Glucocorticoid-binding Proteins of Rat Liver Cytosol

I. SEPARATION AND IDENTIFICATION OF THE BINDING PROTEINS*

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SUMMARY

The specific interaction of glucocorticoid hormones with liver cytosol proteins of adrenalectomized rats was studied by an adsorbent technique for the separation of free and protein-bound steroid. By this rapid procedure a high affinity binding site for glucocorticoids can be detected which is usually overlooked when the slower technique of equilibrium dialysis is used. This metastable binding protein can be quantitatively estimated in whole cytosol preparations because of its high affinity not only for natural glucocorticoids but also for [3H]dexamethasone, which does not bind to the other two more stable glucocorticoid-binding proteins previously reported in rat liver cytosol, the A and B proteins. It can also be distinguished from these two binding proteins for natural glucocorticoids on the basis of its differential fractionation and physical properties. Unlike the A and B proteins, which in sucrose gradients sediment at 4 S independently of ionic strength, the dexamethasone-binding protein sediments in low salt sucrose gradients largely as a heavy complex, 7 S, which reverts to a lighter form, 4 S, in the presence of 0.3 M KCl. A similar salt-dependent behavior is observed upon chromatography of the dexamethasone-binding protein through Sephadex G-200 columns. The fact that specific dexamethasone binding can only be detected in the cytosol of glucocorticoid target cells, in conjunction with previously presented evidence on its relation to hormonal enzyme induction in vivo, makes it reasonable to assume that this binding protein, which we will call the G protein, may indeed represent the physiologically significant hepatic glucocorticoid receptor.

It is becoming increasingly evident that one of the most remarkable and consistent features in the interaction of steroid hormones with their target cells is the formation of specific complexes with soluble cellular proteins generally called “receptors.” The presence of these hormonal receptors appears to be a distinguishing characteristic of target cells, and evidence is accumulating indicating that they may initiate the cellular response to the hormones. Therefore, investigation of the intracellular fate of the hormonal receptors after administration of the corresponding steroid will provide insight into the mechanisms of hormonal regulation. Furthermore, the identification and characterization of these receptor molecules appears as a necessary condition for the development of appropriate in vitro model systems for the study of hormone action.

The induction of specific enzyme synthesis in rat liver by glucocorticoids has been thoroughly explored in an endeavor to ascertain the biochemical processes by which steroid hormones modulate cellular events. Alternative models involving hormonal control at the transcriptional or the translational levels have been proposed for explaining the hormonal enzyme induction (1-3). One way of elucidating this mechanism is to study the interaction of radioactive labeled glucocorticoids with different cellular components and relate it to the processes underlying hormonal enzyme induction. Twenty minutes after intraperitoneal injection of [3H]cortisol unmetabolized radioactivity can be found in the cytosol and in the nucleus of the liver cell forming complexes with soluble protein (4). Two proteins of rat liver cytosol which specifically bind natural glucocorticoids, but not dexamethasone, have been identified previously and referred to as the A and B binding proteins (5, 6). In this paper we will present evidence showing the existence in the liver cytosol of another binding protein with high affinity and specificity for natural as well as biologically active synthetic glucocorticoids, and showing properties clearly distinguishable from the A and B binding proteins. We will refer to this new glucocorticoid-binding protein as the G protein. In a recent paper we have reported a close correlation between the degree of saturation of the G protein in vivo and the extent of hormonal enzyme induction after cortisol injection (7). Some of the molecular properties and the characteristics of the glucocorticoid-binding reaction to these three binding proteins of liver cytosol, A, B, and G, are reported in an accompanying paper (8).

METHODS

Materials

Reagents—[1,2-3H]Cortisol (specific activity, over 40 Ci per mmole) was obtained from New England Nuclear Inc. [1,2,4-

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† The trivial names for steroids used are: cortisol, 11β,17α,21-trihydroxyprogren-4-ene-3,20-dione; corticosterone, 11β,21 dihydroxyprogren-4-ene-3,20-dione; dexamethasone, 9α-fluoro-16α-methyl-11β,17α,21-trihydroxyprogrena-1,4-diene-3,20-dione.
\( ^{3} \text{H} \)Dexamethasone (specific activity, 12 Ci per mmole) was obtained from Schwarz-Mann Inc. The radiochemical purity of the stock solutions was checked periodically by thin layer chromatography as previously described (4). The nonlabeled steroids were obtained either from Steraloids, Inc., New York, or from Sigma Inc. Activated charcoal was purchased from Mallinkrodt Co. Dextran-500 and the Sephadex gels were obtained from Pharmacia, Sweden. DEAE-cellulose, DE-32 microparticulate, was obtained from Whatman. Male Sprague-Dawley rats weighing 150 to 200 g were used. The animals were adrenalectomized 5 to 8 days before the beginning of the experiments and maintained on Purina Chow and 0.9\% NaCl solution \textit{ad libitum}.

**Preparation of Cytosol**

The animals were killed by cervical dislocation and the liver perfused in vitro with 15 ml of ice-cold homogenization buffer (200 mM sucrose, 25 mM KCl, 10 mM MgCl\(_{2}\), 1 mM mercaptoethanol, in 50 mM Tris-HCl pH 7.5). All subsequent operations were performed at 0–4\(^{\circ}\)C unless otherwise specified. After weighing the livers were minced and the minced washed twice with an excess of cold buffer. Homogenization was performed after adding 2 ml of buffer per g of liver, wet weight, in a motor-driven Potter-Elvehjem homogenizer at 1,000 rpm. The homogenate was centrifuged at 240,000 \( \times \) g for 30 min. After aspirating the upper lipoprotein layer, the remaining supernatant (cytosol) was carefully removed and used for the binding studies and protein estimation (9).

**Binding Assays**

**Equilibrium Dialysis**—This method is the most accurate procedure for measuring binding parameters of stable proteins, for it allows simultaneous determination of the concentrations of free and bound steroid without disturbing the equilibrium conditions. We used plexiglass microchambers of 250-\( \mu \)l capacity separated by cellulose membranes pretreated as previously described (5). Aliquots of 200 \( \mu \)l, containing either the proteins or the radioactive steroid, were placed with a microsyringe on each side of the membrane. After closing the microchambers with parafilm, equilibrium was allowed to proceed for 60 to 72 hours at 0–4\(^{\circ}\)C with continuous rotatory shaking. Aliquots of 100 \( \mu \)l were then taken from both sides of the dialysis membrane and the radioactivity counted after adding 10 ml of Bray’s scintillation solution (10). Control experiments showed that over 90\% of the initial radioactivity was recovered. The difference in disintegrations per min between the radioactivity in the protein side and in the original ligand side, represents the protein-bound steroid.

**Adsorbent Technique** (11, 12)—Aliquots of 200 \( \mu \)l of the cytosol preparation were incubated with appropriate concentrations of tritiated glucocorticoid dissolved in homogenization buffer in the presence of either 50 \( \mu \)l of ethylene glycol or a 5000-fold excess of unlabeled glucocorticoid dissolved in 50 \( \mu \)l of ethylene glycol. The ethylene glycol at this concentration (17\%) did not affect the binding of [\( ^{3} \text{H} \)]dexamethasone to the cytosol proteins. After 2 hours of incubation in an ice bath to allow binding to go to completion, the unbound steroid was removed by addition of 100 \( \mu \)l of dextran-coated charcoal (3.75 g of activated charcoal and 0.375 g of dextran-500 in 100 ml of 10 mM Tris-HCl buffer, pH 8.0, prepared weekly). The samples were agitated in a Vortex mixer for 10 sec and then allowed to stand 0 at\(^{\circ}\) for 10 min. After centrifugation at 3000 \( \times \) g for 10 min, 200 \( \mu \)l of the supernatant were used for radioactivity determination. The total amount of radioactivity in the incubation mixture was determined in duplicate assays in which, instead of the charcoal, 100 \( \mu \)l of Tris buffer were added. Correction for quenching was based on the external standard channel ratio. The difference in disintegrations per min bound to cytosol proteins in the absence and presence of the 5000-fold excess of unlabeled steroid is taken as a measure of the high affinity saturable binding sites for the corresponding steroid.

**Sucrose Gradient Centrifugation**

Replicate 5 to 20% sucrose gradients were prepared in homogenization buffer containing various concentrations of KCl. Where indicated the sucrose solutions were supplemented with [\( ^{3} \text{H} \)]dexamethasone or [\( ^{3} \text{H} \)]cortisol to a final concentration of 2 \( \times \) 10\(^{-10}\) M. Aliquots of the hepatic cytosol were incubated with the corresponding radioactive glucocorticoid (2 \( \times \) 10\(^{-8}\) M) and passed through Sephadex G-25 columns, equilibrated with homogenization buffer without sucrose, to remove the free steroid. Two hundred microliters of the macromolecular peak were applied to the sucrose gradients, which were centrifuged in the SW 56 spinco rotor at 55,000 rpm for 15 hours at 0\(^{\circ}\). At the end of the run, 3-drop fractions were collected starting from the top of the gradient and 100-\( \mu \)l aliquots were used for radioactivity determination. Ovalbumin, bovine serum albumin, rabbit muscle aldolase, and beef liver catalase were used as standards. The radioactivity in the pellet was measured after incubation with 0.5 ml of Nuclear Chicago Solubilizer for 3 hours at room temperature and addition of 20 ml of a toluene-based scintillation mixture (13).

**RESULTS**

When [\( ^{3} \text{H} \)]dexamethasone is used in equilibrium dialysis experiments against whole liver cytosol only nonsaturable low affinity binding is observed, in contrast to the high affinity saturable binding sites previously described for natural glucocorticoids (6). However, when liver cytosol is incubated with [\( ^{3} \text{H} \)]dexamethasone for 2 hours and passed through a column of Sephadex G-25 a considerable fraction of the radioactivity is found in the macromolecular peak, suggesting a relatively tight binding of the steroid to a macromolecular component. To account for the discrepancy between the results of equilibrium dialysis and gel filtration two explanations appear possible: either the long shaking time required for equilibrium dialysis may inactivate an hypothetically unstable binding site, or the high affinity binding site can be masked by an excess of unspecific binding. We therefore turned to a charcoal absorption technique for studying macromolecular binding of steroids (see "Methods") which was more rapid and gentle (11, 12). This method has the additional advantage of involving a 10 min incubation period with charcoal, during which most of the unspecifically bound steroid will dissociate and be bound by the adsorbent. Therefore the background of unspecifically bound radioactivity is much lower than in the equilibrium dialysis experiments, thus allowing the detection of even relatively low concentrations of high affinity binding sites. As shown in Fig. 1a, in addition to a small degree of nonspecific binding, a high affinity saturable binding site for [\( ^{3} \text{H} \)]dexamethasone can be readily shown by this procedure. The Scatchard plot of these data (Fig. 1b) is linear and, when analyzed as representing an homogenous class of binding sites, yields an apparent association constant of 2.5 \( \times \) 10\(^{8}\) M\(^{-1}\) at 0\(^{\circ}\). When the binding of [\( ^{3} \text{H} \)]cortisol to liver cytosol is similarly evaluated, the apparent
association constant at 0° is $1.8 \times 10^8 \text{M}^{-1}$ (Fig. 2). In the same cytosol the concentration of binding sites is $1.2 \times 10^{-8} \text{M}$ for [3H]dexamethasone, and $2.2 \times 10^{-8} \text{M}$ for [3H]cortisol (Figs. 1b and 2b). This last value thus, in addition to the binding sites which can be detected with [3H]dexamethasone, and which will also bind [3H]cortisol, includes the two other binding proteins, A and B, specific for natural glucocorticoids, which are not detected with [3H]dexamethasone (8). The fact that the Scatchard plot of [3H]cortisol binding to whole cytosol approaches linearity, is due to the similar affinities for cortisol of the three gluco- corticoid-binding proteins at low temperatures (8). A more detailed analysis of the interaction of various gluco- corticoids with these three binding proteins, including kinetics of association and dissociation as well as thermodynamic parameters and steroid specificity are presented in the accompanying paper (8).

As mentioned above, the failure to detect a high affinity binding site for [3H]dexamethasone in equilibrium dialysis experiments with whole cytosol, could be partially attributed to the instability of the binding activity. Fig. 3 shows the results of an experiment designed to compare the relative stabilities of the binding proteins for [3H]dexamethasone and [3H]corticosterone in a liver cytosol preparation from adrenalectomized rats. As can be seen, the dexamethasone-binding activity decreases linearly with time and is almost absent after 4 days of storage at 0°, whereas more than half of the [3H]corticosterone-binding activity persists at this time. The biphasic decay of [3H]corticosterone binding reflects the presence in the cytosol of two classes of binding sites, one very labile with affinity for natural as well as synthetic glucocorticoids and the other, more stable, with affinity only for the natural hormones.

Ammonium Sulfate Fractionation—In order to determine whether these different binding sites actually correspond to distinct species of proteins, we undertook fractionation of the cytosol proteins with ammonium sulfate (Table I). At 33% saturation with respect to ammonium sulfate a pellet was obtained, containing 12 to 15% of the cytosol proteins, which was more than 2-fold enriched for [3H]dexamethasone binding and markedly depleted with respect to [3H]corticosterone binding. The pellet obtained by bringing the 33% ammonium sulfate supernatant to 66% saturation, showed low dexamethasone-binding activity and was enriched with respect to [3H]corticosterone binding. It has to be pointed out that the passage of cytosol from adrenalectomized rats through a Sephadex G-25 column, results in the loss of its dexamethasone-binding activity, while this activity is preserved if the cytosol is incubated with dexamethasone before applying to the column. This allowed the determination of the percentage of the dexamethasone-binding and corticosterone-binding proteins, from those more stable which bind corticosterone and not dexamethasone.

Sucrose Gradient Studies—Liver cytosol macromolecules labeled with [3H]cortisol in vitro band in low ionic strength sucrose density gradients as an homogeneous peak with a sedimentation coefficient about 4 S (Fig. 4a). As can be seen in Fig.

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**Fig. 1.** Binding of [3H]dexamethasone ([3H]DEX.) to rat liver cytosol proteins as determined by the charcoal technique. Thirty aliquots (200 μl) of cytosol from adrenalectomized rats were incubated for 2 hours at 0° with 100 μl of homogenization buffer containing 15 different concentrations of [3H]dexamethasone alone or supplemented with a 5000-fold excess of unlabeled dexamethasone. The concentration of protein-bound [3H]dexamethasone was determined after adsorption of the free steroid to dextran-coated charcoal (see "Methods"). The concentration of specifically bound [3H]dexamethasone (△—△) was calculated from the difference between the values obtained in the absence (O—O) and in the presence (●—●) of an excess of unlabeled dexamethasone. a, linear plot of the concentration of protein-bound steroid versus the concentration of free steroid. b, representation of the data according to Scatchard (14).

**Fig. 2.** Binding of [3H]cortisol to rat liver cytosol proteins as determined by the charcoal technique. Experimental setup similar to that of Fig. 1, using the same cytosol preparation, but instead of [3H]dexamethasone, [3H]cortisol and a 5000-fold excess of unlabeled cortisol were added.

**Fig. 3.** Stability of glucocorticoid binding activities of rat liver cytosol at 0°. Aliquots of freshly prepared liver cytosol from adrenalectomized rats were checked for specific binding of either [3H]dexamethasone (●—●) or [3H]corticosterone (Δ—Δ) using the dextran-coated charcoal procedure. The amount of protein-bound radioactivity at saturating concentrations of free steroid was taken as 100% binding, and the values obtained at different times after storage of the cytosol at 0° were expressed as per cent of the initial value.
Table I

Ammonium sulfate fractionation of glucocorticoid-binding activities of rat liver cytosol

Duplicate 30 ml aliquots of liver cytosol were incubated for 2 hours at 0° with either 2 x 10⁻⁴ M [³H]dexamethasone or buffer. To 20-ml aliquots of each incubation, saturated ammonium sulfate, adjusted to pH 7.5, was added dropwise with stirring to reach 33% saturation. After standing for 30 min at 0° the precipitates were collected by centrifugation at 15,000 x g for 20 min. To the remaining 33% saturated supernatants, enough saturated ammonium sulfate was added to reach 60% saturation, and the precipitates similarly recovered. Both the 0 to 33% and the 33 to 60% ammonium sulfate precipitates were resuspended in 10 ml of homogenization buffer. Five-milliliter aliquots of these fractions, as well as of the original cytosol, unlabeled or labeled with [³H]dexamethasone, were passed through identical columns of Sephadex G-25 and the excluded macromolecular peaks were collected. The radioactivity and protein content of the three macromolecular fractions that had been labeled with [³H]dexamethasone were determined and are depicted as counts per min of [³H]dexamethasone per mg of protein. The unlabeled macromolecular fractions were used for determining the [³H]cortisosterone-binding capacity by the charcoal procedure, and are depicted as counts per min of [³H]cortisosterone per mg of protein.

<table>
<thead>
<tr>
<th>Macromolecular fraction</th>
<th>Specific steroid binding</th>
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<tbody>
<tr>
<td></td>
<td>[³H]Dexamethasone</td>
</tr>
<tr>
<td>Cytosol</td>
<td>2,840 (100%)</td>
</tr>
<tr>
<td>0 to 33% ammonium sulfate precipitate</td>
<td>6,005 (212%)</td>
</tr>
<tr>
<td>33 to 60% ammonium sulfate precipitate</td>
<td>1,460 (51%)</td>
</tr>
</tbody>
</table>

4b, this sedimentation pattern is not affected by the presence in the gradient solution of 0.3 M KCl. The same way, when labeled with [³H]dexamethasone in vitro and similarly applied to low ionic strength sucrose gradients, sediments as two distinct radioactive entities one in the region of 4 S and the other at about 7 S (Fig. 4a). This heavier moiety disappears when the sucrose gradient solution is supplemented with 0.3 M KCl (Fig. 4b). Moreover, only in low salt sucrose gradients is a significant fraction of the radioactivity recovered in the pellet of the centrifuge tube, thus, showing a sedimentation coefficient over 11 S. Little or no radioactivity is found in the pellets of the sucrose gradients when the cytosol is labeled with [³H]cortisol. It therefore appears that at low salt concentration, dexamethasone and not cortisol forms a 7 S complex with cytosol macromolecules which tends to aggregate. At high salt concentration, the heavy dexamethasone-macromolecular complex either dissociates or is otherwise converted to a 4 S form.

In any case, both radioactive peaks represent saturable steroid binding sites, for the presence of an excess of unlabeled dexamethasone in the gradient solution almost completely eliminates both labeled peaks. Moreover, although neither [³H]cortisol nor [³H]cortisosterone give rise to the heavy form of the complex with cytosol macromolecules, the presence of an excess of unlabeled cortisol or corticosterone in the gradient solution prevents the formation of both the heavy and the light forms of the complex, when the cytosol is labeled with [³H]dexamethasone. These findings could be explained by assuming an equilibrium between the heavy and the light form of the labeled complex which is affected by ionic strength and by the structure of the liganding steroid.

To check the physiological significance of the observed sedimentation patterns after labeling the cytosol in vitro, we prepared cytosol from the livers of animals injected with [³H]dexamethasone 15 min before killing. After passing through Sephadex G-25 the macromolecular peak was analyzed by sucrose gradient centrifugation (Fig. 5). In the low salt sucrose gradient, a clear 7 S-labeled peak was observed and considerable amount of the radioactivity was found in the pellet of the centrifuge tube. The 4 S peak was virtually absent. However, after sucrose gradient centrifugation at 0.3 M KCl, very little radioactivity is found in the pellet and in the 7 S region, but a very clear radioactive peak at 4 S is present. It, thus, appears that the 7 to 4 S interconversion can be observed with cytosol labeled either in vivo or in vitro. When instead of [³H]dexamethasone, [³H]cortisol is injected, only the 4 S-labeled complex is observed independent of the salt concentration in the gradient (4).

Sephadex G-200 Filtration—If freshly prepared liver cytosol is incubated with [³H]dexamethasone and the 0 to 35% ammonium sulfate precipitate is applied to a column of Sephadex G-200 equilibrated with low ionic strength buffer (below 0.1 M) radioactive peaks elute with the excluded volume and in the region corresponding to the free steroid (Fig. 6a). Thus, at low ionic strength, the G protein is not retained by the gel and therefore its molecular weight appears to be greater than 200,000. As has been reported previously, the A and B binding proteins from liver cytosol are retained by Sephadex G-200, eluting in regions
methasone per 100 g of body weight. Proteins present in rat liver cytosol. In "Methods," except that 200 µl per fraction were used. a, no conditions and the radioactivity determination were as indicated 0.3 M in respect to KCl. 

The centrifugation peak were applied to linear sucrose gradients (5 to 20%, w/v) prepared in buffer without added steroid. The centrifugation buffer without sucrose. Aliquots of 200 µl of the macromolecular peak were applied to linear sucrose gradients (5 to 20%, w/v) prepared in buffer without added steroid. The centrifugation conditions and the radioactivity determination were as indicated in "Methods," except that 200 µl per fraction were used. a, no salt added to the gradient buffer. b, the gradient buffer was made 0.3 M in respect to KCl.

corresponding to molecular weights of 40,000 and 70,000, respectively, and show no detectable affinity for [3H]dexamethasone in vitro (6, 8). Thus, Sephadex gel filtration at low ionic strength provides a procedure to separate the three glucocorticoid-binding proteins present in rat liver cytosol.

When the Sephadex G-200 column is equilibrated and eluted with buffer containing 0.3 M NaCl, the G protein is partially retained in the gel and elutes in a region corresponding to a molecular weight of 60,000 to 80,000 (Fig. 6b). This agrees with the behavior of the dexamethasone binding activity in sucrose gradients, where at high salt a form of lower sedimentation coefficient was observed.

A similar elution pattern was observed when cytosol labeled in vitro for 15 min with intraperitoneally injected [3H]dexamethasone was passed through a column of Sephadex G-200. In both cases, the amount of radioactivity found in the macromolecular peak was considerably lower when the column was eluted with high salt as compared with the low salt column, pointing to a more rapid dissociation of the labeled complex at high salt.

**DEAE-cellulose Column Chromatography**—We previously reported that the A and B binding proteins from rat liver cytosol can be separated upon chromatography on DEAE-cellulose columns (6). In our search for analytical procedures to distinguish the G protein from the A and B glucocorticoid binders we studied the behavior of cytosol macromolecules labeled with [3H]dexamethasone on DEAE-cellulose columns (Fig. 7). As can be seen, some of the radioactivity elutes with the wash and probably represents free steroid that dissociates during the washing procedure. When the NaCl gradient is applied, a very sharp peak of radioactivity with a maximum at 0.085 M NaCl is eluted, which is not adsorbed to dextran-coated charcoal and therefore represents protein-bound radioactivity. Thus, the G protein elutes at an intermediary salt concentration between the A and B proteins, showing that not only the molecular weight but also the ionic charge of these three proteins are different.

**Tissue Specificity**—Table II summarizes the data on specific dexamethasone binding obtained with the cytosol prepared from different organs of adrenalectomized rats. In some of the cytosols, the presence of the A and B proteins was also investigated by analyzing the eluate from DEAE-cellulose columns (6). It is clear, that the liver is the organ with the highest concentration of specific dexamethasone binding sites in its cytosol. The kidney cytosol shows about one-third the concentration of dexamethasone binding sites as the liver cytosol. The thymus cytosol shows about one-third the concentration of dexamethasone binding sites as the liver cytosol. The kidney cytosol shows about one-third the concentration of dexamethasone binding sites as the liver cytosol. The kidney cytosol shows about one-third the concentration of dexamethasone binding sites as the liver cytosol. The kidney cytosol shows about one-third the concentration of dexamethasone binding sites as the liver cytosol. The kidney cytosol shows about one-third the concentration of dexamethasone binding sites as the liver cytosol. The kidney cytosol shows about one-third the concentration of dexamethasone binding sites as the liver cytosol.
eluted with a linear gradient of NaCl consisting of 2 column volumes of the initial buffer. The absorbance of the eluate at 280 nm was recorded continuously and 3-ml fractions were collected from each column of precycled DEAE-cellulose equilibrated with the same buffer. After washing with 2 column volumes the proteins were eluted with a linear gradient of NaCl consisting of 2 column volumes of the initial buffer and an equal volume of 0.2 M NaCl in the initial buffer. The absorbance of the eluate at 280 nm was recorded continuously and 3-ml fractions were collected from which 100-μl aliquots were taken for radioactivity determination. The positions where the A and B binding proteins from liver cytosol eluted are marked with arrows.

![FIG. 7. DEAE-cellulose column chromatography of rat liver cytosol labeled with [3H]dexamethasone in vitro.](image)

Liver cytosol prepared from adrenalectomized rats and labeled with 2 × 10^-8 M [3H]dexamethasone for 2 hours at 0° was passed through a column of Sephadex G-25 equilibrated with 10 mM Tris-HCl buffer, pH 8.3, containing 1 mM MgCl₂, 1 mM Na₂-EDTA, and 1 mM mercaptoethanol. The macromolecular peak was pooled and applied to a column of precycled DEAE-cellulose equilibrated with the same buffer. After washing with 2 column volumes the proteins were eluted with a linear gradient of NaCl consisting of 2 column volumes of the initial buffer and an equal volume of 0.2 M NaCl in the initial buffer. The absorbance of the eluate at 280 nm was recorded continuously and 3-ml fractions were collected from which 100-μl aliquots were taken for radioactivity determination. The positions where the A and B binding proteins from liver cytosol eluted are marked with arrows.

### TABLE II

Specific [3H]dexamethasone binding in cytosol of different rat tissues

Cytosol prepared from different tissues of adrenalectomized rats was used for determination of protein content and specific dexamethasone binding (see "Methods"). The values represent the average and standard deviation of two experiments with duplicate determinations.

<table>
<thead>
<tr>
<th>Source of cytosol</th>
<th>Specific [3H]dexamethasone binding</th>
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<tbody>
<tr>
<td></td>
<td>dpm/mg protein</td>
</tr>
<tr>
<td>Liver</td>
<td>5800 ± 220</td>
</tr>
<tr>
<td>Kidney</td>
<td>1715 ± 192</td>
</tr>
<tr>
<td>Thymus</td>
<td>1038 ± 85</td>
</tr>
<tr>
<td>Spleen</td>
<td>95 ± 62</td>
</tr>
<tr>
<td>Lung</td>
<td>14 ± 83</td>
</tr>
<tr>
<td>Heart</td>
<td>118 ± 160</td>
</tr>
<tr>
<td>Testis</td>
<td>12 ± 102</td>
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</table>

interfered with the detection of a specific binding site present at relatively low concentrations.

The B binding protein, i.e. transcortin, was present in all of the tissues studied as would be expected from blood contamination (6). The relative amount of this material in the different cytosols probably reflects the degree of vascularization of the corresponding organs. The A protein was only found in the liver cytosol.

**DISCUSSION**

In previous studies we investigated the binding of natural glucocorticoids, cortisol, or corticosterone, to rat liver cytosol, either after in vitro injection or by labeling the cytosol in vitro (4-6, 17). The equilibrium dialysis technique, selected for quantitative studies, allowed us to identify two protein factors in the liver cytosol with high affinity and specificity for natural glucocorticoids (6, 18). These two proteins, that we have called the A and B binding proteins, can be separated by DEAE-cellulose columns and gel filtration and are relatively stable in buffer containing 10% glycerol. However, neither of these proteins was able to bind [3H]dexamethasone in vitro (8). Since this synthetic glucocorticoid is a very potent inducer of specific liver enzymes, the functional relevance of the A and B proteins in this connection appeared uncertain (6). We therefore undertook a search for other binding factors in the liver cytosol with the ability to specifically combine [3H]dexamethasone. With the adsorbent technique for separating free and protein-bound steroid, a new class of binding sites with high affinity for both dexamethasone and natural glucocorticoids could be detected. The fact that this binding entity could not be detected in previous studies with equilibrium dialysis, is probably due to its instability and masking by unspecified binding. Thus, hepatic cytosol contains three glucocorticoid-binding proteins, one of which, the G protein, is labile and binds dexamethasone as well as cortisol and corticosterone, and the other two, the A and B proteins, are more stable and only bind natural glucocorticoids.

From the data in Figs. 1 and 2 the apparent equilibrium association constant of cytosol for [3H]dexamethasone at 0° is about 2.5 × 10^9 M⁻¹, and that for [3H]corticosterone seems to be slightly lower. Since we know that dexamethasone is only binding to the G protein while corticosterone is also binding to the A and B proteins, the association constants in whole cytosol cannot be compared as reflecting differences in the affinity of the same binding sites for dexamethasone and corticosterone. Two limitations of our charcoal technique have to be kept in mind when quantitative calculations are made; first, it measures the specific binding of the steroid to the whole cytosol, where unspecified binders may affect the actual concentration of free steroid, and secondly, the technique involves a disturbing of equilibrium, caused by the addition of the charcoal solution, which may lead to an underestimation of the equilibrium association constant. Moreover due to the thermal instability of dexamethasone binding sites (8), no experimental values for the equilibrium association constant at 37° could be obtained, although this is the physiologically significant temperature. Even if the concentration of free glucocorticoids could be determined in the blood, the association constant at 0° does not allow an estimation of the degree of saturation of the dexamethasone binding sites in vitro, since it is known that the affinity of these sites for glucocorticoids is markedly affected by temperature (8). Since the concentration of binding sites is independent of temperature (8), the data obtained at 0° can be used for calculating the number of binding sites per cell. This figure varies between 80,000 and 120,000 in adrenalectomized rats, depending on the cytosol preparation. Both the equilibrium association constant and the number of binding sites per cell are in good agreement with the values reported by Baxter and Tomkins for cultured hepatoma cells (19). The similarity between the liver and the hepatoma dexamethasone-binding proteins is supported by the sucrose gradient analysis. As in the cultured hepatoma cells, a heavy form of the dexamethasone-protein complex sedimenting at about 4 S can be detected in rat liver cytosol when the sucrose gradients are prepared in buffers of low salt concentration (below 0.1 M KCl). In these gradients the labeled complexes tend to aggregate and are partially recovered from the pellet of the centrifuge tubes. When the sucrose gradients are prepared in buffer containing 0.3 M KCl, a lighter form of the labeled complex sedimenting at about 4 S becomes dominant with very little label left in the 7 S region or in the pellet. That...
this behavior really reflects a change in the Stokes radius of the macromolecule, is shown by the results of gel filtration on Sephadex G-200 (Fig. 6).

The presence of the dexamethasone binding sites exclusively in the cytosol of glucocorticoid target tissues, as well as their specificity for natural and synthetic glucocorticoids make them good candidates for a functional role in glucocorticoid action. We recently reported a very close correlation between the degree of saturation in vivo of the dexamethasone binding sites of liver cytosol after injection of cortisol, and the extent of specific enzyme induction (7). A similar relationship has been previously reported in hepatoma cell cultures (19). These findings, together with the characteristics of the binding reaction (8), bring us to consider the dexamethasone-binding protein reported here as the most plausible hepatic glucocorticoid receptor. The presence of the G protein exclusively in the cytosol of glucocorticoid target cells may provide the basis for a biochemical definition of target tissues.

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Glucocorticoid-binding Proteins of Rat Liver Cytosol: I. SEPARATION AND IDENTIFICATION OF THE BINDING PROTEINS
Miguel Beato and Philip Feigelson


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