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# STABILIZATION OF GLUCOCORTICOID RECEPTOR ASSOCIATION WITH RNA BY A LOW MOLECULAR WEIGHT FACTOR FROM RAT LIVER CYTOSOL

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**Summary**—A small (Mr < 500) anionic, heat-stable molecule has been identified in rat liver cytosol which prevents the RNase-induced decrease in the glucocorticoid receptor sedimentation properties which we described previously. This factor, which can be removed by dialysis, molecular exclusion chromatography, or ultrafiltration, functions as a true stabilizer of the RNA–glucocorticoid receptor association, and not as a RNase inhibitor. Preliminary characterization shows that the factor is not a protein, nucleic acid, or nucleotide, is not absorbed by activated dextran-charcoal, and is unaffected by extraction with organic solvents. This factor prevents activation of the glucocorticoid receptor by dilution. The relationship of this stabilization factor to a low molecular weight activation inhibitor described by others is discussed.

## INTRODUCTION

Steroid hormones appear to exert their tissue effects by first binding to a specific receptor protein, then in some fashion interacting with the genome to regulate the level of transcription [1–4]. In some cases, specific sequences in the DNA with a particular affinity for the steroid receptor have been identified [5, 6], although it is still far from clear whether transcriptional regulation can be explained on the basis of steroid receptor complex interaction with DNA alone. Particular chromosomal proteins may play important roles, for instance, in defining or limiting the degree of steroid receptor–DNA interactions [4].

Neither the means by which the receptor itself functions nor the actual nature of the functional receptor is yet clear. For instance, the exact number and type of subunits of the receptor is still unknown, and a number of different factors have been suggested as possible modulators of receptor function [7, 8]. These factors have been postulated to play roles in the ability of the receptor to bind to the hormone, to become activated, and to bind to DNA. In particular, a group of low molecular weight inhibitors of activation have been identified [9–16]. These are small molecules (generally less than a few thousand Daltons) of rather ill-defined chemical characteristics.

We have recently suggested that RNA may be associated with the glucocorticoid receptor when it is isolated in low salt buffers. We have also shown that, following RNase treatment, the sedimentation profile of the receptor is shifted from 7–8S to 3–4S, and there is an increase in DNA-binding [17]. Further, we have presented evidence which implies that the receptor may bind DNA and RNA concurrently, suggesting that there may be separate RNA and DNA binding domains [18].

Here we report on the identification of a low molecular weight factor which stabilizes the glucocorticoid receptor to RNase digestion, causes the complex to sediment as the 9–10S form, and prevents binding to DNA.

## EXPERIMENTAL

All reagents were of analytical grade. Dexamethasone [6,7-$^3$H(*N*)], 48.9/Ci/mmol was purchased from New England Nuclear. RNase A (3002 U/mg), and RNase-free DNase I (1872 U/mg) were purchased from Cooperbiomedical. Alkaline phosphatase (1090 U/mg), chymotrypsin (62 U/mg), and proteinase K (16.3 U/mg) were purchased from Sigma.

### *Preparation of radioactive glucocorticoid receptor and stabilizing factor.*

Receptor was prepared from the livers of Sprague–Dawley male rats 4–21 days after adrenalectomy. The animals were sacrificed by cervical dislocation and the livers were perfused *in situ* with ice-cold 0.9% NaCl. All steps of the preparation were performed at 0–4°C. The livers were removed and homogenized in 1 vol of 20 mM Tris–HCl, pH 7.5, – 1.5 mM EDTA-1 mM dithiothreitol. The homogenate was centrifuged first at 600 *g* for 10 min, and then at 105,000 *g* for 60 min. The supernatant was made to 28 nM with dexamethasone [6,7-$^3$H(*N*)], 48.9 Ci/mmol and incubated on ice for 2 h. Specific binding was at least 90% as judged by the dextran-charcoal technique [19].

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The low molecular weight stabilizing factor was prepared as described above except that the homogenization occurred in 2 vol of the Tris–EDTA–dithiothreitol buffer.

*Dilution assay for the presence of the low molecular weight factor*

In the assay to determine the presence of the stabilizing factor, 0.025 ml of the receptor (12,000–27,000 cpm/0.1 ml) was diluted with either 0.2 ml of Tris–EDTA–dithiothreitol buffer, or with 0.2 ml of a fraction containing the factor. The mixture was incubated at 20°C for 10 min and layered onto 5–20% sucrose gradients in Tris–EDTA–dithiothreitol buffer containing 10 mM sodium molybdate. Centrifugation was performed in a VTi 65 rotor (65,000 rpm for 97 min) at 2°C. Following centrifugation, the tubes were punctured at the bottom and 0.2 ml fractions collected. In the presence of the factor, the diluted receptor sediments at the 9–10S region of the gradient, while in its absence most of the receptor sediments at the 7–8S region. Ovalbumin (3.7S) and sweet potato $\beta$-amylase (9.4S) were used as external markers.

When the gradient analyzed receptor was to be assayed for DNA-binding ability, the fractions from 3 gradients were pooled, and each of these was tested for the ability to bind to DNA. Total radioactivity from the DNA-cellulose assay (unbound and bound) was used to determine the gradient profile of the glucocorticoid receptor.

*DNA-cellulose column assay*

DNA-cellulose was prepared as described by Alberts and Herrick [20], and contained 0.250 mg DNA per ml (packed volume) of DNA-cellulose.

All steps of the assay were performed at 0–4°C. Combined fractions from the gradients (0.6 ml) were mixed with DNA-cellulose (0.4 ml of a 50% slurry) and incubated on ice for 15 min with frequent stirring. The mixture was poured into glass columns, and the columns were washed 4 times with 1 ml Tris–EDTA–dithiothreitol buffer. The initial eluant and the washes constituted the unbound fraction. The bound glucocorticoid receptor was removed with 3 ml of Tris–EDTA–dithiothreitol buffer containing 0.6 M KCl. Aliquots of each fraction were processed for scintillation counting.

## RESULTS

We previously reported evidence which suggested that the glucocorticoid receptor from rat liver is isolated as a ribonucleoprotein complex [17, 18]. The basis for this suggestion was that the large 7–8S glucocorticoid receptor could be converted to the 3–4S by treatment with RNase A or RNase $T_1$. During the course of these studies, we noted that the ability of the nuclease to convert the large form to the small form varied with receptor preparation, from lows of 20% conversion to highs of 100% conversion to the 3–4S form. The unconverted receptor would invariably run at the 9–10S region of sucrose gradients without molybdate. This is the reported sedimentation value of the unactivated receptor when centrifuged in gradients containing molybdate [21]. This suggested to us that the receptor preparation consisted of a mixture of RNase-sensitive 7–8S material and RNase-insensitive 9–10S material.

In order to determine the nature of the RNase sensitivity, we attempted to convert RNase-insensitive material to the RNase sensitive form. We had previously reported that dilution of the receptor preparation enhanced RNase-induced conversion to the 3–4S form, suggesting that some factor was dissociating from the receptor [17]. To ascertain whether this factor was a small molecule, we passed unactivated receptor prepared in the absence of molybdate over a G-25 column and then determined the sedimentation value of the receptor. As can be seen in Fig. 1, the control receptor sediments predominantly as the 9–10S form, with a shoulder in the 7–8S region, in gradients containing molybdate. It should be noted that amount of glucocorticoid receptor which sediments at the 7–8S varies with the receptor preparation, but is always less than 9–10S form. The

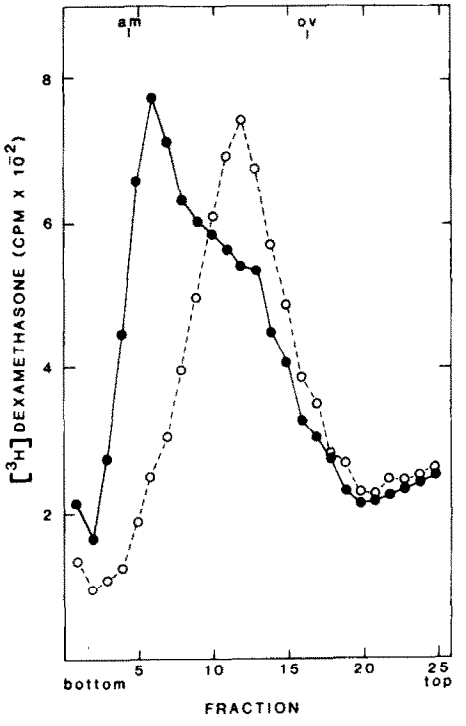


Fig. 1. Effect of passage through Sephadex G-25 on glucocorticoid receptor sedimentation profile. The glucocorticoid receptor was passed through a Sephadex G-25 column (21 × 0.9 cm) in Tris–EDTA–dithiothreitol buffer. The Sephadex-treated receptor and control receptor were adjusted to 10,400 cpm/0.1 ml and centrifuged as described in Experimental. Closed circles: control receptor; open circles: receptor which has been passed through G-25.

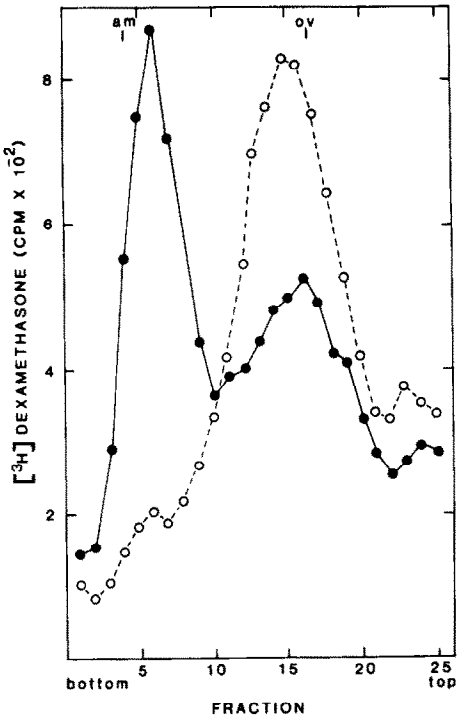


Fig. 2. RNase A treatment of G-25 treated and untreated glucocorticoid receptor. Conditions were as described in the legend to Fig. 1, except that prior to centrifugation the receptor preparations were treated with 50 $\mu$g of RNase A for 10 min at 20°C. Closed circles: control receptor; open circles: receptor which has been passed through G-25.

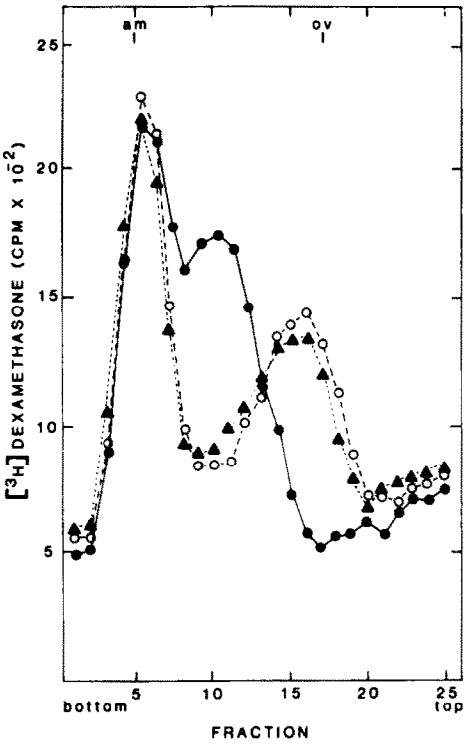


Fig. 3. Effect of various amounts of RNase A on the glucocorticoid receptor. Glucocorticoid receptor (13,700 cpm/0.15 ml) was treated with buffer only (closed circles), 250 $\mu$g of RNase A (open circles) or 1000 $\mu$g of RNase A (closed triangles) and centrifuged as described in Experimental.

ratio of receptor in the 9–10S region to that in the 7–8S region is sometimes as high as 8, but never lower than 2. This variation is presumably due to varying degrees of dissociation of a small molecule during receptor preparation. The receptor that had been passed through the G-25 column sedimented at 7–8S. This data imply that a small molecule (MR < 5000) is required to form the 9–10S form of the receptor.

These results are consistent with the observations of others who have hypothesized the existence of a small molecule which is an inhibitor of glucocorticoid receptor activation [8–16]. We confirmed the small molecule nature of this factor by showing that a substantial quantity of receptor could be converted to the 7–8S form by dialysis for as little as 30 min and by passage of the factor through an Amicon YCO5 filter, a filter which reportedly excludes molecules with Mr less than 500 (data not shown).

We next determined whether conversion to the 7–8S form coincided with an increase in RNase sensitivity. As shown in Fig. 2, the G-25 treated receptor can essentially be completely converted to the 3–4S form by RNase treatment, with only a small amount of 9–10S material remaining, while in the case of the control receptor, only the 7–8S shoulder is processed. The remaining receptor sediments as a sharp 9–10S peak. As we reported previously, this effect is seen with RNase that has been boiled for 15 min and is not prevented by the addition of a variety of protease inhibitors, but is prevented by human placental RNase inhibitor [17]. This suggests that if the receptor is associated with a low molecular weight factor, it is refractory to RNase treatment. It is unlikely that the factor is an RNase inhibitor, since large amounts of RNase convert only the 7–8S shoulder to the 3–4S form (Fig. 3). Even when the amount of RNase is quadrupled, there is no loss of radioactivity from the 9–10S region of the gradient. Furthermore, the cytosol is unable to prevent the digestion of radioactive poly U by RNase A, whereas human placental RNase inhibitor completely prevents the digestion of the polynucleotide (data not shown). This implies that the factor is not an RNase inhibitor, but instead stabilizes the receptor–RNA association.

We wished to further characterize the factor. The work of others [9] has suggested that a low molecular weight factor dissociates from the receptor when it is diluted, while dilution of the receptor in the presence of the factor prevents dissociation. If our receptor is diluted, there is a decrease in the 9–10S material with a concomitant increase in the 7–8S material (Fig. 4). If the dilution of the steroid-bound receptor is made in the presence of cold cytosol, there is no dilution

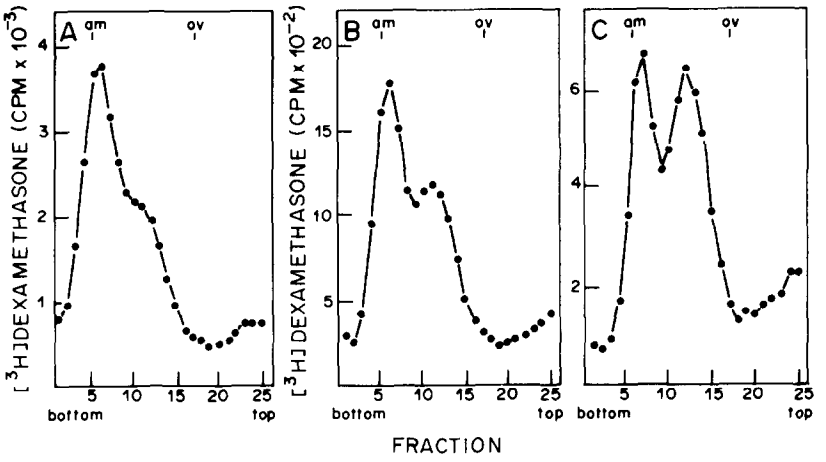


Fig. 4. Effect of dilution on the sedimentation profile of the glucocorticoid receptor. Glucocorticoid receptor (39,600 cpm/0.2 ml) was diluted with Tris–EDTA–dithiothreitol buffer and 0.2 ml was centrifuged as described in Experimental. A: undiluted receptor; B: receptor diluted with 1 vol of Tris–EDTA–dithiothreitol buffer; C: receptor diluted with 4 vol of Tris–EDTA–diethiothreitol buffer.

relative to the factor and no shift in the sedimentation value. In fact, the small amount of 7–8S receptor that is present in the undiluted receptor can be converted to 9–10S material in the presence of uncharged supernatant (data not shown). This suggested to us that the association of the factor with the receptor is reversible. We confirmed this by passing the steroid-bound receptor through a G-25 column to generate the 7–8S form. If this receptor is then incubated with uncharged cytosol, a significant portion of the receptor now sediments as the 9–10S form (Fig. 5). These results indicate that the association of the receptor with the low molecular weight factor is at least partially reversible. The reason why all of the 7–8S receptor could not be converted to the 9–10S form is under investigation.

Using the dilution assay, we were able to show that the receptor diluted in buffer to generate the 7–8S form, was sensitive to RNase (Fig. 6B) while receptor diluted with cold cytosol to maintain the 9–10S form, was not digested by the nuclease (Fig. 6A). In the experiment shown, a small amount of 7–8S material as well as 9–10S material remained following the digestion.

This dilution assay provided us with a means by which we could partially characterize the low molecular weight factor. As mentioned above, the factor can be removed from the cytosol by dialysis. The factor is retained on DEAE-cellulose, suggesting that it is negatively charged. This is confirmed by the fact that the receptor is not retained on CMC-cellulose. The factor is heat stable, retaining all activity when boiled for 15 min. The factor is not absorbed by dextran-charcoal, suggesting that it is neither a steroid nor a nucleotide as these are strongly adsorbed by activated dextran-charcoal [9, 16].

Further characterization involved treatment with various hydrolytic enzymes (Table 1). The factor is apparently not a protein, RNA, or DNA. Others have shown that pyridoxal phosphate [22] and adenine nucleotides [23] may play a role in modulating receptor activity. None of these compunds prevented the dilution effect observed in the absence of the low molecular weight factor (Table 1). Finally, a factor

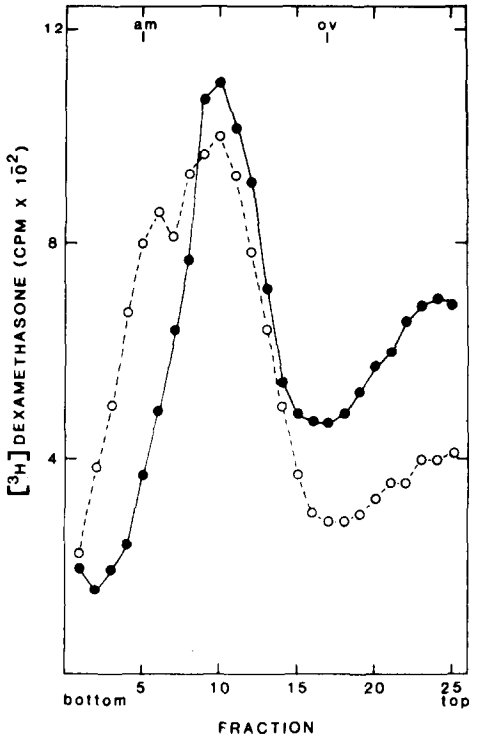


Fig. 5. Regeneration of the 9–10S form of the glucocorticoid receptor. Glucocorticoid receptor was passed through a G-25 Sephadex column as described in the legend to Fig. 1. The receptor (25,000 cpm/0.1 ml) was then diluted with 4 vol of Tris–EDTA–dithiothreitol buffer (closed circles) or with 4 vol of cold supernatant (open circles), and 0.2 ml was centrifuged as described in Experimental.

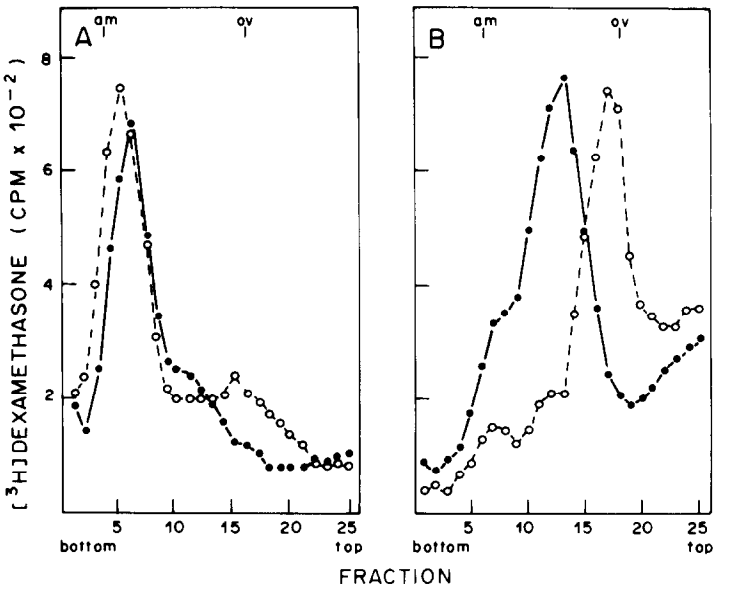


Fig. 6. Susceptibility of diluted receptor to RNase A treatment. A: Glucocorticoid receptor was diluted with 4 vol of unlabelled cytosol and incubated at 20°C for 10 min with buffer only (closed circles) or with 50 $\mu$g of RNase A (open circles). B: Glucocorticoid receptor was diluted with 4 vol of Tris–EDTA–dithiothreitol buffer and incubated at 20°C for 10 min with buffer only (closed circles) or with 50 $\mu$g of RNase A (open circles). Following these incubations, 8000 cpm/0.2 ml was centrifuged as described in Experimental.

with similar properties can be found in the kidney, spleen, thymus, and testes, but interestingly, not in the ventral prostate or seminal vesicle (data not shown).

The properties of this factor are similar to those of other low molecular weight inhibitors of activation which have been described by others [9–16]. Since activation of the receptor can be achieved by dilution [9], we tested whether our factor inhibited activation by dilution. We diluted the unactivated receptor with either buffer or cold cytosol, centrifuged it on a sucrose gradient, and then assayed the

Table 1. Attempts to destroy or mimic the effects of low molecular weight factor

| Controls | 9–10S/7–8S (Avg) | Number of trials | Range |
|---|---|---|---|
| Receptor only (no dilution) | 4.73 | 9 | (2.65–6.50) |
| Receptor diluted with low molecular weight factor | 5.05 | 12 | (2.44–10.30) |
| Receptor diluted with buffer | 0.45 | 13 | (0.32–0.71) |
| *Dilution with factor treated with:* | | | |
| Alkaline phosphatase | 3.81 | 3 | (1.72–6.07) |
| Proteinase k | 3.50 | 3 | (2.12–6.11) |
| DNase I | 3.91 | 5 | (1.75–5.87) |
| RNase A | 4.60 | 3 | (3.70–5.91) |
| Chymotrypsin | 3.61 | 6 | (1.90–5.21) |
| *Dilution with buffer containing:* | | | |
| AMP (1 mM) | 0.49 | 2 | (0.39–0.59) |
| AMP (5 mM) | 0.64 | 1 | |
| ADP (1 mM) | 0.41 | 1 | |
| ADP (5 mM) | 0.43 | 2 | (0.38–0.48) |
| ATP (1 mM) | 0.46 | 2 | (0.43–0.48) |
| ATP (5 mM) | 0.49 | 2 | (0.48–0.51) |
| ATP (10 mM) | 0.48 | 2 | (0.39–0.56) |
| *c*AMP (1 mM) | 0.36 | 1 | |
| *c*AMP (5 mM) | 0.51 | 2 | (0.36–0.65) |
| Pyridoxal 5′-phosphate (1 mM) | 0.58 | 3 | (0.29–0.73) |
| Pyridoxal 5′-phosphate (5 mM) | 0.45 | 3 | (0.25–0.56) |
| Pyridoxal 5′-phosphate (10 mM) | 0.34 | 3 | (0.16–0.44) |

The low molecular weight factor was treated with 100 $\mu$g of the indicated enzyme for 60 min at 37°C. Prior to using the treated factor in the dilution assay, the enzymes were removed by filtering through an Amicon YM5 filter (which retains molecules with Mr < 5000), or the enzyme activity was destroyed by boiling for 15 min. In the case of the nucleotides, the receptor was diluted in the presence of the indicated concentration of nucleotides in Tris–EDTA–dithiothreitol buffer. Results are presented as the ratio of the radioactivity located in the 9–10S region of the gradient to that found in the 7–8S region.

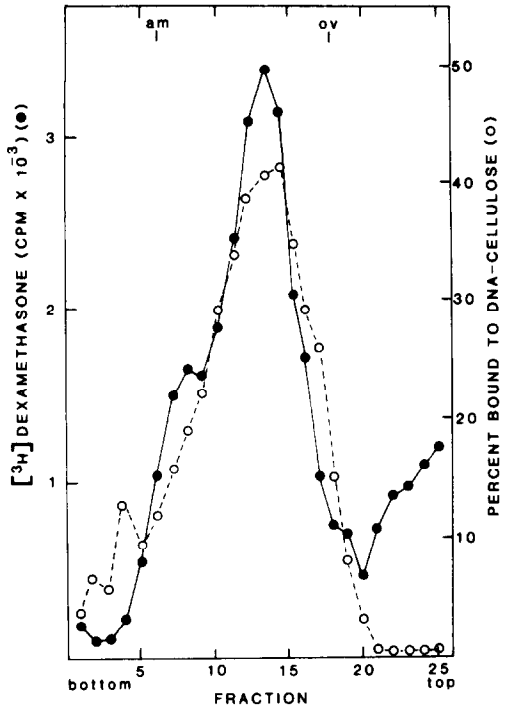


Fig. 7. Gradient profile and DNA-binding capacity of glucocorticoid receptor diluted with buffer. Glucocorticoid receptor (21,800 cpm/0.1 ml) was diluted with 8 vol of Tris–EDTA–dithiothreitol buffer and analyzed by gradient centrifugation and the gradient fractions were assayed for the ability to bind DNA-cellulose as described in Experimental.

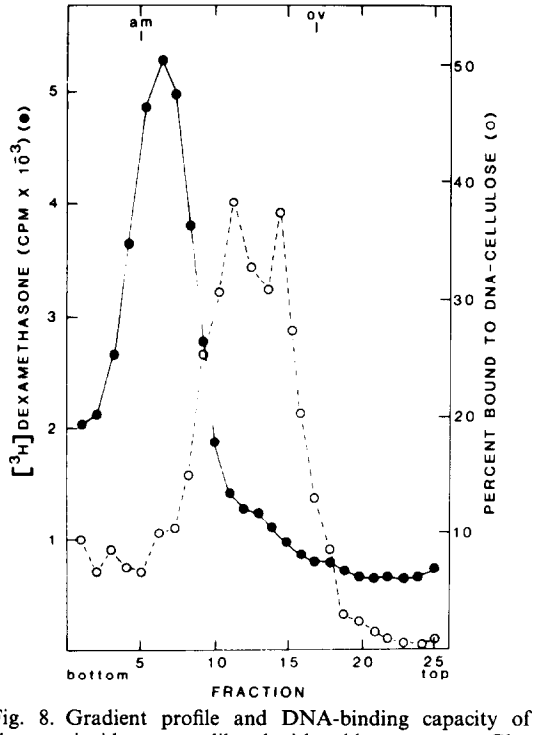


Fig. 8. Gradient profile and DNA-binding capacity of glucocorticoid receptor diluted with cold supernatant. Glucocorticoid receptor (21,800 cpm/0.1 ml) was diluted with 8 vol of cold supernatant and analyzed by gradient centrifugation, and the gradient fractions were assayed for ability to bind to DNA-cellulose as described in Experimental.

gradient fractions for the ability to bind to DNA. As can be seen in Fig. 7, when the receptor is diluted with buffer, the majority of the radioactivity as well as all of the DNA-cellulose binding sediments in the 7–8S region. These results are in keeping with our previous work which demonstrated that the 7–8S receptor is capable of binding to DNA [18]. However, if the receptor is diluted in cold cytosol, the majority of the receptor sediments at the 9–10S region and furthermore displays little DNA binding (Fig. 8). The DNA-binding that is present sediments exclusively in the 7–8S region. Note that although the percent binding is approximately the same in both cases, the overall extent of binding to DNA is higher in the sample diluted with buffer. These results thus suggest that there exists a factor in the cytosol which prevents the conversion of the glucocorticoid receptor from the 9–10S form which is incapable of binding DNA, to the 7–8S form, which can bind to DNA. This factor apparently also protects the receptor from digestion by RNase. Whether these activities reside in one factor can be determined only after further purification and characterization of the low molecular weight factor.

## DISCUSSION

We have demonstrated that the ability of our glucocorticoid–receptor complex to be converted to the 3–4S form by RNase treatment is determined by whether the complex is associated with a low molecular weight factor. If the receptor is associated with this factor, it is refractory to RNase digestion and sediments at 9–10S in sucrose gradients. The factor apparently functions by stabilizing the receptor–RNA association thus preventing the formation of the 7–8S form, rather than acting as an RNase inhibitor since it is not capable of preventing the digestion of poly U by RNase A. The factor is a dialyzable, heat-stable anion of $Mr < 500$ which is present not only in the rat liver cytosol, but also in spleen, testes, thymus, and kidney cytosol.

The 9–10S form of the receptor that is formed via association with the factor is not capable of binding to DNA. The existence of low molecular weight substances which prevent activation of the steroid-receptors to forms which can associate with DNA have been described by others [8–16], and many of the physical properties of these factors are similar to those of the factor described here. Bailly *et al.*[9] and Leach *et al.*[16] describe low molecular weight activation inhibitors from rat liver cytosol that are heat stable and not adsorbed by activated charcoal with apparent molecular weights less than 500. The former group reports that their factor is cationic whereas the latter describe it as being anionic at pH 7.5. The latter workers show that the low molecular weight factor also stabilizes the steroid-free receptors from inactivation. Others have also described low molecular weight inhibitors of activation in glucocorticoid

systems [11–13, 15], androgen systems [11], and estrogen systems [10, 11, 14]. Additionally, results have been presented which suggest that low molecular weight factors can modulate the degree of receptor association with other molecules, although their effect on receptor association with RNA was not examined [15].

The fact that we can isolate the receptor in an unactivated form that is also inaccessible to digestion by RNase suggests that the RNA–receptor interaction may play a role in the activation process. The results reported here imply that a receptor with a stabilized association with RNA cannot be activated, and consequently, cannot bind to DNA. Activation may dissociate the low molecular weight factor which in turn would make the receptor–RNA association labile. The fact that we have previously shown that the 7–8S receptor can bind to DNA [18] demonstrated that the presence of the RNA itself does not prevent activation and DNA-binding. Work has been reported by others [25] confirming our earlier results on the effects of RNase A on the glucocorticoid receptor [17]. In line with our work presented previously [18] and here, these workers suggest that the RNA itself is not an activation inhibitor. It is possible that the low molecular weight factor, in conjuction with the RNA, stabilizes an unactivated conformation of the receptor such that the DNA-binding site is blocked and the RNA is inaccessible to nuclease. Possibly, neither the low molecular factor nor the RNA alone would be sufficient to maintain this conformation.

We have previously suggested that RNA may play a role in modulating the association of the glucocorticoid receptor with DNA [17, 18, 24, 26] by binding to a regulatory site and diminishing the affinity of the receptor for DNA. The fact that we have demonstrated that the receptor can bind DNA and RNA concurrently is consistent with a separate regulatory site [18], and the existence of such a site has also been postulated by others [27]. The work that we present here suggests that other factors may be involved in stabilizing the RNA–receptor interaction, perhaps further decreasing the affinity of the receptor for DNA. If the factor that stabilizes the receptor–RNA interaction is identical to the activation inhibitor, it would suggest that the activation might consist of the conversion of the receptor from a form in which tight receptor–RNA association precludes any association with DNA. With removal of the factor upon activation, the receptor conformation becomes more open, permitting dissociation of the RNA and maximal association with DNA. Whether the above hypothesis has any physiological significance will depend upon further characterization of the receptor–RNA association, including a detailed examination of the stabilizing factor.

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