Characterization of Nonactivated and Activated Glucocorticoid-Receptor Complexes from Intact Rat Thymus Cells*

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In cells exposed to glucocorticoids at 37 °C activated glucocorticoid-receptor complexes (complexes with affinity for nuclei and DNA) are formed after nonactivated complexes. Activation thus appears to be an obligatory physiological process. To investigate this process we have characterized cytoplasmic complexes formed in rat thymocytes at 0 and 37 °C. Complexes in cytosols stabilized with molybdate were analyzed by sucrose gradient centrifugation and by chromatography on DNA-cellulose, DEAE-cellulose, and agarose gels. Two major complexes were observed: the nonactivated complex, eluted from DEAE at ~200 mM KCl, was formed at 0 and 37 °C, gave $s_{20,w}=9.2$ S, Stokes radius = 8.3 nm, and calculated $M_\text{r}=330,000$; the activated complex, eluted from DEAE at ~50 mM KCl, appeared only at 37 °C, gave $s_{20,w}=4.8$ S, Stokes radius = 5.0 nm, and $M_\text{r}=100,000$. A third, minor complex, probably mero-receptor, which appeared mainly at 37 °C, bound to neither DNA nor DEAE, and gave $s_{20,w}=2.9$ S, Stokes radius = 2.3 nm, and $M_\text{r}=27,000$. With three small complexes of DNA-cellulose, DEAE-cellulose and hydroxylapatite, the three complexes can be separated in 5–10 min. By this method we have examined the stability of complexes under our conditions. We conclude that in intact thymus cells glucocorticoid-receptor complexes occur principally in two forms, nonactivated and activated, and that activation is accompanied by a large reduction in size. The origin of the mero-receptor complex remains uncertain.

Primary actions of glucocorticoids and other steroid hormones are thought to be mediated by specific cytoplasmic receptors via a two-step mechanism. Upon entry into a target cell, the steroid binds to the receptor forming a cytoplasmic complex; this complex is then translocated to the nucleus. From studies in cell-free systems translocation appears to require a process called "activation," through which cytoplasmic complexes acquire affinity for nuclei (1–4). Activated receptor complexes can be separated from nonactivated complexes by their enhanced affinity not only for nuclei but for DNA (5, 6) and ATP-Sepharose (7), and by their altered mobility on phosphocellulose (8), DEAE-Sephadex (9), and DEAE-cellulose (10).

Most studies on activation have been performed under cell-free conditions, starting with cytosols incubated at low temperature (0–4 °C) with radiolabeled steroid to produce nonactivated complexes. Activation of these complexes can be achieved by various methods including brief warming (usually to 15–25 °C) (4), exposure to high ionic strength (5, 11), gel filtration, and dilution (12, 19).

Recently, we and others have shown that activation occurs in intact cells exposed to steroid at 37 °C (14, 15). Furthermore, a glucocorticoid-resistant mutant of CEM cells has been shown to be activation defective (16). Since it therefore is likely that activation is an obligatory step in the response of normal cells to glucocorticoids, our goal in the present study has been to characterize the nonactivated and activated cytoplasmic glucocorticoid-receptor complexes and their transformations in intact thymus cells.

Physicochemical characterization of glucocorticoid-receptor complexes is difficult because of their liability and the lengthy procedures required for their analysis. Methods used to activate under cell-free conditions themselves can result in dissociation and proteolytic degradation of complexes (17–20), giving rise to multiple forms. Hence, it is difficult to distinguish complexes present in intact cells from those which appear after cells are broken.

Recent studies by Sherman and co-workers (17–19), based on the earlier work of Pratt and co-workers (21–23), have demonstrated the remarkable ability of molybdate to stabilize glucocorticoid-receptor complexes during long chromatographic procedures, preventing both steroid-receptor dissociation and proteolytic degradation. Molybdate, moreover, inhibits activation, and is therefore useful for preventing spontaneous activation during gel filtration and other procedures (24, 25).

We have applied these findings to obtain stabilized cytosols from cells exposed to glucocorticoids by breaking the cells in medium containing molybdate. We have then characterized cytosolic complexes by DNA- and DEAE-cellulose chromatography, agarose gel filtration, and sucrose gradient centrifugation, all conducted in the presence of molybdate. Our results indicate that the transformation of nonactivated to activated complexes in thymus cells is accompanied by a substantial decrease in apparent molecular weight. In addition to these complexes, mero-receptor, defined by Sherman et al. (26) as the smallest unit or fragment of receptor containing the steroid-binding site, may also be present in these cells, but our evidence on this point remains tentative. To assay these three complexes rapidly and reliably, we have developed a mini-column procedure which separates the complexes in a few minutes, and have applied this method to study the stability of activated and nonactivated complexes under cell-free conditions.
EXPERIMENTAL PROCEDURES

Materials

[3H]TA (37 Ci/mmole) and [6,7-3H]dexamethasone (50 Ci/mmole) were purchased from New England Nuclear. Dexamethasone was purchased from Steraloids, Wilton, NH, and TA from Sigma. Calf thymus DNA was obtained from P-L Biochemicals, DE-52 DEAE-cellulose from Whatman, and agarose (A-50m, 100-200 mesh) from Bio-Rad. The calibration standards bovine pancreas chymotrypsinogen A, hen egg ovalbumin, bovine serum albumin, rabbit muscle aldolase, beef liver catalase, and ferritin, were obtained from Boehringer Mannheim. Equine skeletal myoglobin and sweet potato β-amylase were obtained from Sigma.

Methods

General Procedures—Thymuses were obtained from 100-200 g male Sprague-Dawley rats (Charles River CD strain; Wilmington, MA) that had been bilaterally adrenalectomized at least four days prior to sacrifice and maintained on 0.9% NaCl. Thymus cell suspensions were prepared in Krebs-Ringer bicarbonate buffer supplemented with 10 mM glucose equilibrated with 95% oxygen:5% CO2 as described previously (27).

Concentrated solutions (50-100 mM) of [3H]dexamethasone and [3H]TA were dissolved in Krebs-Ringer bicarbonate buffer supplemented with 10 mM glucose, after removing the benzenc:ethanol solvent by evaporation. Suspensions of thymus cells at a cytoticrit (milliliters of packed cells/milliliters of cell suspension) of 0.3 to 0.4 were added to the steroid solutions to give final concentrations of 500 or 500 μM for analysis of receptor complexes binding to DNA-cellulose.

Cell-free Activation—Cell-free activation was accomplished by incubating the cytosols at 25°C for 15 min. This time period gave maximum activation of receptor complexes. DNA-cellulose, DEAE-cellulose and hydroxylapatite columns were prepared separately at 0°C for 15 min. The cell suspensions and steroid solutions were first chilled at 0°C 0°C for 2 h to obtain nonactivated complexes. For the 0°C co-oxidations the cell suspensions and steroid solutions were first chilled at 0°C by adding 1 volume of cell suspension to 5 volumes of 1.5 mM TDM buffer. Free steroid and unbound glucocorticoid-receptor complexes were removed from the column with a 25-ml wash buffer, which consisted of 10 mM TES, 50 mM KCl, 10% glycerol, 4 mM EDTA, 20 mM molybdate and 0.02% sodium azide, pH 7.5 at 3°C. After molybdate was omitted from the buffer, additional KCl (30 mM) was included to maintain the same conductivity. Cytosols was combined with glycerol (final concentration, 15%) and 0.5 ml layered under the eluting buffer. The column was run at a flow rate of 10 ml/cm2/h. About 12 h elapsed from the time the cytosol was applied to the column until free steroid was eluted. The elution positions (Vs) of the various peaks of radioactivity were compared to that (V0) of blue dextran 2000, which was applied to the column before the cytosol. Radiographic Gel Filtration—Gel filtration studies were performed using a 1.5 × 68 cm column of Bio-Gel HTP from Bio-Rad. The calibration standards bovine pancreas chymotrypsinogen A, hen egg ovalbumin, bovine serum albumin, rabbit muscle aldolase, beef liver catalase, and ferritin, were obtained from Boehringer Mannheim. Equine skeletal myoglobin and sweet potato β-amylase were obtained from Sigma.

Calculation of Apparent Molecular Weights—Apparent molecular weights and frictional ratios due to shape, were calculated from the Stokes radius and sedimentation coefficient as described by Siegel and Monty (39), using values of 0.74 ml/g for the partial specific volume (40) and assuming 0.2 g of solvent/g of protein for the solvent density factor (41). Axial ratios for ellipsoidal molecules were derived from the values listed by Schachman (42).

Mini-Column Chromatographic Procedure—This rapid chromatographic procedure employs DNA- and DEAE-cellulose chromatography for separation of activated, nonactivated, and receptor-bound complexes. DNA-cellulose, DEAE-cellulose and hydroxyapatite columns of 0.3, 0.3, and 0.2 ml, respectively, were prepared separately in 1-ml tuberculin syringes. The three columns were then connected one to the other (DNA-cellulose column on top, DEAE-cellulose in the middle, and hydroxyapatite on the bottom) through 3-way stopcocks and equilibrium with TDM buffer. The columns were well in excess of those required to retain the complexes with the volumes of cytosol used. A 0.1 ml cytosol sample obtained from cells incubated with [3H]TA or [3H]dexamethasone was applied to the DNA-cellulose column. To determine nonspecific binding, a similar cytosol sample from cells exposed to 1 μM unlabeled dexamethasone, was applied to a second mini-column. After the samples had settled into the bed, 8 ml of TDM buffer to recover unbound complexes. DNA-bound complexes were eluted with 400 ml of 0.3 M NaCl. After incubation with the time cytosols were applied to the column until unbound complexes were recovered by the wash procedure. An additional 20–30 min was required for elution of DNA-bound complexes. 

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buffer in a 10-ml syringe inserted into the top column was passed through the three columns by applying a steady manual pressure to the syringe plunger. The buffer was followed by air to remove most of the liquid. This procedure leaves the activated, nonactivated, and mero-receptor complexes respectively, on the DNA-cellulose, DEAE-cellulose and hydroxylapatite columns. Elution generally took 6-8 min, but the rate at which buffer was run through the columns had little effect on the results obtained; we have observed essentially identical results with passage times ranging from 4 to 15 min. The contents of the DNA-cellulose, DEAE-cellulose, and hydroxylapatite column beds were then placed directly in vials with 4 ml of scintillation fluid and counted to give the amount of complex associated with each column bed. A sample of the eluate was also counted to determine the amount of free (unbound) steroid.

Results are expressed as percentage of total receptor-bound cpm retained on the three columns. Receptor-bound cpm were derived separately for each of the three column beds by subtracting cpm bound in presence of unlabeled steroid (nonsaturable binding), from that bound in the absence of unlabeled steroid. These values were then added to give total receptor-bound cpm. Nonsaturable binding amounted in all cases to less than 10% of the receptor-bound cpm for each column bed. Results of replicate analyses for a single sample by this procedure are very consistent, the range of receptor-bound cpm retained on any one column generally being less than 3% of the sum of cpm on all three columns. Removal of activated complexes by the DNA-cellulose column is highly efficient. In experiments in which we have connected two such columns in series, at least 90% of activated complexes are bound to the first column and less than 10% are retained by the second. From similar experiments, we have found the DEAE-cellulose and hydroxylapatite columns to be even more efficient in retaining complexes.

Radioactivity Measurements—Radioactivity was assayed with a Packard 3390 liquid scintillation counter, using Hydrofluor scintillation fluid (New England Nuclear) at about 44% efficiency for tritium.

RESULTS AND DISCUSSION

DEAE-chromatography of Molybdate-stabilized Cytoplasmic Glucocorticoid-Receptor Complexes from Rat Thymus Tissue—Fig. 1 illustrates the KCl gradient elution profiles from DEAE-cellulose for glucocorticoid-receptor complexes in cytosols from rat thymus cells incubated with [3H]dexamethasone at 0 °C (Fig. 1A) and 37 °C (Fig. 1B). In this figure, total cpm/ fraction are represented by open circles and bound cpm (measured by binding to hydroxylapatite), by closed circles. Under nonactivating conditions (0 °C, 2 h) we find a single major peak which elutes late in the gradient (~200 mM KCl). In keeping with earlier studies (10, 14, 29) we refer to this peak as Peak II. A small hydroxylapatite binding peak that elutes with free steroid before the gradient is due largely, if not entirely, to binding of free steroid to hydroxylapatite; in control experiments, we found about 5% of free steroid bound to hydroxylapatite. In contrast, cytosol from cells exposed to glucocorticoid under conditions known to produce both nonactivated and activated receptors (37 °C, 30 min) elutes as three peaks during DEAE-chromatography. The major peak (Peak I) elutes early in the gradient (~60 mM KCl). In addition to a small peak in the same position as Peak II in Fig. 1A, a third peak of radioactivity (Peak III) is eluted before the gradient starts. This peak, which varies considerably in relative amount from experiment to experiment, is present in much greater proportion than can be accounted for by binding of free steroid to hydroxylapatite. Experiments in which cells were incubated with [3H]dexamethasone in the presence of 1 μM unlabelled dexamethasone, showed loss of Peak III as well as Peaks I and II (data not shown), indicating that all three peaks are composed of saturaably bound dexamethasone. It is likely that the complex in Peak III corresponds to the complex Ia identified in our earlier studies (29) in which columns were eluted with phosphate buffer, for like Ia, it has no affinity for DNA (shown below). In the earlier studies no molybdate was used.

A point to be kept in mind regarding results such as those in Fig. 1B, where cells were incubated with steroid at 37 °C, is that 50-80% of the total receptor complexes formed in those cells are translocated to the nuclei and do not appear in the cytosol. This fact accounts largely for the generally lower receptor-bound counts in those cytosols.

The receptor complexes in Peaks I, II, and III were further characterized with respect to their DNA-binding affinity. Previously (29) we demonstrated by KCl gradient chromatography on DNA-cellulose without molybdate, that cytosol complexes from cells incubated with [3H]dexamethasone at 0 °C did not bind to DNA, whereas 60-80% of the complexes from cells incubated at 37 °C, did bind to DNA. With molybdate, we have since obtained essentially identical results (not shown). The DNA-binding ability of complexes in Peaks I-III was determined by observing which complexes were removed from cytosols by initial passage through a DNA-cellulose column at low ionic strength (TDM buffer) before DEAE-chromatography. With cytosol from cells incubated at 0 °C, prior passage over DNA-cellulose (Fig. 2A) left the DEAE-cellulose gradient elution pattern unchanged from that in Fig. 1A, showing that the complex in Peak II does not bind to DNA. On the other hand, as shown in Fig. 2B, with cytosol from cells incubated at 37 °C, passage through DNA-cellulose before DEAE-chromatography eliminated most of Peak I seen in Fig. 1B, but left Peaks II and III intact.

We also examined the relationship of the three peaks to the activation process under cell-free conditions. Cytosols with nonactivated complexes were prepared without molybdate from cells incubated with [3H]dexamethasone at 0 °C for 2 h. When warmed to 25 °C, these cytosols, before and after passage through a DNA-cellulose column, gave elution profiles on DEAE-cellulose (not shown) indistinguishable from those in Figs. 1B and 2B obtained with cytosols from cells incubated at 37 °C. When molybdate was added to the cytosol before warming to block activation (24, 25), the DEAE-gradient elution patterns (not shown) were like those in Figs. 1A and 2A.

Taken together, these data, in agreement with earlier results (10, 14, 29) obtained under different conditions, indicate that Peak II contains the nonactivated complex, and Peak I contains the activated complex. Peak III possesses properties
of the mero-receptor complex described by Sherman et al. (18, 26) in that it binds to neither DNA- nor DEAE-cellulose. Further support for this characterization is provided in experiments below.

**Agarose Filtration of Molybdate-stabilized Cytoplasmic Glucocorticoid-Receptor Complexes**—Because of the time required for agarose filtration, [3H]TA, which dissociates from the receptor more slowly than [3H]dexamethasone, was used. With [3H]dexamethasone this procedure gave essentially the same result as [3H]TA except that more free steroid was formed.

Cytosol from cells incubated at 0 °C (Fig. 3A) gave a major peak that eluted with an estimated Stokes radius of 8.3 nm. The small peak which eluted after myoglobin (M) was composed of free steroid. Incubation at 37 °C (Fig. 3B) gave several peaks. A small amount of radioactivity (fraction 46) that probably represents aggregated complexes, appeared in the void volume. The major peak which eluted between ferritin (F) and β-amylase (B) with a Stokes radius of 5.0 nm will be shown to correspond to the activated complex. It was followed by a smaller peak with a Stokes radius of 2.3 nm, the mero-receptor complex. The peak after myoglobin (M) again contained free steroid. In these preparations, we also frequently observed a shoulder on the leading edge of the 5.0 nm peak, consistent with a Stokes radius of 8.3 nm. This shoulder, as will be shown below, contains nonactivated complexes.

The elution buffer used in these studies did not include any sulphydryl-protecting reagent, but we have found in other experiments that addition of 2 mM dithiothreitol to the buffer did not alter the results. Results similar to those in Fig. 3, A and B were also obtained using hypertonic buffers (400 mM KCl) with 20 mM molybdate to elute the agarose columns (not shown). Furthermore, exposure of cytosal from cells incubated at 0 °C to 400 mM KCl with 20 mM molybdate for 1 h, followed by agarose gel filtration with hypertonic buffer (400 mM KCl), also gave results like those in Fig. 3A. In other experiments (data not shown), we found that cell-free heat activation gave complexes similar to those obtained from cells incubated at 37 °C, though with proportionately more of the 2.3 nm form. Inclusion of 20 mM molybdate during the heating prevented formation of both the 5.0 nm and 2.3 nm complexes, leaving the pattern essentially like that in Fig. 3A. A reduction in Stokes radius from 8.0 to 6.0 nm on cell-free activation has been reported for [3H]TA glucocorticoid-receptor complexes from glucocorticoid-sensitive P1798 cells; this reduction was blocked by molybdate (43).

**Sequential Chromatography of Molybdate-stabilized Receptor Complexes on DNA-cellulose and Agarose Columns**—To correlate the three receptor forms seen on DEAE- and DNA-chromatography with those seen on agarose filtration, we performed sequential chromatography of the receptor complexes with various combinations of these columns.

First, cytosol from cells incubated with [3H]TA at either 0 °C for 2 h, or 37 °C for 30 min, was passed through a DNA-cellulose column to remove DNA-binding complexes. The remaining complexes were then analyzed by agarose filtration. Passage over DNA-cellulose did not alter the elution profile on agarose of cytosols from cells incubated at 0 °C for 2 h (Fig. 4A), which remained like that in Fig. 3A. The 8.3 nm
peak obtained under these conditions can therefore be identified with the nonactivated complex in Peak II on DEAE-cellulose (Fig. 1A). Similar treatment of cytosol from cells incubated with steroid at 37 °C (Fig. 4B), removed the major 5.0 nm peak normally seen (Fig. 3B) between ferritin (F) and β-amylase (B). The 8.3 nm complex, which appeared as a leading shoulder in untreated cytosol (Fig. 3B), is now clearly seen, as is the 2.3 nm complex.

In another type of experiment, cytosol from cells incubated at 37 °C was first applied to the agarose column, and the fractions comprising the 5.0 nm peak tested for binding to DNA. More than 50% of the radioactivity in these fractions was bound to DNA-cellulose (data not shown), as expected if this peak consists of activated complexes. In the reverse experiment, cytosol was passed through a DNA-cellulose column, the DNA-bound complexes eluted with 400 mM KCl, and the eluate analyzed on the agarose column. As seen in Fig. 5, only complexes with Stokes radii of less than 5.0 nm were present. Most were in the 4.3 nm range (peak fraction), although smaller forms were also present. The 4.3 nm peak probably represents an altered form of the 5.0 nm complex. It has not been established whether the high-salt elution from DNA affected the conformation of the 5.0 nm complex or increased its susceptibility to enzymatic cleavage.

**Sucrose Gradient Centrifugation of Molybdate-stabilized Glucocorticoid-Receptor Complexes**—Sucrose gradient analysis of cytosols from cells incubated with [3H]TA at 0 °C revealed a single sharp peak which sedimented as a 9.0–9.5 S complex (Fig. 6A). In contrast, complexes from cells incubated at 37 °C with [3H]TA gave two separate peaks (Fig. 6B), one of which corresponded to the 9.0–9.5 S complex seen in Fig. 6A. The predominant peak was much broader and sedimented at 4.8 S. These results are consistent with studies on cell-free activation (44, 45) suggesting that the glucocorticoid-receptor complex is converted to a slower sedimenting form as it becomes activated. That this 4.8 S peak does indeed correspond to a DNA-binding form was shown by the fact that passing the cytosol through DNA-cellulose before sucrose gradient analysis, removes the major 4.8 S peak (Fig. 7). Fig. 7 also reveals a 2.9 S peak, undetectable in Fig. 6B because it is buried under the 4.8 S peak. This sedimentation coefficient for the non-DNA binding complex is consistent with that reported for the mero-receptor complex (17, 44).

Based on the data derived from agarose filtration and sucrose gradient centrifugation with molybdate, we have calculated the apparent molecular weights and other characteristics for the receptor complexes in rat thymus cells. These results, summarized in Table I and discussed further below, indicate that activation of glucocorticoid-receptor complexes results in about a 70% decrease in the size of the receptor protein.

**Gel Filtration and Sucrose Gradient Centrifugation of Glucocorticoid-Receptor Complexes in the Absence of Molybdate**—The experiments with molybdate described thus far indicate clear differences in Stokes radii and sedimentation coefficients for nonactivated and activated complexes in rat thymus cells. Several reports have suggested that molybdate itself causes alterations in the conformation of glucocorticoid-receptor complexes (45, 46). Therefore, it was important to determine whether the characteristics found with molybdate...
could also be observed in its absence. The results are shown in Figs. 8 and 9. Without molybdate during agarose gel filtration, the free steroid in preparations from cells incubated with [3H]TA at 0 °C and 37 °C, was increased as compared to results with molybdate (Fig. 3, A and B). In the 0 °C preparations, although there were complexes of a comparable size to the molybdate-stabilized form (8.3 nm), there was also a continuous range of smaller sizes from 8.3 nm to 2.3 nm or less. Similarly, in the 37 °C preparations, although some receptor complexes eluted in the 5.0 nm size range, most counts were associated with smaller forms, the majority of which corresponded to the 2.3 nm complex. The smaller complexes probably result from limited proteolysis, although in the 0 °C preparations, gel filtration may also have produced some degree of activation (12). Thus, these results imply that the large receptor complexes that predominate in the presence of molybdate are stabilized native forms rather than molybdate-induced artifacts.

Additional evidence for this point comes from experiments in which cytosols from 0 °C and 37 °C cell incubations were prepared without molybdate, left for 6 h at 0 °C, then supplemented with 20 mM molybdate and analyzed by gel filtration in the presence of molybdate. The patterns obtained under these conditions (data not shown) were similar to those in Fig. 8, A and B, indicating that molybdate does not convert small complexes to large ones, but rather, stabilizes the large complexes.

Consistent with these results, on sucrose gradients without molybdate (Fig. 9), the peaks representing nonactivated and activated complexes are broader and sediment more slowly with sedimentation coefficients of about 7.6 S and 3.4 S instead of the 9 S and 4.5 S seen with molybdate (Fig. 6). We have also recently analyzed by agarose filtration, cytoplasmic glucocorticoid-receptor complexes cytosols of WEHI-7 mouse thymoma cells. The analysis could be done without molybdate, because little proteolysis of receptor complexes occurs in these cytosols during gel filtration. As in rat thymus cells, activation is accompanied by a decrease in Stokes radius of the predominant peak from ~7.8 nm (nonactivated) to ~5.6 nm (activated). Even with these relatively stable preparations, however, the gel chromatograms show much broader peaks and considerably greater amounts of free steroid compared to those obtained with molybdate.

**Analysis of Glucocorticoid-Receptor Complexes Using a Rapid Mini-Column Chromatographic Technique**—Based on the studies above, we have developed a rapid mini-column procedure by which activated, nonactivated, and mero-receptor forms of glucocorticoid-receptor complexes can be separated and quantitated within minutes in a single step. To establish the validity of this method, we have compared it to the full column procedures described above. Table II shows the relative distribution of complexes in cytosols from cells incubated with [3H]dexamethasone at 0 °C and 37 °C, measured with mini-columns and with sequential chromatography on full DNA- and DEAE-cellulose columns as described for Fig. 2. Results obtained with the two methods are in general agreement. By either method, virtually all complexes from cells incubated at 0 °C were in the nonactivated form, and most complexes from cells incubated at 37 °C were in the activated form. Both procedures showed that there was con-
considerable variation in the proportions of the three complexes from one 37 °C preparation to the next. However, the mini-columns in general gave slightly less activated and slightly more nonactivated complex than the full column procedures. This difference is probably due primarily to the fact that nonactivated complexes are determined in the mini-column procedure from total cpm bound to DEAE-cellulose, but in the full column procedure from the amount of complex which binds to hydroxylapatite in individual fractions eluted from DEAE-cellulose with high ionic strength buffer. In the latter procedure more losses are incurred. The mini-column procedure offers the major advantage over full column techniques of separating the various complexes in less than 10 min, thereby minimizing artifacts due to degradation and other time-dependent processes; in addition it concentrates all the radioactivity from each complex into a single sample, and is adaptable to simultaneous analysis of many cytosols.

**Effect of Molybdate on Cell-free Changes in the Distribution of Cytoplasmic Complexes**—In the experiments above, we have used molybdate to stabilize complexes as well as to prevent spontaneous activation during analysis. Our results, in agreement with those of others (17–19, 24, 25) show that molybdate is effective in both these functions.

To investigate the stability of cytosols more precisely, we have used the mini-columns to examine the time-course of changes in cytosols after cell lysis with and without molybdate. A single suspension of rat thymus cells was divided into two portions, one incubated at 0 °C with [3H]TA, the other at 37 °C. After the incubations, each suspension was further divided into two groups and broken in MgCl₂ with or without 20 mM molybdate to obtain cytosols. The four cytosol preparations were left at 0 °C for up to 8 h. Samples from each group were assayed on the mini-columns at 0, 4, and 8 h. The results (Fig. 10) clearly demonstrate that molybdate stabilizes the cell-free complexes. In 0 °C preparations, which contain only nonactivated complexes at the time of cell lysis, molybdate maintains this distribution for up to 8 h (Fig. 10A, solid lines) preventing the spontaneous activation and formation of mero-receptor complexes which occur in its absence (Fig. 10A, dashed lines).

![Figure 10](http://www.jbc.org/)  
*Fig. 10.* Mini-column analysis of the effect of 20 mM molybdate on cell-free changes in the distribution of gluocorticoid-receptor complexes in rat thymus cytosols. Cytosols were prepared with or without molybdate from cells incubated with 20 nM [3H]TA at 0 °C for 2 h (A) or 37 °C for 20 min (B). Cytosols were kept at 0 °C for up to 8 h, and 0.1-ml samples were removed at 0, 4, and 8 h, and assayed on mini-columns. Times given are h after the first sample (0 h) was placed on a mini-column. The 0 h samples were assayed immediately. The amount of DNA-bound complexes in cytosols with molybdate; Δ--Δ, without molybdate. •••, DNA-bound complexes in cytosols with molybdate; ○--○, without molybdate. ●●●, hydroxylapatite-bound complexes in cytosols with molybdate; □--□, without molybdate.

Molybdate also stabilizes complexes in cytosols from cells incubated at 37 °C (Fig. 10B, solid lines), although over long time periods it does not completely prevent the loss in DNA binding seen in mero-receptor complexes. Without molybdate (Fig. 10B, dashed lines), there is more rapid loss of DNA binding complexes and increase of mero-receptor complexes. The increase in DEAE-binding seen with molybdate is apparently not due to reversal of activation and accumulation of nonactivated complexes, since warming of the cytosol after 8 h does not increase the proportion of DNA-cellulose binding complexes. Furthermore, analysis of these cytosols by gel filtration showed that the increase in DEAE-binding is not accompanied by an increase in the Stokes radius to the 8.3 nm form. Thus, the DEAE-binding complexes are probably altered activated complexes with decreased affinity for DNA.

The rates at which receptor complexes undergo such changes during gel filtration and sucrose gradient centrifugation, are probably considerably slower than those in Fig. 10. For the experiment in Fig. 10, cytosols were prepared in MgCl₂ with or without molybdate, and left undiluted until assayed. During the gel filtration and other characterization procedures, which employ buffers containing other stabilizing agents besides molybdate (e.g., glycerol, monothioglycerol, EDTA, and dithiothreitol), cytosol components are progressively diluted and separated. Nonetheless, some degradation undoubtedly takes place; the relative amount of mero-receptor complex in Fig. 3B, for example, is somewhat greater than the amounts in Table II (37 °C) measured by mini-columns and full-column DNA- and DEAE-chromatographic procedures.

The mini-column procedure with molybdate appears to provide a reliable measure of the amounts of activated and nonactivated complexes in intact cells, as little change in the distribution of complexes occurs up to 1–2 h after cell breakage, and the analysis can be completed within 15–20 min after the cells are broken (including the time required for lysis). Our results suggest that the mero-receptor complex may exist in small amounts in the intact cell at physiologic temperatures, because as shown both in Fig. 10B and Table II,
detectable amounts are present by mini-column analysis at the earliest times after cells are broken.

The correlation between formation of activated and mero-receptor complexes, consistently observed throughout these studies, is also seen in Fig. 10. When activation is prevented, no mero-receptor is formed (Fig. 10A). However, once activated complexes are present in the cytosol (Fig. 10B), molybdate cannot completely prevent mero-receptor formation.

General Discussion—Addition of 20 mM molybdate to buffers has allowed us to characterize the cytoplasmic glucocorticoid-receptor complexes in cytosols from rat thymus cells incubated with steroid. Table I summarizes the properties of these complexes. Cytosols from cells incubated with [3H]dexamethasone or [3H]TA at 0 °C, contained only the nonactivated complex. Cytosols from cells incubated with these steroids at 37 °C contained three different complexes: the nonactivated and activated complexes, which accounted for about 20 and 70% of total bound steroid respectively, and the mero-receptor complex, which accounted for about 10%.

Activation has long been thought to involve a change in the conformation of the receptor protein, consistent with observed changes in the sedimentation properties (44, 45). The present study provides the first clear evidence that activation in the intact cell is also accompanied by a significant decrease in the size of the receptor complex, as determined from Stokes radius and sedimentation coefficient analysis. Together, these parameters indicate about a 70% decrease in the apparent molecular weight of the complex on activation. Such a change in size could occur either by enzymatic cleavage, with removal of part of the receptor protein, and/or by dissociation of similar or dissimilar subunits. In other experiments, we found that activation occurs in vitro in the presence of a variety of protease inhibitors including leupeptin, antipain, phosphoramidon, aprotinin, α-antitrypsin, and trypsin inhibitors (soy bean, lima bean, and egg). Thus, proteolysis seems unlikely as a mechanism of activation, unless it involves a specific protease that is insensitive to these inhibitors. However, the possibility that activation is accompanied by dissociation of a multimeric nonactivated complex into subunits is compatible with known structural properties of the complexes. The molecular weight of both the nonactivated and activated complex of rat liver is ~90,000 when analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (47). This value obtained under denaturing conditions is similar to that we have obtained for the activated complex (Mr = ~100,000) under nonnaturating conditions.

The Stokes radius for the activated glucocorticoid-receptor complex has generally been reported to be in the range of 5.0-6.0 nm (44, 48-52), in agreement with our results. We have found that the activated complexes from rat thymus cells undergo considerable dissociation and conversion to smaller complexes when analyzed using molybdate-free buffers (the conditions used for most earlier studies), but even without molybdate, some of the complexes are in the 5.0 nm range. Fewer reports are available concerning the size of the nonactivated complex. The 8.3 nm nonactivated complex seen in our study is similar to the molybdate-stabilized forms (7.5-8.5 nm) identified by Sherman et al. (18) in molybdate-stabilized cytosols from a variety of tissues incubated with steroids at low temperature. Vedeckis (53) has similarly reported a Stokes radius of 7.7 nm for the molybdate-stabilized glucocorticoid-receptor complex of the mouse AT-20 pituitary cell line. Again, the fact that even without molybdate in rat thymus cytosols, some receptor complexes are found in the 8 nm size range, suggests that the 8.3 nm form represents the native nonactivated complex rather than a molybdate-induced artifact. We have also found a similar complex in cytosols from WEHI-7 cells analyzed by agarose filtration without molybdate.

On the other hand, Cidlowski (44), has reported that the Stokes radius for both nonactivated and cell-free activated glucocorticoid-receptor complexes from rat thymus cells is 5.6 nm. The disagreement with the above results probably reflects differences in the chromatographic conditions employed. In particular, the studies of Cidlowski (44) were performed without molybdate.

Recently, Currie and Cidlowski (54) reported that the Stokes radius for cytoplasmic receptor complexes in cytosols from HeLa S cells incubated at 0 °C, varied considerably with the salt concentration employed in column elution buffers; increasing KCl concentrations (ranging from 0 to 400 mM KCl) were associated with decreasing Stokes radii (from 9.5 nm to 6.9 nm). Since increased ionic strength is known to activate the receptor (3, 9) and these studies were done without molybdate, it is not clear to what degree the reduced Stokes radius they observed can be attributed to activation. As mentioned above, even with 400 mM KCl, we obtained gel filtration results essentially identical to those in Fig. 3 (obtained with hypotonic buffers), as long as molybdate was present throughout.

Several studies using sedimentation centrifugation analysis have suggested that molybdate alters the conformation of the glucocorticoid-receptor complexes. Maki et al. (46) reported that the nonactivated glucocorticoid receptor from rat mammary gland was altered from 6-7 S to 9-10 S by 10 mM molybdate. This change was accompanied by enhanced stability of the complex. Similarly, Hutchens et al. (45) reported that molybdate altered the sedimentation coefficient of activated receptor complexes of a Syrian hamster melanoma cell line from a combination of 12-13 S and 7-8 S to a single 9-10 S form. They also found that the activated complex sedimented as a 4-5 S form in the absence or presence of molybdate. In view of the results of Sherman and co-workers (17-19) and our own results, we interpret these findings to reflect the stabilizing influence of molybdate on the native nonactivated 9-10 S form.

The significance of the mero-receptor complex in the cytosol from rat thymus cells incubated at 37 °C is not clear. Because the levels of mero-receptor in freshly prepared cytosols are so low, we cannot be sure whether this complex is present in the intact thymus cell or is generated in a burst during cell lysis. Cidlowski (44) has found a similar complex in cytosols from rat thymus cells in the absence of molybdate or other stabilizers. Sherman et al. (17) demonstrated that mero-receptor complex is formed from larger receptor complexes by proteolysis under cell-free conditions, and that molybdate can completely block its formation at 0-4 °C. Our results with rat thymus cytosols obtained by low speed centrifugation are not strictly comparable to those of Sherman et al. (17) obtained with high speed cytosols from rat kidney, but they are consistent for preparations which contain only the nonactivated complex. In thymus cytosols which contain activated complexes, however, mero-receptor accumulates even in the presence of 20 mM molybdate. We interpret the association between mero-receptor and activated complex to be due to the relative instability of the activated complex (compared to the nonactivated complex). An alternative interpretation, that generation of mero-receptor is coupled to activation, is probably not correct, since in studies with WEHI-7, a mouse thymoma cell line, we find virtually no detectable mero-receptor in cytosols containing activated complexes even after the cytosols are left at 0 °C for up to 24
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h with or without molybdate. Thus, although our data suggest that mero-receptor may be present in low amounts in intact thymus cells at physiologic temperatures, its role remains uncertain.

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