Glucocorticoid Receptors in Lung

I. SPECIFIC BINDING OF GLUCOCORTICOIDS TO CYTOPLASMIC COMPONENTS OF RABBIT FETAL LUNG*

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SUMMARY

Specific binding of glucocorticoids in fetal lung extracts was studied to investigate the mechanism of glucocorticoid action in the developing lung. The cytosol fraction of rabbit fetal lung contains a macromolecule with high affinity (3.8 × 10^-7 M) for dexamethasone and with a limited number of binding sites which are saturated with low hormone concentrations (1 × 10^-8 M). A much higher concentration of specific dexamethasone-binding substances is present in fetal lung cytosol than in the cytosol of fetal liver. With the exception of 21-deoxycortisol, the ability of various steroids to compete with labeled dexamethasone for the binding sites correlates well with their glucocorticoid potency.

The participation of protein in the steroid binding site was inferred from the destruction of the complex by β-chloromercuribenzoate and by Pronase, but not by nuclease. Several other properties of the dexamethasone-protein complex were studied. The complex is excluded from Sephadex G-200 and sediments near 7 S in sucrose gradients at low ionic strength. In the presence of 0.4 M KCl, the complex sediments near 4 S. Dexamethasone has a stabilizing effect upon the binding protein since the complex is much less thermolabile than the protein in the free state.

Cortisol inhibits the formation of the 7 S dexamethasone-protein complex indicating that both steroids interact with the same binding sites. However, no 7 S cortisol-protein complex could be detected in fetal lung extracts. Significant amounts of cortisol are associated with components sedimenting near 4 S and having properties similar to serum corticosteroid-binding globulin. A possible explanation of these results is that the 7 S cortisol-protein complex is unstable and dissociates during prolonged centrifugation or gel filtration. An alternative possibility, supported by our preliminary data, is that cortisol and dexamethasone may bind to different induced or pre-existing conformational states of the binding protein. This may lead to qualitative differences between the effects exerted by the two steroids. Thus a comparison of the effects and mechanism of action of the synthetic and natural corticosteroids deserves further investigation.

Although the fetal lung has not been recognized as a target tissue for glucocorticoids in the past, very recently a number of observations indicate that these hormones may play an important role during lung maturation and biochemical differentiation. Administration of exogenous glucocorticoids to mammalian fetuses at appropriate stages of gestation accelerates morphological development of the lung and causes precocious appearance of pulmonary surfactant (1-6). In addition, endocrine ablation, such as decapitation of rat fetuses, results in inhibition of normal lung maturation and decreased levels of surfactant in fetal lungs (7). A deficiency of surfactant in lungs of human infants is considered to be the primary cause of respiratory distress syndrome (8), a major cause of death in premature infants.

The mechanism by which glucocorticoids stimulate the early appearance of surfactant in developing lung is not known. In particular, it is not known whether glucocorticoids influence the lung directly or as a consequence of their effects on other tissues. It is generally believed that the first step in the cellular action of steroid hormones involves the interaction of the hormone with specific cytoplasmic receptors (9, 10). The steroid-receptor complex then migrates to the nucleus, where it binds to specific acceptor sites on the chromatin (9-12). As a result of this interaction, the transcriptional apparatus is activated to produce specific new RNA and finally yield new proteins. This is thought to initiate the characteristic hormonal response. If fetal lung is a target organ for glucocorticoids, the hormones may exert their effects by inducing the synthesis of key enzymes involved in the biosynthesis of the phospholipid components of surfactant. To test this hypothesis our initial approach has been to examine rabbit fetal lung for the presence of specific cytoplasmic and nuclear receptors.

In a preliminary communication (13), we have reported that rabbit fetal lung nuclei contain macromolecules which have the properties of physiological receptors for cortisol by the criteria of specificity of binding and saturation of binding sites at low concentrations of the hormone. It was also shown that the concentration of nuclear binding sites increases between Day 20 and Day 28 of gestation. These results correlate with reported changes in pulmonary epithelial cell maturation and surfactant concentrations in lung extracts. Preliminary evidence for the presence of cytoplasmic cortisol-binding components in fetal lung was also presented (13). In a recent report, Ballard and Ballard (14) have also demonstrated specific binding of dextra-
methasone in fetal lung extracts. This report describes the isolation and properties of macromolecular-glucocorticoid complexes extracted from the cytosol fractions of fetal lung and fetal liver. Data will be presented which show that binding of glucocorticoids in fetal lung involves proteins with properties similar to those reported for other steroid-receptor systems (10). A preliminary report of these studies has been presented (15).

**MATERIALS AND METHODS**

**Steroids**—[1,2-3H]Cortisol (44 Ci per mmole) was purchased from New England Nuclear, [1,2-3H]dexamethasone (22 Ci per mmole) from Amerham-Searle, and [1,2,4-3H]triamcinolone acetonide (10.7 Ci per mmole) was obtained from Schwarz-Mann. The radiochemical purity of the steroids was verified by paper chromatography. Dexamethasone, triamcinolone acetonide, fluocinolone, and 6α-methylprednisolone were gifts from the Upjohn Co. and all other steroids were purchased from Iakpharm.

**Enzymes**—Bovine RNase, DNase, and Pronase were purchased from Sigma.

**Buffers**—The following buffers were used: 0.01 M Na₂EDTA, pH 7.6 (Buffer A); 0.01 M Tris-0.0015 M Na₂EDTA-0.4 M KCl, pH 8.9 (Buffer B); Krebs-Ringer 0.1 M phosphate buffer, pH 7.4.

**Preparation of Serum and Cytosplasmic Supernatant Fractions**—Pregnant white New Zealand rabbits at 28 to 31 days of gestation were operated under local anesthesia. Two milliliters of 1% Xylocaine were injected into the anterior abdominal wall and laparotomy was performed. The gravid uterus was exposed and the fetuses were removed and immediately decapitated. Fetal blood was collected, allowed to clot, and centrifuged at 1,000 x g for 15 min to obtain the serum fraction. Excised fetal organs (lung and liver) were washed in cold Krebs-Ringer phosphate buffer, blotted, weighed, sliced, and homogenized (glass barrel, Teflon pestle) in batches of 1 g in 5 ml of Buffer A at a temperature close to 0°C. The homogenate was centrifuged for 30 min at 224,000 x g at 2°C to obtain the cytoplasmic supernatant fraction (cytosol). Protein concentrations in the cytosol preparations were determined by the Lowry method (16).

**Preparation of Cytosol from Lungs Frozen in Liquid Nitrogen**—Lung tissue (0.5-g aliquots) was stored in plastic vials in liquid nitrogen. Frozen tissue was pulverized (Pulverizer, Thermovac Industries, Inc.) and homogenized in 2 ml of Buffer A (Polytron P-10, Rhoestat Setting 6) by three 10-s bursts with 1-min cooling periods between bursts. The homogenate was then centrifuged for 30 min at 224,000 x g to prepare the cytosol.

**Formation of Steroid-Macromolecule Complexes**—Appropriate aliquots of [3H]cortisol, [3H]dexamethasone, or [3H]triamcinolone acetonide, dissolved in redistilled ethanol were pipetted into tubes and the ethanol was evaporated under nitrogen. Aliquots of cytosol or diluted serum were then added, mixed in a Vortex, and incubated at 0–4°C for 2 hours. At the concentrations used, all the steroids were soluble in cytosol or serum and incubation for 2 hours was adequate to obtain maximum binding.

**Measurement of Specific Binding**—Separation of bound from free steroid was achieved by the activated charcoal assay of Korenman et al. (17). Specific binding of [3H]dexamethasone was measured as follows. Aliquots of the cytosol containing [3H]dexamethasone (up to 1 x 10⁻⁷ M) were incubated at 0°C for 2 hours. Samples containing the same concentration of [3H]dexamethasone plus a 100-fold excess of nonlabeled dexamethasone as a competitor were run in parallel for “background” determination. Following incubation, 1 ml of charcoal suspension (0.5% activated charcoal (Malinkrodt Co.) and 0.05% dextran 500 suspended in Buffer A) was added to the samples (0.2 ml) which were immediately agitated for 5 s in a Vortex mixer. After standing at 0°C for 10 min the samples were centrifuged at 800 x g for 10 min. An aliquot of the supernatant (0.5 ml) was mixed with 10 ml of scintillation fluid (0.5% Omnifluor in toluene) and counted in a Packard Tri-Carb model 3002 spectrometer after a pre-equilibration period of at least 2 hours. Counting efficiency under these conditions was 42 to 45%. The amount of steroid specifically bound by the cytosol was calculated by subtracting the radioactivity of samples containing the competitor from the radioactivity of the corresponding competitor-free sample. The amount of charcoal used adsorbed quantitatively all of the steroids tested in the absence of a complex-forming agent. The same method was used to measure specific binding of [3H]cortisol and [3H]triamcinolone acetonide in cytosol as well as specific binding of the three labeled steroids in fetal serum. In order to estimate the relative binding affinity of various nonlabeled steroids for the cytosol, aliquots of the cytosol were incubated with [3H]dexamethasone (1 x 10⁻⁸ M) in the presence or absence of a 10-fold excess of the nonlabeled steroid.

**Enzymatic Release Studies**—Aliquots of the cytosol (1 ml) charged with 1 x 10⁻⁸ M [3H]dexamethasone were incubated for 30 min at 0°C with (a) no additions, (b) 1 mg of Pronase, (c) 1 mg of bovine RNase, or (d) 1 mg of DNase plus 5 µl of 1 M magnesium acetate. Specifically bound radioactivity in treated samples was measured by the charcoal assay.

**Succrose Density Gradient Analysis**—The samples to be analyzed (0.2 ml) were layered on 3.6-ml linear gradients of 5 to 20% or 10 to 30% sucrose in Buffer A or B and were centrifuged for time periods varying between 7½ and 16 hours at 297,000 x g in a Beckman model L2-65B ultracentrifuge using a SW 56 rotor. Following centrifugation, the tubes were pierced and 32 fractions were collected directly in counting vials. Following the addition of 3 ml of ethanol and 10 ml of scintillation fluid, the radioactivity in each sample was determined in a Packard Tri-Carb model 3002 spectrometer with a 26% efficiency. Approximate sedimentation coefficients of steroid-binding components were estimated by comparison with that of bovine serum albumin.

**Fractionation of Cytosol and Serum by Gel Filtration**—For gel filtration, Sephadex G-200 was swollen in Buffer A over boiling water for 4 hours and fines were removed by aspiration. The swollen gel was cooled at 4°C and packed in the cold in a column (2.5 x 30 cm) using Buffer A. A 2-cm layer of Sephadex G-25 coarse was packed on the top to prevent disturbance of the gel surface upon sample application. Sample solutions (2 ml) were applied and eluted with Buffer A. Two-milliliter fractions were collected at a flow rate of 0 ml per hour. In other experiments, Buffer B was used for the preparation of the Sephadex elution and for elution of the samples.
**RESULTS**

**Dexamethasone-binding Components in Lung and Liver Cytosols**

Due to the high concentration of corticosteroid binding globulin in blood, studies on the binding of glucocorticoids in tissues are accompanied by technical problems of distinguishing between tissue-specific binding and binding observed in tissue fractions as a result of blood contamination. Fortunately, we have found that fetal rabbit serum has a high binding affinity for cortisol but no affinity for the potent synthetic glucocorticoid dexamethasone. In contrast, lung and liver cytosols have a binding capacity for both cortisol and dexamethasone (Table I). Similar results were obtained with triamcinolone acetonide. Since we cannot detect any specific binding of dexamethasone or triamcinolone acetonide to serum components, it can be said with a reasonable degree of certainty that the observed binding of these steroids to lung and liver cytosols is indicative of an interaction of the hormones with tissue components and not to blood contaminants. For this reason, in most of the studies described in this report [3H]dexamethasone and [3H]triamcinolone acetonide were used. The use of the synthetic glucocorticoids in binding studies appears to be justified since these compounds as well as cortisol seem to interact with the same sites. This is indicated from the observation that the binding of [3H]dexamethasone or [3H]triamcinolone acetonide to lung and liver cytosol is virtually eliminated in the presence of 100-fold excess of nonlabeled cortisol.

Strong interactions of [3H]dexamethasone with macromolecular components of lung and liver cytosols is revealed by sucrose gradient centrifugation (Fig. 1). A low dissociation constant is indicated by the low concentration of steroid required, and the low rate of release of the steroid at 2°C is indicated from the persistence of binding when the complex sediments away from the free steroid at the meniscus. Even when the cytosol, preincubated with [3H]dexamethasone, is first treated with charcoal to remove the free steroid and then centrifuged on sucrose gradients for 16 hours, the rate of dissociation of the complex is slow and most of the hormone remains bound to macromolecules (Fig. 1).

**Table I**

<table>
<thead>
<tr>
<th>Steroids</th>
<th>Amount of bound labeled steroid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Serum</td>
</tr>
<tr>
<td>[3H]Cortisol</td>
<td>400 ± 50</td>
</tr>
<tr>
<td>[3H]Dexamethasone</td>
<td>20 ± 3</td>
</tr>
<tr>
<td>[3H]Dexamethasone + dexamethasone</td>
<td>18 ± 4</td>
</tr>
<tr>
<td>[3H]Dexamethasone + cortisol</td>
<td>17 ± 3</td>
</tr>
<tr>
<td>[3H]Triamcinolone acetonide</td>
<td>22 ± 5</td>
</tr>
<tr>
<td>[3H]Triamcinolone acetonide + dexamethasone</td>
<td>20 ± 2</td>
</tr>
<tr>
<td>[3H]Triamcinolone acetonide + cortisol</td>
<td>18 ± 4</td>
</tr>
</tbody>
</table>

The sedimentation coefficient of the [3H]dexamethasone-macromolecule complex of lung or liver cytosol is about 7 S. A similar pattern was obtained with [3H]triamcinolone acetonide (Fig. 1). Only a limited number of binding sites is present in lung cytosol since the addition of a 10-fold excess of nonlabeled dexamethasone reduces binding of [3H]dexamethasone to the 7 S component by about 90%. Binding of [3H]dexamethasone to the 7 S macromolecule is also inhibited by an excess of nonlabeled cortisol indicating that both steroids interact with the same sites. It is interesting that the cytosol of the fetal lung contains a much greater amount of the 7 S [3H]dexamethasone-binding component than the cytosol of the fetal liver. The fetal serum does not contain any detectable amounts of 7 S dexamethasone-binding macromolecules (Fig. 1).

**Protein Nature of Dexamethasone-binding Site of Lung Cytosol**—To explore the chemical nature of the dexamethasone-binding

![Fig. 1. Sucrose density gradient patterns of fetal lung and liver cytosols and of fetal serum preincubated at 4°C with 1 × 10^{-8} M of [3H]dexamethasone or [3H]triamcinolone acetonide in the presence or absence of 1 × 10^{-4} M of nonlabeled steroid. The steroid-macromolecule complexes were resolved from free steroid by centrifugation for 16 hours at 297,000 × g at 2°C in 10 to 30% sucrose gradients prepared in Buffer A. Panel A, fetal lung cytosol incubated with: [3H]dexamethasone (O--O); [3H]dexamethasone + dexamethasone (A--A); [3H]dexamethasone + cortisol (A--A). Panel B, fetal lung cytosol incubated with [3H]triamcinolone acetonide. Panel C, fetal liver cytosol incubated with [3H]dexamethasone. Panel D, diluted fetal serum incubated with [3H]dexamethasone.**
components, lung cytosol was subjected to the following treatments: brief heating of the cytosol before addition of the hormone, addition of p-chloromercuribenzoate or N-ethylmaleimide during incubation with the hormone, and enzymatic digestion of the complex.

Heating of the cytosol to 70° for 5 min completely prevented subsequent binding of [3H]dexamethasone. Addition of p-chloromercuribenzoate (1 mm) in cytosol also prevented binding. N-ethylmaleimide (1 mm) reduced binding of the hormone by 50%. Treatment with Pronase resulted in release of radioactivity from the complex while RNase and DNase had no effect (Table II). These results indicate not only the protein nature of the hormone-binding site but also the involvement of sulfhydryl groups in either the interaction with the hormone or the maintenance of the active structure of the binding component.

Effect of Various Steroids on Specific Dexamethasone Binding—A number of steroids were tested for their capacity to inhibit specific binding of [3H]dexamethasone to lung cytosol. In these studies, a saturating concentration (1 X 10^{-8} M) of [3H]dexamethasone and a 10-fold excess of the competitors were used. As shown in Table III, the ability of the steroids to compete with [3H]dexamethasone for specific binding correlates well with their glucocorticoid activity. These data are consistent with the hypothesis that steroid hormones exert their biological effects by interacting with specific tissue receptors (10). The only exception noted is 21-deoxycortisol which is a weak glucocorticoid but competes effectively with [3H]dexamethasone for the specific binding sites of lung cytosol.

Properties of Dexamethasone-binding Protein of Lung Cytosol—The concentration of KCI has been shown to influence dramatically the sedimentation coefficients of the estrogen-binding proteins of rat uterus (19) and the progesterone-binding proteins of chick ovicui (20). The analogous sensitivity of the dexamethasone-binding proteins of lung cytosol is apparent from the gradient centrifugation patterns shown in Fig. 3. In the presence of 0.4 M KCI the [3H]dexamethasone-protein complex sediments at 4 S while in the absence of KCI the complex sediments at 7 S. Similar results are obtained following filtration of lung cytosol.

TABLE II

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Amount bound</th>
<th>Radioactivity released</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>2600</td>
<td>0</td>
</tr>
<tr>
<td>DNase</td>
<td>3200</td>
<td>0</td>
</tr>
<tr>
<td>RNase</td>
<td>3000</td>
<td>0</td>
</tr>
<tr>
<td>Pronase</td>
<td>700</td>
<td>73</td>
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Effect of enzymes on [3H]dexamethasone-macromolecule complex of fetal lung cytosol

Aliquots of cytosol (1 ml) were first incubated with 1 X 10^{-8} M [3H]dexamethasone at 0° for 2 hours. This was followed by an additional 30-min incubation at 0° with or without the addition of 1 mg of enzyme. In the incubation mixture containing DNase, 5 u of 1 m magnesium acetate were also added. The charcoal assay was used to separate free from bound steroid. Each value represents the average of five determinations.

Effect of nonlabeled steroids on binding of [3H]dexamethasone to cytosol of fetal lung

Aliquots of cytosol were incubated with 1 X 10^{-8} M [3H]dexamethasone in the presence or absence of 1 X 10^{-7} M of nonlabeled steroid at 0° for 2 hours. The charcoal assay was used to separate bound from free [3H]dexamethasone. Each value is the average of ten determinations.

Competing steroid | Binding
<table>
<thead>
<tr>
<th></th>
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</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>Tetrahydrocortisol</td>
<td>94</td>
</tr>
<tr>
<td>Testosterone</td>
<td>88</td>
</tr>
<tr>
<td>Estradiol-17β</td>
<td>87</td>
</tr>
<tr>
<td>Allo-tetrahydrocortisol</td>
<td>87</td>
</tr>
<tr>
<td>Cortisone</td>
<td>80</td>
</tr>
<tr>
<td>Progesterone</td>
<td>78</td>
</tr>
<tr>
<td>3α,11β,17α,21-Tetrahydroxyprog-n-5-en-20-one</td>
<td>73</td>
</tr>
<tr>
<td>Aldosterone</td>
<td>70</td>
</tr>
<tr>
<td>17α-Hydroxyprogesterone</td>
<td>56</td>
</tr>
<tr>
<td>Deoxycorticosterone</td>
<td>55</td>
</tr>
<tr>
<td>11-Deoxycorticisol</td>
<td>46</td>
</tr>
<tr>
<td>11α-Hydroxyprogesterone</td>
<td>45</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>39</td>
</tr>
<tr>
<td>21-Deoxycorticisol</td>
<td>32</td>
</tr>
<tr>
<td>Cortisol</td>
<td>24</td>
</tr>
<tr>
<td>Fluocinolone</td>
<td>22</td>
</tr>
<tr>
<td>6α-Methylprednisolone</td>
<td>17</td>
</tr>
<tr>
<td>Triamcinolone acetonide</td>
<td>12</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>10</td>
</tr>
</tbody>
</table>

Fig. 2. A, specific and nonspecific binding of [3H]dexamethasone by fetal lung cytosol. Aliquots of cytosol (1.5 mg of protein per ml) were incubated with 1 X 10^{-8} M [3H]dexamethasone in the presence or absence of nonlabeled dexamethasone (1 X 10^{-8} M) for 2 hours at 0°. Then bound [3H]dexamethasone was measured by the charcoal assay. Total binding represents bound [3H]dexamethasone in the presence of a 100-fold excess of nonlabeled steroid. Specific binding was calculated by subtracting the nonspecific from the total binding. B, Scatchard analysis of the data shown in A.
Fig. 3. Effect of KCl on the sedimentation rate of [3H]dexamethasone-binding components of lung cytosol. Gradients without KCl (A) were 10 to 30% sucrose and those containing 0.4 M KCl (B) were 5 to 29% sucrose. Centrifugation was performed as in Fig. 1. BSA, bovine serum albumin.

Fig. 4. Gel filtration on Sephadex G-200 of lung cytosol preincubated with [3H]dexamethasone on Sephadex G-200 columns (Fig. 4). In the absence of KCl the complex is eluted with the void volume while in the presence of 0.4 M KCl it is retained by the gel. No significant amounts of [3H]dexamethasone are associated with macromolecules similar to serum corticosteroid-binding globulin which under these experimental conditions has an elution pattern similar to that of bovine serum albumin.

The dexamethasone-binding protein of lung cytosol is stable at 0–4°C for at least 24 hours and the hormone-protein complex is more stable than the uncomplexed protein. As shown in Fig. 5, in the presence of saturating concentrations of [3H]dexamethasone, no significant difference in the binding capacity of cytosol is observed between 2 hours (when saturation has been achieved) and 12 hours of incubation. Even after 24 hours of incubation, the binding capacity of cytosol is decreased by only 20 to 30%. Storage of lung cytosol at 0–4°C in the absence of hormone results in greater losses of the binding activity. Under these latter conditions, about 50% of the dexamethasone-binding capacity is lost in 24 hours.

The stabilizing effect of dexamethasone on the binding protein is better illustrated by freezing or heating the cytosol in the presence or absence of the hormone. Freezing the cytosol (-15°C) in the absence of [3H]dexamethasone results in complete loss of the binding activity, but no significant losses in binding activity are observed in the presence of saturating amounts (1 × 10^-8 M) of the hormone (Table IV). When cytosol is heated at 25°C for 30 min in the absence of dexamethasone, the binding activity is decreased by 65% and it is completely destroyed after 30 min at 37°C (Fig. 6). In contrast, when cytosol is preincubated with [3H]dexamethasone at 0°C and then heated for 30 min at 25 or

**TABLE IV**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Amount bound[^a] (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None[^b]</td>
<td>3850</td>
</tr>
<tr>
<td>Freezing at -15°C with [3H]dexamethasone[^c]</td>
<td>2900</td>
</tr>
<tr>
<td>Freezing at -15°C in the absence of [3H]dexamethasone[^d]</td>
<td>300</td>
</tr>
</tbody>
</table>

[^a]: Bound steroid was measured by the charcoal assay.
[^b]: Lung cytosol incubated with 1 × 10^-8 M [3H]dexamethasone for 2 hours at 0°C.
[^c]: Lung cytosol incubated with 1 × 10^-8 M [3H]dexamethasone for 2 hours at 0°C and then stored at -15°C for 24 hours. This was followed by thawing and further incubation for 2 hours at 0°C.
[^d]: Lung cytosol was kept frozen at -15°C for 24 hours. It was then thawed and incubated with 1 × 10^-8 M [3H]dexamethasone for 2 hours at 0°C.
Temperature [°C]

**FIG. 6.** Comparison between the stability of the [3H]dexamethasone-protein complex of lung cytosol and the stability of the steroid-free binding protein at various temperatures. ○-○, aliquots of lung cytosol were incubated without steroid for 30 min at the temperatures indicated. Then the samples were transferred at 0° and incubated with 1 × 10^{-8} M [3H]dexamethasone for 2 hours. ▲-▲, aliquots of lung cytosol were incubated with 1 × 10^{-8} M [3H]dexamethasone for 2 hours at 0° followed by a 30 min incubation at the temperature indicated. Then the samples were transferred at 0° and further incubated for 2 hours. Bound [3H]dexamethasone was measured by the charcoal assay. Each point represents the average of six determinations.

37°, the binding activity decreases by only 14 and 55%, respectively (Fig. 6).

At least 50% of the binding activity is maintained by freezing the complex in liquid nitrogen. After thawing and sucrose density gradient centrifugation, the sedimentation pattern of the complex is identical with the pattern of the complex prepared with fresh cytosol (Fig. 7). The binding protein is stable in the absence of hormone when intact lung tissue is stored in liquid nitrogen. Under these conditions full binding activity is preserved and the sedimentation pattern of the complex is not altered (Fig. 7).

**Cortisol-binding Components of Lung Cytosol—**As discussed above, lung cytosol contains a specific dexamethasone-binding protein, distinct from serum corticosteroid-binding globulin, which under conditions of low ionic strength is excluded from Sephadex G-200 (Fig. 4) and has a sedimentation coefficient of about 7 S (Fig. 1). Cortisol appears to bind to the same protein since it inhibits the formation of the 7 S [3H]dexamethasone-protein complex (Fig. 1). However when the cytosol is incubated with [3H]cortisol and then subjected to centrifugation on sucrose density gradients, there is no detectable binding of the hormone to the 7 S component (Fig. 8). Similarly, only minute amounts of radioactivity are associated with substances excluded from Sephadex G-200 during gel filtration of cytosol charged with [3H]cortisol (Fig. 9). A possible explanation for these results is that binding of [3H]cortisol to the 7 S component is not as tight as that of [3H]dexamethasone-protein complex and dissociates during prolonged centrifugation or gel filtration.

Both techniques of sucrose gradient centrifugation and gel filtration show that in lung cytosol significant amounts of [3H]cortisol are associated with macromolecules having a pattern identical with serum corticosteroid-binding globulin (Figs. 8 and 9). Lung cytosol has a much higher binding capacity for [3H]cortisol than for [3H]dexamethasone (Table I). This suggests that at least part of the [3H]cortisol associated with the 4 S components (Fig. 8) may represent binding to serum components. It is also possible that the lung cytosol contains a 4 S cell component which has a binding affinity for cortisol but not dexamethasone. These possibilities are currently under investigation.

**DISCUSSION**

The presence of a specific glucocorticoid-binding macromolecule in the cytoplasmic fraction of rabbit fetal lung has been shown by ultracentrifugal and chromatographic techniques. This macromolecule was unequivocally distinguished from fetal blood corticosteroid-binding globulin by the following criteria. It has a high affinity for the synthetic glucocorticoids dexamethasone and triamcinolone acetonide, sediments near 7 S in low salt sucrose density gradients, it is eluted from Sephadex
FIG. 8. Sedimentation patterns of fetal lung cytosol (■—■) and fetal serum (○—○) preincubated at 0° with 1 × 10⁻⁸ M [³H]cortisol. Samples were applied on 10 to 30% sucrose gradients in Buffer A and centrifuged at 297,000 × g for 16 hours at 2°. BSA, bovine serum albumin.

FIG. 9. Gel filtration on Sephadex G-200 of lung cytosol (■—■) and fetal serum (○—○) preincubated with 1 × 10⁻⁸ M [³H]cortisol. Elution was carried out with Buffer A.

G-200 in low salt media, and it loses its capacity to bind glucocorticoids after short incubation at 37°. In contrast, fetal serum corticosteroid-binding globulin does not bind dexamethasone or triamcinolone acetonide, it has a sedimentation coefficient of about 4 S, it is retained by Sephadex G-200, and it retains its capacity to bind glucocorticoids after heating at 37°.

There is an impressively close correlation between the ability of a given steroid to block [³H]dexamethasone binding and the glucocorticoid potency of the steroid (Table III). These results suggest that the binding component plays a functional role of a specific receptor in fetal lung. One exception noticed was that the weak glucocorticoid 21-deoxycortisol competes effectively with [³H]dexamethasone for the cytosol binding sites. A similar observation has been reported for the rat thymus (21). In this system, 11-deoxycortisol blocks the binding of [³H]cortisol to cytoplasmic proteins. However, it was found that in thymus cells 11-deoxycortisol acts as an effective anti-glucocorticoid since it reduces the effect of cortisol on glucose uptake. It will be of interest to see whether 21-deoxycortisol has an anti-glucocorticoid activity in fetal lung.

The properties of the dexamethasone-binding component of lung cytosol are similar to those described for the binding proteins of several other steroid-responsive tissues (10). That the binding macromolecule of fetal lung is a protein is indicated by direct analysis, susceptibility of the complex to proteolytic enzymes, and dependence of binding on intact -S-H groups. The dexamethasone-protein complex sediments more rapidly at low ionic strength than at increased salt concentrations. In crude extracts, the binding protein is much more stable in a complex with steroid than in the free state (Figs. 5 and 6 and Table IV). Similar observations have been reported for the dexamethasone-binding protein of hepatoma tissue culture cells (22). We also have evidence that in fetal lung the cytoplasmic dexamethasone-binding protein is involved in promoting nuclear localization of specifically bound steroid.

Cortisol inhibits the formation of the 7 S [³H]dexamethasone-protein complex (Fig. 1) indicating that both steroids interact with the same binding sites. However, when the cytosol is incubated with [³H]cortisol and then subjected to centrifugation on sucrose density gradients, there is no detectable 7 S complex (Fig. 8). Similarly, only small amounts of radioactivity are associated with macromolecules excluded from Sephadex G-200 during gel filtration of cytosol preincubated with [³H]cortisol (Fig. 9). By both ultracentrifugal and gel filtration techniques, it was found that significant amounts of cortisol are associated with components similar to serum corticosteroid-binding globulin (Figs. 8 and 9). A possible explanation of these results is that the 7 S [³H]cortisol-protein complex is not stable and dissociates during prolonged centrifugation or gel filtration. Preliminary observations suggest that this may not be the case. For example, lung cytosol kept at 0° with [³H]cortisol for several hours is still active in promoting specific uptake of [³H]cortisol by isolated lung nuclei. After heating the cytosol at 37° for 30 min, specific nuclear uptake of [³H]cortisol was not observed. In addition, heating of the cytosol at 37° reduces the amount of [³H]cortisol bound to the 4 S macromolecules suggesting that lung cytosol contains a 4 S cortisol-binding component distinct from corticosteroid-binding globulin.

Glucocorticoid-binding macromolecules have been reported to be present in extracts of other tissues (23-26). In all cases either dexamethasone or cortisol was used and to our knowledge no comparison of the proteins binding the two steroids has been made in the same system. It is interesting that an 8 S dexamethasone-binding protein has been detected in hepatoma tissue

G. Giannopoulos, manuscript in preparation.

G. Giannopoulos, unpublished results.
culture cells (23) but a 4 S cortisol-binding protein (distinct from corticosteroid-binding globulin) has been isolated from rat liver cytosol (26). An intriguing possibility is that both cortisol and dexamethasone bind to the same sites but each steroid induces different conformational changes which may result in different aggregation states of the binding protein. This raises the possibility that there may be qualitative differences between the cellular effects of the natural and synthetic glucocorticoids. It should be noticed that dexamethasone and triamcinolone ace- tone inactivate pregnant mare serum-induced ovulation in rats (27) and baboons (28) whereas cortisol and corticosterone have no effect. If that were the case, data obtained from studies dealing with specific interactions of synthetic glucocorticoids with cell components should be interpreted with caution and the use of the natural corticosteroids in such studies would be a more desirable choice.

Preliminary data also suggest that at least part of the CBG-like component found in lung cytosol may be intracellular or bound to the cell surface since it is present in lung extracts even after excessive washing of fetal lungs with isotonic buffer to remove blood contaminants. A similar CBG-like component has been detected in the cytosol of adult rat liver (26). However, this is not surprising since the liver is assumed to be the site of synthesis of corticosteroid-binding globulin. If it can be shown conclusively that corticosteroid-binding globulin is present in fetal lung cells, this will be of particular significance since it will suggest a functional role for the blood protein in transporting corticosteroids to their cellular site of action.

Several investigators have reported that administration of glucocorticoids to fetuses at appropriate stages of gestation accelerates morphological development of the fetal lung and causes precocious appearance of pulmonary surfactant (1-6) while endocrine ablation such as fetal decapitation inhibits normal lung maturation (7). However, such studies do not provide proof that glucocorticoids act directly on the fetal lung to stimulate those processes. The findings reported here as well as in previous communications (13, 15) are consistent with a direct role for glucocorticoids in fetal lung development. If the concentration of receptor determines the responsiveness of a tissue to glucocorticoids, it is of interest that the fetal lung contains a much greater concentration of cytoplasmic glucocorticoid-binding protein than the fetal liver (Fig. 1) and several other fetal tissues examined with the exception of the kidney.

At present we can only speculate about the mechanism of glucocorticoid action in fetal lung. Corticosteroids induce specific enzymes in many tissues such as the embryonic retina (29), fetal gut (30), and newborn liver (31). Furthermore, it is believed that enzyme induction by glucocorticoids is mediated by specific cell receptors (22). Thus a possible action of glucocorticoids in fetal lung may be the induction of enzymes involved in the biosynthesis of the components of surfactant. A recent report describing the induction of phospholipid choline glycerol transferase (an enzyme necessary for lecithin synthesis by the choline pathway) by cortisol in fetal lung supports this concept. We have also reported that the number of glucocorticoid-specific nuclear binding sites in rabbit fetal lung increases during the last 10 days of gestation (19), a process which correlates with increased levels of pulmonary surfactant (3, 33, 34) and rising levels of cortisol in the fetal circulation.

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4 The abbreviation used is: CBG, corticosteroid-binding globulin.
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