Inactivation of Mammary Cytoplasmic Glucocorticoid Receptors under Cell-free Conditions*

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The conditions for the inactivation of glucocorticoid receptors in the cytosol from lactating mouse mammary glands have been studied in detail. The dexamethasone-binding capacity of cytosol was variable and decreased to near zero levels by 4 h when incubated without steroid at 4°C. This loss of binding activity was partially prevented by binding of the steroid and was readily reversed by sulfhydryl-reducing compounds such as dithiothreitol. Molybdate and other phosphatase inhibitors were without effect on this dithiothreitol-reversible binding loss.

A second type of loss of specific binding of dexamethasone occurred in mammary cytosol exposed to high ionic strength or elevated temperature, but this loss could not be prevented or reversed by dithiothreitol; a 1-h treatment of cytosol with 0.3 M KCl or a 1/2-h exposure to 25°C eliminated most of the dexamethasone binding. Treatment with the steroid or with 10 mM molybdate partially prevented but did not reverse this inactivation. A molybdate-sensitive change of the steroid-receptor complex was inferred from analysis of the [3H]dexamethasone-labeled cytosol on high ionic strength sucrose gradients; the steroid-receptor complex in the untreated cytosol migrated as a 4.4 S peak while that in the molybdate-treated cytosol migrated as a 5.4 S peak.

Therefore, lactating mouse mammary glucocorticoid receptors are inactivated by two types of in vitro processes, one involving sulfhydryl groups and the other enhanced by high salt or elevated temperature. The possibility that similar in vitro processes may regulate glucocorticoid receptor activity remains to be explored.

In these studies (9-11) and others (12-14), an analysis of the binding of a particular glucocorticoid to the receptor and its biological effect indicated that the receptors may be allosteric proteins. However, glucocorticoid receptors of mammary tissues have not yet been purified to any extent and we felt that this would necessarily preclude any further understanding of the molecular basis for the receptor-mediated regulation of gene expression. During our attempts to purify the native cytoplasmic glucocorticoid receptors of lactating mammary glands, it became apparent that the receptors of these tissues undergo a very rapid inactivation in the absence of ligand. In the present report, we describe two types of inactivation of the receptors: one process can be prevented by sulfhydryl-reducing agents, whereas the second type of inactivation occurs even in the presence of sulfhydryl reagents, but the receptors can be partially protected from this inactivation by pretreatment of the cytosol with sodium molybdate.

MATERIALS AND METHODS

Animals—Female Balb/c mice from our own colony were used and at the time of killing they had been lactating for 7 to 10 days.

Stereoids—[3H]Dexamethasone (36.0 to 41.4 Ci/mmol) and [3H]estradiol (90.0 Ci/mmol) were obtained from New England Nuclear Corp., Boston, MA, and unlabeled dexamethasone, unlabeled estradiol, monothioglycerol, dithiothreitol, B-mercaptoethanol, and levamisole were purchased from Sigma Chemical Co., St. Louis, MO. Sephadex G-25 was from Pharmacia, Uppsala, Sweden, and sodium molybdate was from British Drug Houses, Poole, England. All other chemicals were from Fisher Scientific Co.

Buffers—Tris buffer (50 mM Tris-HCl, containing 12 mM thioglycerol and 10% glycerol (v/v)); phosphate buffer (5 mM phosphate, containing 12 mM thioglycerol and 10% glycerol (v/v)); barbital buffer (20 mM sodium barbital, containing 12 mM thioglycerol and 10% glycerol (v/v)). All buffers were pH 7.4 at 23°C.

Preparation of Cytosol—All preparations were done at 0-4°C. Following excision, mammary tissues were weighed, rinsed twice in buffer, minced, and then homogenized in 1 volume of buffer using a Polytron PT-10 (Brinkmann) homogenizer with two 10-s bursts. The homogenate was centrifuged at 105,000 × g for 1 h and the resulting supernatant (cytosol) was drawn from beneath the lipid layer with a cold Pasteur pipette.

Binding Assay—Aliquots of cytosol were incubated on ice with predetermined concentrations of [3H]dexamethasone with or without a 100-fold excess of unlabeled dexamethasone for defined periods of time. All steroids were added to incubations in ethanol at concentrations which limited the final volume to a maximum of 5%. The amount of steroid bound was determined by a dextran-coated charcoal assay procedure based on that of Korenman (15). Specific binding was defined as the amount of binding occurring in the presence of [3H]-dexamethasone alone minus the amount of binding when a 100-fold excess of unlabeled dexamethasone was also present. Nonspecific binding was consistent among experiments and represented about

† The nomenclature used is: dexamethasone, 3α-fluoro-16α-methyl-11β,17α,21-trihydroxypregna-1,4,9,11-tetraene-3,20-dione; estradiol, 1,3,5(10)estratriene-3,17β-diol; levamisole, levo isomer of 2,3,5,6-tetrahydro-6-phenylnindazole (2,1-b)thiazole hydrochloride.
10% of the total [(3H)dexamethasone binding when the experimental
design permitted maximal binding to be observed.

Sucrose Gradient Analysis—The samples to be analyzed were
exposed to a dextran-coated charcoal pellet (15 min), centrifuged at
800 × g for 5 min, and the resulting supernatant (0.2 to 0.3 ml)
centrifuged on 4.5-ml gradients of 10 to 30% (w/v) sucrose in specified
buffers. Approximate sedimentation coefficients were determined by
the method of Martin and Ames (16) using yeast alcohol dehydrogen-
ase (7.6 S), liver alcohol dehydrogenase (5 S), and bovine serum albumin
(4.5 S) as standards. Standard S values represent average for
the data presented in Ref. 17.

Other Methods—Protein concentration of the cytosol was deter-
mixed by the method of Lowry et al. (18) using bovine serum albumin
as the standard and was usually about 40 mg/ml.

RESULTS

Effect of Dithiothreitol on the Binding Capacity—In our
earlier studies on glucocorticoid receptors of lactating mam-
mary glands (8), monothioglycerol was included in the ho-
mogenizing buffer without special emphasis on the importance
of sulfhydryl-reducing compounds to the binding reaction. Subsequently, Granberg and Ballard (19) demonstrated the need
for such hydrol reagents in order to elicit maximal binding of
4-glucocorticoids in certain target tissues, but their studies
did not include mammary tissues. Therefore, in an effort to
optimize the conditions for glucocorticoid binding in mam-
mary cytosol, we investigated the effect of added sulfhydryl
reductants.

Cytosols were prepared both in the presence and absence
of dithiothreitol and the binding activity was determined as a
function of dithiothreitol concentration. As shown in Fig. 1
(upper panel), dithiothreitol increased the binding activity in a
concentration-dependent manner and at the optimal con-
centration of 10 to 20 mM the binding was approximately 10-
fold higher than that observed in the absence of dithiothreitol.
Similar experiments performed with other sulfhydryl com-
phrases revealed dithiothreitol to be the most effective of the
compounds tested (Fig. 1, lower panel). The amount of binding
observed in the absence of dithiothreitol was variable from
one experiment to another (not shown), probably reflecting
differring extents of sulfhydryl reduction by cytoplasmic com-
ponents and by the 12 mM thioglycerol present in the homog-
ization buffer.

The kinetics for the association of dexamethasone with the
receptor was nearly identical in cytosols prepared with or
without dithiothreitol (Fig. 2). Thus, dithiothreitol appears
to augment the binding capacity of the cytosol without altering
the rate of association. It is also evident from Fig. 2 that once
the steroid-receptor complex is formed, the stability of the
complex is the same in cytosols with or without the addition
of dithiothreitol, at least up to 8 h after the addition of steroid.
By 24 h a considerable amount of dexamethasone dissociates
from the receptors as the specific binding decreases to about
50% of the 4-h value (data not shown).

It has been shown that the inactivation of cytoplasmic
4-glucocorticoid receptors in liver was affected by the speed
of centrifugation used to prepare the cytosol (20). However, for
mammary tissue this was not a significant factor. The results
shown in Table I indicate that dithiothreitol was required in
the various supernatants to yield maximal binding. Although
it may appear that with higher speeds of centrifugation there
is a slight increase in the binding activity expressed on the
basis of protein, the binding activities expressed per unit
volume of the various supernatants were quite similar. The
effect of dithiothreitol was identical whether added directly
4 to homogenates or to cytosols (data not shown). Furthermore,
dithiothreitol could exert its effect on the binding capacity
even when added to the labeled cytosol at later times and in
all cases, the binding with the addition of dithiothreitol be-
came equivalent to that observed when dithiothreitol was
added in the beginning with the dexamethasone (data not
shown).

Effect of Dithiothreitol on the Stability of the Unbound
Receptor—The results of the foregoing experiments indicated
that (a) dithiothreitol could increase the binding capacity of
the cytosol without altering the rate of association of steroid
with the receptor and (b) that once the steroid-receptor
complex was formed, its stability was not significantly de-
pendent upon the presence of dithiothreitol. It remained to
be determined whether the stability of the unbound receptor
was affected by dithiothreitol. Cytosol was prepared without
dithiothreitol and the binding capacity was determined at
various times. As shown in Fig. 3, the unbound receptor
undergoes rapid inactivation in the absence of dithiothreitol
but this inactivation is readily and almost completely reversed
by the addition of dithiothreitol.

Inability of Inhibitors of Phosphatase Activity to Replace

Fig. 1. Specific binding of [(3H)dexamethasone in lactating
mouse mammary gland cytosol containing increasing concen-
trations of various sulfhydryl reagents. To aliquots of mam-
mary cytosol prepared in Tis buffer, dithiothreitol (.),β-mercaptoethanol
(©), or thioglycerol (©) were added at indicated concentrations. The
cytosol contained a background level of 12 mM Dithiothreitol in the
homogenization buffer and the data present for thioglycerol have
been corrected accordingly. Duplicate samples of each preparation
were incubated for 4 h at 4°C with 50 nM [(3H]dexamethasone alone
or in the presence of a 100-fold excess of unlabeled dexamethasone.
Bound radioactivity was determined by dextran-coated charcoal
adsorption.
Dithiothreitol—It has been proposed that the instability of glucocorticoid receptors in the absence of ligand in cell-free preparations of rat liver and mouse fibroblasts is due to dephosphorylation mechanisms involving phosphatases (20-22); in these studies, the receptor was stabilized by the addition of inhibitors of phosphatase activity such as fluoride or molybdate. We therefore compared our observed effect of dithiothreitol with the effect of known inhibitors of phosphatase activity; molybdate, fluoride, and levamisole (1 to 50 mM) were unable to replace dithiothreitol in increasing the binding capacity of the unlabeled cytosol (data not shown).

Ability of Dithiothreitol to Restore Binding Activity Lost during Gel Filtration—The cytoplasmic glucocorticoid receptors of liver appear to lose most of their binding activity if subjected to gel filtration in the absence of ligand (23). To ascertain whether the loss of binding activity during gel filtration is analogous to receptor inactivation due to lack of sulfhydryl reagents, we studied the effect of gel filtration and dithiothreitol on the binding activity of the unoccupied cytoplasmic glucocorticoid receptors of mammary gland (Table II). In the absence of added dithiothreitol, the unlabeled cytosol loses much of its binding capacity upon gel filtration. If dithiothreitol is added to the cytosol prior to gel filtration, the binding capacity is still lost if the elution buffer does not contain dithiothreitol. However, if dithiothreitol is added prior to gel filtration and also added to the elution buffer, most of the binding activity can be recovered. Furthermore, in all

![Figure 2](image1.png)

**FIG. 2.** Association kinetics for the specific binding of [H]dexamethasone to lactating mouse mammary gland cytosol in the presence (■) or absence (○) of dithiothreitol. Cytosol was prepared with or without 20 mM dithiothreitol in Tris buffer and incubated with 50 nM of [H]dexamethasone alone or in the presence of a 100-fold excess of unlabeled dexamethasone. At the times indicated, the bound radioactivity present in 1 ml samples of the incubations was determined by dextran-coated charcoal adsorption. The inset represents an analysis of the kinetic data for the association of [H]dexamethasone with the receptors in the presence or absence of dithiothreitol. The free dexamethasone concentrations determined by subtracting the specifically bound steroid from the total dexamethasone concentration. The total receptor concentration was assumed to be equal to the specifically bound [H]dexamethasone present at equilibrium (4 h). The calculated association rate constants for this experiment were both 3.2 x 10^4 M^-1 min^-1.

**TABLE I**

The requirement of dithiothreitol for glucocorticoid-binding activity in mammary cytosol prepared by various speeds of centrifugation

Mammary gland homogenate prepared in Tris buffer, as described under “Materials and Methods,” was divided into three samples and centrifuged as indicated below. Aliquots of the resulting supernatants with or without 20 mM dithiothreitol were incubated in duplicate for 4 h at 4°C with 50 nM [H]dexamethasone alone or in the presence of a 100-fold excess of unlabeled dexamethasone. Bound radioactivity was determined by dextran-coated charcoal adsorption.

![Figure 3](image2.png)

**FIG. 3.** Time course for the loss of specific binding of [H]dexamethasone in cytosol incubated in the presence of steroid at 4°C (○) and the subsequent reappearance of binding following the addition of 20 mM dithiothreitol (□). Cytosol prepared in Tris buffer was incubated at 4°C. At the indicated times, duplicate samples were removed and incubated for 4 h with 50 nM [H]dexamethasone alone or in the presence of a 100-fold excess of unlabeled dexamethasone. Bound radioactivity was determined by dextran-coated charcoal adsorption.

**TABLE II**

Effect of dithiothreitol and Sephadex G-25 chromatography on binding of [H]dexamethasone in mammary cytosol

Samples (1.0 ml) of mammary cytosol prepared in Tris buffer were chromatographed through columns (1 x 20 cm) of Sephadex G-25 eluted with Tris buffer ± dithiothreitol and assayed (±dithiothreitol) for steroid binding as indicated. Dithiothreitol when added was present at 10 mM. The protein concentrations of the chromatographed samples were roughly half those of the control samples. All samples were incubated with steroids and assayed for specific binding as in Fig. 1.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Presence (+) or absence (−) of dithiothreitol</th>
<th>Specific binding capacity (fmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytosol + dithiothreitol (Without chromatography)</td>
<td>229</td>
<td></td>
</tr>
<tr>
<td>Cytosol</td>
<td>−</td>
<td>47</td>
</tr>
<tr>
<td>Cytosol + dithiothreitol</td>
<td>+</td>
<td>213</td>
</tr>
<tr>
<td>Cytosol + dithiothreitol</td>
<td>−</td>
<td>60</td>
</tr>
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<td>Cytosol + dithiothreitol</td>
<td>−</td>
<td>175</td>
</tr>
<tr>
<td>Cytosol + dithiothreitol</td>
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<td>189</td>
</tr>
<tr>
<td>Cytosol + dithiothreitol</td>
<td>+</td>
<td>164</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sample</th>
<th>During chromatography</th>
<th>During steroid-binding assay</th>
<th>Specific binding capacity (fmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytosol</td>
<td>−</td>
<td>−</td>
<td>229</td>
</tr>
<tr>
<td>Cytosol</td>
<td>+</td>
<td>+</td>
<td>47</td>
</tr>
<tr>
<td>Cytosol + dithiothreitol</td>
<td>−</td>
<td>−</td>
<td>213</td>
</tr>
<tr>
<td>Cytosol + dithiothreitol</td>
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<td>+</td>
<td>60</td>
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<td>Cytosol + dithiothreitol</td>
<td>+</td>
<td>+</td>
<td>175</td>
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<tr>
<td>Cytosol + dithiothreitol</td>
<td>+</td>
<td>−</td>
<td>189</td>
</tr>
<tr>
<td>Cytosol + dithiothreitol</td>
<td>+</td>
<td>+</td>
<td>164</td>
</tr>
</tbody>
</table>
cases where the binding activity was lost due to the absence of dithiothreitol in the elution buffer, addition of dithiothreitol to the eluate restored most of the binding capacity of the filtered cytosol.

**Inactivation Due to High Ionic Strength**—As stated earlier, the initial objective of these studies was to partially purify the native cytoplasmic glucocorticoid receptor of mammary glands. After ascertaining the importance of sulfhydryl reagents to the steroid-binding reaction, attempts were made to partially purify the receptor by salt precipitation. In previous studies from our laboratory, we have demonstrated that steroid-free estrogen receptor from lactating mammary glands can be successfully precipitated with ammonium sulfate (24). Similar experiments on glucocorticoid receptors revealed that while receptors formed in a complex with dexamethasone could be precipitated with good recovery, ammonium sulfate precipitates from steroid-free cytosol had no binding activity (data not shown). Since in all of these studies, dithiothreitol was included in the buffer, the observed inactivation could not be attributed to lack of sulfhydryl reagents.

It has been reported that glucocorticoid receptors in rat thymocytes (25), mouse fibroblasts (26), and rat liver (22) can be inactivated by buffers containing salt. Accordingly, the effect of salt on the stability of the glucocorticoid receptor in mammary cytosol was investigated. To distinguish the salt inactivation from the inactivation due to lack of sulfhydryl reagents, all experiments were performed with cytosols containing dithiothreitol. The results of a typical experiment are shown in Fig. 4; the unlabeled receptor is highly unstable in the presence of potassium chloride; the extent of inactivation was dependent on the salt concentration and almost all the receptor activity was lost with 0.3 M KC1. Sodium chloride gave similar results at the same concentrations and ammonium sulfate (20% saturation) also completely inactivated the receptor (results not shown). Next, the salt was removed from the reaction mixture by dialysis, dilution, or Sephadex G-25 filtration and the subsequent ability of the receptor to bind dexamethasone was tested; the results of these experiments revealed that while the procedures used to reduce the salt concentration did not in themselves affect the binding capacity of control cytosol greatly, the inactivation of the receptor by KC1 was irreversible (data not shown). Several known inhibitors of phosphatase activity were tested for their ability to protect the unlabelled receptor from inactivation by KC1. Fluoride and levamisole, tested at concentrations ranging from 0.1 to 100 mM, did not protect the receptor from inactivation due to KC1 (data not shown). However, as shown in Fig. 5, molybdate was effective in protecting the receptor from inactivation due to KC1 and its effect varied with concentration, the optimal concentration being 5 to 10 mM. In the absence of KC1, molybdate at all concentrations tested had no effect on the binding capacity of the cytosol (Fig. 5). Studies on the time course of the effect of molybdate in protecting the receptor against inactivation due to KC1 indicated that the effect of molybdate was instantaneous (data not shown). Thus, molybdate, even when added just prior to the addition of KC1, was able to protect against receptor inactivation but, as shown in Fig. 6, if added after KC1, molybdate had no effect. The inability of molybdate to reverse the inactivation of the receptor by salt also persisted even after the salt concentration had been considerably reduced by the techniques of dialysis, dilution, or Sephadex G-25 filtration (data not shown).

**Inactivation Due to Temperature**—In a recent article on the glucocorticoid receptors of thymocytes, Sando et al. (27) have demonstrated that molybdate can prevent the inactivation of glucocorticoid receptors occurring at elevated temperature. Therefore, to assess whether the observed inactivation of mammary glucocorticoid receptors by ionic strength was analogous to the heat-induced inactivation of receptors from thymocytes, we investigated the effect of temperature on the stability of ligand-free glucocorticoid receptors of mammary tissues. If mammary cytosol is heated, the glucocorticoid receptor loses its binding capacity progressively as a function of time and temperature (Table III); at all temperatures, molybdate greatly reduced the inactivation of the receptor (Table III) but fluoride (10 to 50 mM) had no effect on this temperature-mediated inactivation (not shown).
Table III

Molybdate protection of temperature-mediated receptor inactivation

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Specific binding capacity (%)</th>
<th>No molybdate</th>
<th>With molybdate</th>
</tr>
</thead>
<tbody>
<tr>
<td>0°C</td>
<td></td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>15</td>
<td></td>
<td>78.8</td>
<td>91.8</td>
</tr>
<tr>
<td>20</td>
<td></td>
<td>52.8</td>
<td>89.9</td>
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<td></td>
<td>18.5</td>
<td>85.0</td>
</tr>
<tr>
<td>30</td>
<td></td>
<td>0.8</td>
<td>62.5</td>
</tr>
<tr>
<td>37</td>
<td></td>
<td>0.2</td>
<td>9.1</td>
</tr>
</tbody>
</table>

Relative Abilities of Dexamethasone and Molybdate in Preventing Receptor Inactivation—An important observation in previous studies on glucocorticoid receptor inactivation was the ability of the steroid to partially protect the receptor from inactivation (20, 28). The receptor inactivation which occurs as a function of time in the absence of sulphydryl reagents (Fig. 3) can be partially prevented if the receptor is formed in the presence of dexamethasone (Fig. 2). Since molybdate can also partially prevent receptor inactivation (Fig. 5 and Table III), it was of interest to compare the relative efficacy of dexamethasone versus molybdate for this purpose. The results shown in Table IV indicate that, similar to molybdate, dexamethasone is able to partially prevent receptor inactivation in the presence of dithiothreitol. Thus, it appears that receptors which have bound dexamethasone are partially protected against both the effects of oxidation of sulphydryl groups and the effects of exposure to high salt or temperature. Dithiothreitol is required to give maximal dexamethasone binding and hence protection, by the steroid, against inactivation, but unbound receptors are inactivated by KC1 or heat both in the presence and absence of dithiothreitol (data not shown). Also, receptors protected by molybdate against KC1 or temperature inactivation exhibit identical dexamethasone-binding activities whether dithiothreitol is present throughout the treatment or for the steroid-binding reaction only (data not shown). That is, dithiothreitol affects the steroid-binding reaction but has no apparent effect on the inactivation of steroid-free receptors by KC1 or temperature.

Sucrose Gradient Analysis of Glucocorticoid Receptor Interactions with Molybdate—The experiments conducted so far clearly established that molybdate as well as steroid can protect the mammary glucocorticoid receptor from inactivation occurring in the presence of dithiothreitol. Steroid receptors may undergo conformational changes upon binding the steroid (9, 11-13) and, since the protective effect of molybdate may be analogous to that of the steroid, it was of interest to see if molybdate could alter the physical properties of the receptor. Therefore, sucrose gradient analysis of glucocorticoid receptor interaction with molybdate was performed.

Previous studies from our laboratory and others (7, 8) have demonstrated that the cytoplasmic glucocorticoid-receptor of rodent mammary tissues sediments as a 6 to 7 S complex on low ionic strength gradients. As shown in Fig. 7A, the resolution of the receptor-bound radioactivity on low ionic strength gradients is considerably affected by the choice of buffer although the rate of sedimentation was the same as reported previously, 6.6 ± 0.1 S (mean ± S.E., n = 4). Resolution of the receptor-bound radioactivity was worst when cytosol was prepared in Tris buffer and centrifuged on sucrose gradients in Tris buffer and best when cytosol prepared in phosphate buffer was centrifuged on sucrose gradients in phosphate buffer. However, it appeared that the buffer of gradient centrifugation rather than homogenization was responsible for the better resolution since cytosol prepared in Tris buffer but layered on gradients in phosphate buffer yielded a sedimentation profile nearly identical with that of the cytosol prepared in phosphate buffer (Fig. 7B). The choice of homogenization buffer also had no effect on the observed specific binding of [3H]dexamethasone in cytosols assayed by the dextran-coated charcoal technique (data not shown).

When cytosol prepared in Tris buffer was pretreated with molybdate prior to adding the steroid, the resolution of receptor-bound radioactivity on Tris gradients was similar to that seen on phosphate gradients (Fig. 7C); it is also evident from Fig. 7C that the sedimentation rate of the steroid-receptor complex in molybdate-treated cytosol was not greatly different from that in the control cytosol.

The rate of sedimentation of the steroid-receptor complex in the control versus molybdate-treated cytosol was markedly different on high ionic strength gradients (Fig. 7D). The average rate of sedimentation of the receptor in control cytosol was 4.4 ± 0.1 S (mean ± S.E., n = 3) while that in the molybdate-treated cytosol was 5.4 ± 0.1 S (mean ± S.E., n = 3); this effect of molybdate was apparent only if the molybdate preceded KC1 in the treatment sequence. Similar results were obtained if the KC1 treatment was replaced by exposure of the cytosol to 25°C for 30 min (data not shown).

Effect of High Ionic Strength and Elevated Temperature on the Inactivation of Glucocorticoid Receptor versus Estrogen Receptor in the Same Cytosol—It is well established that the cytosol of rodent lactating mammary glands contains estrogen receptors (24, 29, 30) in addition to glucocorticoid receptors. Previous studies from our laboratory have demonstrated that even in the absence of ligand, partial purification of estrogen receptor from lactating mammary cytosol can be successfully achieved by precipitation with ammonium sulfate (24). This would suggest that, in contrast to glucocorticoid receptors, estrogen receptors without steroid do not undergo inactivation at high ionic strength. Nevertheless, the experimental conditions used for the glucocorticoid receptors in this report differed from those used for estrogen receptors in our previous work. It seemed possible that the processes of inactivation described here for glucocorticoid receptors might have been caused non-specifically by various components present in Tris buffer and best when cytosol prepared in phosphate buffer was centrifuged on sucrose gradients in phosphate buffer. However, it appeared that the buffer of gradient centrifugation rather than homogenization was responsible for the better resolution since cytosol prepared in Tris buffer but layered on gradients in phosphate buffer yielded a sedimentation profile nearly identical with that of the cytosol prepared in phosphate buffer (Fig. 7B). The choice of homogenization buffer also had no effect on the observed specific binding of [3H]dexamethasone in cytosols assayed by the dextran-coated charcoal technique (data not shown).

<table>
<thead>
<tr>
<th>Samples</th>
<th>Specific binding capacity (%)</th>
<th>No treatment (control)</th>
<th>0.4 M KC1</th>
<th>Temperature 25°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytosol</td>
<td>100</td>
<td>0.4</td>
<td>4.4</td>
<td></td>
</tr>
<tr>
<td>Cytosol + dexamethasone</td>
<td>100</td>
<td>96.3</td>
<td>70.6</td>
<td></td>
</tr>
<tr>
<td>Cytosol + molybdate</td>
<td>100</td>
<td>52.1</td>
<td>60.8</td>
<td></td>
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</tbody>
</table>
Fig. 7. Sucrose gradient profiles for the binding of $[^3H]$dexamethasone in the cytosol of lactating mammary tissue. Cytosol, prepared in the indicated buffer (20 mM dithiothreitol), was incubated for 4 h at 4°C with 50 nM $[^3H]$dexamethasone alone or in the presence of a 100-fold excess of unlabeled dexamethasone. Samples were assayed by charcoal-treated, layered on gradients (see "Materials and Methods"), and centrifuged at 189,000 × g for 18 h (Panels A to C) or 216,000 × g for 22 h (Panel D) at 4°C. Extreme standards were centrifuged on parallel gradients to estimate the sedimentation coefficients ("Materials and Methods"). The radioactive peaks of the low salt gradients (Panels A to C) correspond to 6 to 7 S forms while those of the high salt gradients (Panel D) are 4.3 S (○) and 5.4 S (□). α, $[^3H]$dexamethasone-labeled Tris cytosol (○) on sucrose gradient in Tris buffer; $[^3H]$dexamethasone-labeled barbital cytosol (△) on sucrose gradient in barbital buffer; $[^3H]$dexamethasone-labeled phosphate cytosol (□) on sucrose gradient in phosphate buffer; $[^3H]$dexamethasone plus excess unlabeled dexamethasone cytosol (×) on sucrose gradient in Tris buffer. B, $[^3H]$dexamethasone-labeled Tris cytosol (○) or phosphate cytosol (□) on sucrose gradients in phosphate buffer. C, $[^3H]$dexamethasone-labeled Tris cytosol with (●) or without (○) preincubation with 10 mM molybdate (4 h) and $[^3H]$dexamethasone-labeled plus excess unlabeled dexamethasone cytosol (×) on sucrose gradients in Tris buffer. D, phosphate cytosol labeled with $[^3H]$dexamethasone alone (○) or in the presence of a 100-fold excess of unlabeled dexamethasone (×) for 4 h and treated with 0.4 M KCl for 1 h, and phosphate cytosol treated with 10 mM molybdate (4 h), 0.4 M KCl (1 h), and $[^3H]$dexamethasone (4 h) (△) centrifuged on sucrose gradients in 0.4 M KCl phosphate buffer.

TABLE V
Effect of high ionic strength and temperature on steroid-free estrogen receptors of mammary cytosol

<table>
<thead>
<tr>
<th>Sample</th>
<th>Treatment</th>
<th>Specific binding capacity</th>
<th>% control</th>
</tr>
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<tbody>
<tr>
<td>Cytosol</td>
<td>None</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Cytosol</td>
<td>0.4 M KCl</td>
<td>83.3</td>
<td></td>
</tr>
<tr>
<td>Cytosol</td>
<td>25°C</td>
<td>79.3</td>
<td></td>
</tr>
<tr>
<td>Cytosol + 10 mM molybdate</td>
<td>0.4 M KCl</td>
<td>93.2</td>
<td></td>
</tr>
<tr>
<td>Cytosol + 10 mM molybdate</td>
<td>25°C</td>
<td>85.7</td>
<td></td>
</tr>
</tbody>
</table>

ent in the lactating mammary tissue cytosol. Therefore, steroid-free estrogen receptors of mammary cytosol were exposed to the inactivating conditions effective against the glucocorticoid receptors. The results in Table V indicate that only about 20% of the binding capacity of estrogen receptors is lost upon exposure to high ionic strength or elevated temperature and prior treatment of the cytosol with molybdate partially prevented the inactivation. Thus, consistent with the earlier observation, the estrogen receptors are relatively resistant to inactivation by high salt or temperature in the absence of steroid. This suggests that the inactivation of glucocorticoid receptors presented in this report is not due to nonspecific destruction of the receptor by various cellular components.

DISCUSSION

The data presented in this report demonstrate that mammary glucocorticoid receptors can undergo two types of inactivation in the absence of steroidligand. A major loss in binding activity occurs apparently through oxidation of sulphydryl groups as this inactivation is readily reversed with the addition of a sulphydryl-reducing agent such as dithiothreitol. A second type of inactivation, elicited by high ionic strength or high temperature, occurs even in the presence of dithiothreitol but can be partially prevented by molybdate. Dexamethasone-bound receptors are relatively resistant to both types of inactivation.

The importance of reduced sulphydryl groups for maximal steroid binding to cytoplasmic glucocorticoid receptors has

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been documented previously (19, 31), but the studies did not include mammary tissues. Our present report, therefore, is the first to demonstrate that glucocorticoid receptors of mammary glands can undergo inactivation in the absence of sulfhydryl reagents and that these reagents are crucial for maximal binding activity in tissue extracts. We have also demonstrated that dithiothreitol is much more effective than either mercaptoprotohol or monothioglycerol and, in light of the results presented here, it is clear that the concentration of monothio-

hydroperoxide in previous studies on mammary tissues was not sufficient to estimate the total number of glucocorticoid rece-

ptors present. In this context, it is necessary to point out that our finding for a requirement of 10 mM dithiothreitol for mammary gland receptor is 5 times in excess of the 2 mM used by Granberg and Ballard (19) for estimating the glucocorticoid-binding capacity of various other tissue extracts. Since it is possible that 10 mM dithiothreitol is required for estimating the binding capacity of lactating mammary tissues only, it may be necessary to investigate the optimal concentration of dithiothreitol required to estimate the concentration of glu-
corticoid receptors in various types of mammary tissues includ-
ing mammary tumors.

In a series of articles (20–22), Pratt and his colleagues have proposed that the inactivation of glucocorticoid receptors occurring in the absence of ligand may occur through a phos-
phorylation-dephosphorylation mechanism; the support-
ing evidence was that (a) calf intestinal alkaline phosphatase could inactivate glucocorticoid receptors, (b) addition of phos-
phatase inhibitors could inhibit the inactivation, and (c) re-

moval by centrifugation of a membrane-bound inactivating enzyme, which was sensitive to phosphatase inhibitors, re-

sulted in a slower rate of receptor inactivation. The inactiva-
tion of the glucocorticoid receptors in mammary cytosol oc-
curring in the absence of dithiothreitol does not appear to be mediated through phosphatascs since the simple addition of dithiothreitol is capable of restoring dexamethasone binding, while known inhibitors of phosphatase activity cannot replace dithiothreitol in preventing or reversing the binding loss.

A comparison of our data on mammary receptor inactiva-
tion with previous data for other tissues (20–22, 27, 32) reveals that the cytoplasmic mammary glucocorticoid receptor may be most similar to that of thymocytes. One notable difference between thymocytes and mammary tissue was the behavior of the receptor during gel filtration in the absence of ligand; while receptors in both tissues lost binding activity upon gel filtration, dithiothreitol alone was enough to restore the bind-
ing activity in mammary cytosol but molybdate in addition to dithiothreitol was necessary for restoring the binding activity of thymocytes. The most significant features of the phenom-

enon of mammary glucocorticoid receptor inactivation result-

ing from a lack of sulfhydryl reagents are the ready reversi-

bility by dithiothreitol and the inability of inhibitors of phos-
phatase activity, including molybdate, to prevent or reverse this inactivation. These features distinguish this inactivation from that resulting from exposure of cytosol to high ionic strength or elevated temperature.

The inactivation of glucocorticoid receptor by high ionic strength or elevated temperature can occur whether sulfhy-
dryl reagents are present or not. However, addition of 10 to 20 mM dithiothreitol is necessary in order to obtain a reliable measurement of the number of binding sites present under any given experimental condition. Thus, the binding was the same whether dithiothreitol was added before or after the salt or heat treatment and, similarly, the protection by molybdate against heat or salt inactivation did not differ whether dithi-

othreitol was added before or after any of the other treatments utilized. The major distinction between the effect of dithio-

threitol on the sulfhydryl-related inactivation, and molybdate on the salt- or heat-induced inactivation, is that while dithio-

threitol is effective whether added before or after inactivation of the receptor, molybdate can only exert its effect when added before the receptor inactivation. That is, molybdate cannot reverse the high ionic strength or temperature-effected inactivation.

Our analysis of the glucocorticoid receptors by sucrose gradients clearly revealed that molybdate can affect the con-
formation of the receptor and also protect the steroid-recep-
tor complex from dissociation or aggregation. However, the effect of molybdate on the receptor may be direct or indirect and the molecular basis for the molybdate effect must await future research. In any case, it appears that molybdate is not exerting its effect simply by virtue of its property as a phos-
phatase inhibitor.

Extensive studies on various target tissues have docu-

mented that cytoplasmic glucocorticoid receptors, when bound to steroid can undergo in vitro conformational changes resulting in several altered physicochemical properties of the receptor. Such changes have been termed "transformation" or "activation" and are thought to be related to the processes involved in the in vitro cytoplasm-to-nucleus translocation of receptors (33–35). Elevated temperature and high ionic strength enhance the transformation process (33–36) and the "transformed" steroid-receptor complexes exhibit the ability to bind to DNA (23, 35, 37).

The differentiation between the behavior of the receptor under the conditions of elevated temperature and high ionic strength, as described in this report, and that classified under transformation of the steroid-receptor complex, is the absence of ligand in the present studies. In preliminary experiments, we have found that the conditions of exposure to 0.4 M KCl or 25°C, if used on steroid-free receptor, will lead to irreversible inactivation of the receptor, while the same treatment of the steroid-bound receptor will lead to enhanced DNA binding. Furthermore, molybdate, when added to steroid-bound recep-
tor prior to exposure to salt or elevated temperatures, will inhibit the binding of the steroid-receptor complex to DNA apparently by interfering with the transformation process. A similar effect of molybdate on the binding of receptor com-
plexes to DNA has recently been observed for rat liver glu-
cocorticoid receptors (38) and chick ovovid progesterone receptors (39). It has been proposed that the transformation of steroid-receptor complex may involve exposure of posi-
tively charged groups on the surface of the receptor (34) which results in the increased affinity of the steroid-receptor com-
pex to DNA. If, in fact, the interaction of molybdate with the receptor is a direct one, molybdate-treated receptors can be used as a probe to study the mechanism of receptor interaction with DNA.

The in vitro transformation of hormone-bound steroid re-
ceptors to their DNA-binding form provides a basis for un-

derstanding the in vivo processes which result in the nuclear translocation of steroid-receptor complexes. We have shown herein that the conditions for in vitro transformation also inactivate unbound glucocorticoid receptors but we do not know if a similar inactivation can occur in vivo; that is, it would be of interest to determine whether a proportion of in vitro cytoplasmic glucocorticoid receptors can exist in a form unable to bind steroid. Such inactivation would be of impor-
tance to the cellular responsiveness to glucocorticoids. In any case, our observations regarding the in vitro inactivation of the mammary glucocorticoid receptor and the prevention of inactivation will be useful for the eventual purification and detailed characterization of this receptor. The prevention of inactivation will also allow for a more accurate quantitation
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of glucocorticoid receptor levels in mammary tumors. Mammary tumor regression occurs in about 15% of patients receiving glucocorticoids (40) and recently it has been reported that about 50% of human mammary tumors contain glucocorticoid receptors (41). Although the quantity of estrogen receptors in these tumors is known to be indicative of patient response to endocrine therapy (42), it is not known whether knowledge of glucocorticoid receptor levels can be used to predict the effectiveness of glucocorticoid therapy.

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