, diameter 0.8 cm). Washings were performed with 12 ml of Tris-EDTA and 12 ml of Tris-EDTA-KCl, 0.1 M buffers. The following buffers were applied successively for elution: 10 ml of Tris-EDTA-KCl, 0.4 M, and 6 ml of Tris-EDTA-KCl, 2 M. Fractions of 0.75 ml were collected. Optical density at 280 nm was measured (results were identical for heated and nonheated cytosols) and 100-μl fractions were counted for radioactivity.

steroid-receptor complexes with acceptors (nuclei, chromatin, DNA) is studied, since only the equilibrium between acceptor-bound and free activated complexes is observed. Without chromatographic separation a second equilibrium (between activated free complexes and nonactivated free complexes) is added.

The reason why prolonged contact of nonactivated complexes with phosphocellulose leads to activation is not clearly understood. One explanation is a displacement of the equilibrium between activated and nonactivated forms by the presence of the acceptor, which binds only the latter. However, the rate of activation is exceedingly slow at 0 °C and a displacement of the equilibrium at 0 °C would take days. This is also shown by the fact that the presence of nuclei at 0 °C does not shift the equilibrium (Atger and Milgrom, 1976). It thus appears likely that phosphocellulose exerts an effect through its charged phosphate groups similar to that of activation by temporary exposure to high ionic strength. This phenomenon does not allow the use of phosphocellulose binding for measurement of concentration of activated complexes. However, if our explanation of activation by phosphocellulose is correct and since the capacity of the gel for activated complexes is very high, it should be possible to synthesize a phosphocellulose with fewer phosphate moieties that could retain a sufficient binding capacity without provoking the activation of nonactivated complexes.

It is also possible to use the differential affinity of activated and nonactivated complexes for the purification of receptor. Cytosol containing nonactivated complexes is chromatographed on phosphocellulose-retaining proteins with affinity for this gel but not complexes. The latter are retained during a second chromatography that is performed after activation. Advantage was also taken of the "amphoteric" behavior of the receptor that binds to both DEAE-cellulose and phosphocellulose. If a molecular weight of approximately 100 000 (see Raspe 1971) is admitted for the receptor binding unit then a totally pure and totally active receptor preparation should have a specific activity of 10 000 pmol/mg of protein. In this respect, our preparation would be about 4% pure.

A better result could theoretically be obtained with the help of affinity chromatography (Failla et al., 1975; Kuhn et al., 1975; Sica et al., 1973; Truong et al., 1974). We have tried to purify the glucocorticoid receptor using the C_{20} acid derived from corticosterone by periodic acid oxidation. This compound was conjugated to Sepharose. However, when applying crude cytosol to the affinity gel a rapid saturation of the latter was observed. This may possibly have been due to the presence of at least two other glucocorticoid binding proteins in liver cy-

tosol (Koblinsky et al., 1972). All our attempts to fractionate the proteins in the absence of hormone (in order to use such partially purified preparations for affinity chromatography) failed. The glucocorticoid receptor seems to be specially labile in the absence of hormone. Thus, progress could be made either by synthesizing a more receptor-specific affinity gel or by finding a way of stabilizing nonhormone-bound receptor.

The preparation procedure described in this paper may probably be improved by further purification steps using other techniques based, for instance, on size properties (Litwack et al., 1973; Koblinsky et al., 1972). Gradient ultracentrifugation at low and high ionic strength could also be helpful. Such a preparation would, however, make it necessary to start with very large amounts of target tissue.

The sedimentation behavior of partially purified receptor is striking: at low ionic strength it sediments as a very heavy aggregate, markedly heavier than that observed in the crude cytosol. This aggregate is dissociated into 4S subunits by 0.3 M KCl. It is unknown if the aggregate is composed only of receptor molecules or if other protein molecules are included. Both hypotheses appear to be plausible. The activated receptor probably has surface regions of both positive and negative charge, as shown by its binding properties to various polyanions (Milgrom et al., 1973) and its electrofocusing and chromatographic behavior. Autoaggregation through ionic interactions is thus possible. Alternatively, the purification technique that has been used isolates, through both DEAE and phosphocellulose binding, proteins having an amphoteric behavior. Such proteins could form heterogeneous aggregates. The receptor–steroid complexes purified by this procedure have kept their capacity to bind to nuclear acceptor. The kinetics of this interaction have been studied (M. Atger and E. Milgrom, manuscript in preparation).

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