Progesterone-binding Components of Chick Oviduct

I. PRELIMINARY CHARACTERIZATION OF CYTOPLASMIC COMPONENTS*

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SUMMARY

Induction of the synthesis of the specific protein avidin by a single administration of progesterone has been demonstrated previously in chicks in vivo and in tissue minces and monolayer cultures of chick oviduct. To investigate the mechanism of induction, macromolecular components of oviduct cytoplasm which bind $^3$H-progesterone in vitro were isolated and characterized by sucrose gradient centrifugation, polyacrylamide gel electrophoresis, enzymatic digestion, and gel filtration on Agarose (Bio-Rad Laboratories, Richmond, California).

The radioactive steroid in the isolated complex was identified as progesterone, not a metabolite, by paper chromatography. The interaction with $^3$H-progesterone has an apparent dissociation constant $k_d \approx 8 \times 10^{-10} \text{ M}$ in 0.3 M KCl at $1^\circ$ and is reversed by mild heating and by unlabeled progesterone > testosterone > 20α-hydroxy-4-pregnene-3-one > 17β-estradiol > cortisol > estrone > androstenedione. The participation of protein in the steroid-binding site was inferred from the destruction of the complex by $10^{-3} \text{ M } p$-hydroxymercuribenzoate and Pronase, but not by ribo- or deoxyribonucleases.

The apparent number and size of the cytoplasmic binding components vary with the concentration of KCl and the technique of isolation and detection. In the absence of KCl, the major components are characterized by sedimentation coefficients, $s_{20, w} \approx 5$ S and $8$ S, molecular weights of about 1.0 and $3.6 \times 10^5$ (estimated from the variation of electrophoretic mobility with gel concentration), and sufficiently large effective radii to be eluted in, or near, the void volume of columns of Agarose A-0.5m. Under the same conditions the corticosteroid-binding globulin (CBG) of chick plasma behaves as a single component with $s_{20, w} \approx 3.7$ S and mol wt $\approx 6.0 \times 10^5$. In solutions containing 0.3 M KCl, the cytoplasmic components and CBG all sediment at about the same rate but may be distinguished from each other by the distribution coefficients on Agarose A-0.5m. From the latter results, molecular Stokes radii of 55 and 63 Å can be calculated for the cytoplasmic components, compared with 37 Å for CBG. The progesterone-binding components of chick oviduct cytoplasm are thus distinguishable from CBG by all physicochemical methods tested.

A functional role for these components in the induction of avidin synthesis by progesterone is supported by (a) the parallel order of effectiveness of various steroids in competing with progesterone binding and in potency as inducers, and (b) the analogous effects of treating the chicks with diethylstilbestrol on progesterone-binding activity and on avidin induction.

Although the sequence of events which mediate the effect of a steroid hormone on its target tissue is not yet known, an early step is thought to be the interaction between the hormone and a specific macromolecular component of the target tissue (3–5). The distribution of such “receptor” molecules could determine tissue specificity, and the hormone-receptor complex could participate in the regulation of RNA and protein metabolism in the target tissue.

The receptor hypothesis is supported by recent results from several laboratories. These studies include preliminary characterizations of receptor molecules for estrogens (5–8), aldosterone (9, 10), and androgens (11, 12). In each case the target tissue has been shown to contain proteins that are unique to these tissues, are present in small concentrations, and are recognizable by the ability to bind the respective steroid specifically and with high affinity.

Estrogen binding to uterine target cells has been the subject of intensive study. The uptake and distribution of radioactively labeled estradiol by the uterus has been followed by cell fractionation (13) and by radioautographic techniques (8, 14). The hormone appears to be bound to cytoplasmic macromolecules initially and then transported to the nucleus, where stimulation of RNA synthesis takes place (8). The affinities of the cyto-

* Preliminary reports of this investigation have been published (1, 2).
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plasmic receptor for estradiol and several analogues correlate well with the respective biological activities (13, 15). It therefore appears quite probable that the receptors play a central role in the mechanism of action of estrogens in the uterus.

Less is known about the binding and localization of progesterone in target tissues. A progesterone- and cortisol-binding protein has been isolated from the uterus of castrated rats, but the investigators did not demonstrate its distinction from the corticosteroid-binding globulin of plasma or its intracellular origin, although simple plasma contamination was excluded (16). A substance capable of binding pregnenolone and progesterone has also been reported to be present in the cytoplasm of rat prostate, but the progestins serve no known function in this gland (17). More recently, a specific progesterone-binding macromolecule has been demonstrated in the supernatant fraction of the corpus luteum of the pregnant cow, an organ which synthesizes and secretes progesterone, as distinguished from a target organ (18).

Since the study of steroid-receptor interactions may be an effective and necessary approach to the mechanism of hormone action, experiments were undertaken in the chick oviduct, a hormone-responsive system which is simpler and more specific than many now available. In this organ, progesterone induces de novo synthesis of a single, specific protein, avidin (19–21). We have reported elsewhere the subcellular distribution and de novo synthesis of a single, specific protein, avidin (19–21).

Preparation of Plasma and Cytosolic Supernatant Fractions

Female Rhode Island Red chicks received either no hormone treatment for 20 days, starting at 4 days old (unstimulated), or 5 mg of diethylstilbestrol in sesame oil subcutaneously for 20 days (on diethylstilbestrol), or 20 days of diethylstilbestrol injections, followed by 10 days without treatment (off diethylstilbestrol). Chick plasma was prepared by cardiac puncture into heparinized syringes and centrifugation at 1,000 × g for 15 min. Excised organs (oviduct, lung, and spleen) were washed 10 min in NaCl solution, weighed, sliced, and homogenized (glass barrel, Teflon pestle) in batches of 2 to 2.4 g in 6 ml of 0.25 m sucrose, 0.01 m Tris, 1.5 mm EDTA, pH 7.4, at temperatures close to 0°. Centrifugation for 15 min at 1,000 × g to remove the nuclear-endoplasmic reticulum-myofibrillar fraction was followed by centrifugation for 1 hour at 105,000 × g at 1° to obtain the cytoplasmic supernatant fraction (cytosol). Protein concentrations in the cytosol preparations were determined by the Lowry method (23).

The samples of human corticosteroid-binding globulin obtained from the Cancer Chemotherapy National Service Center, Bethesda, Maryland, were concentrated and adsorbed to Sephadex G-50, eluted with 0.01 m MgCl₂, pH 7.4, and collected into 1-ml fractions, all at 4°. The labeled steroid

**Materials and Methods**

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The samples of human corticosteroid-binding globulin consisted of unfractonated plasma from pregnant women who had been treated for 3 days with 8 mg of dexamethasone.

Enzymes and protein standards were obtained commercially with the exception of human fibrinogen (courtesy of Dr. John Finlayson, National Institute of Health), human thyroglobulin (courtesy of Dr. Harold Edelhoch, National Institutes of Health), and phycoerythrin (courtesy of Dr. H. W. Siegelman, Brookhaven).

**Steroids**—Unlabeled steroids were obtained from the Cancer Chemotherapy National Service Center, Bethesda, Maryland. Concentrations of ethanolic solutions were determined spectrophotometrically (24).

**Progesterone-7α-H (10.1 Ci per mmole), progesterone-1,2-3H (33.5 Ci per mmole), cortisol-1,2-3H (25 Ci per mmole), and tolune-3H for instrument calibration were obtained from New England Nuclear. Radiochemical purity of the steroids was verified by chromatography on Silica Gel 60F254 (Brinkmann Instruments, Inc., Westbury, New York) developed with benzene-ethyl acetate (60:40, v/v). Radioactivity was usually measured with a Packard Tri-Carb scintillation spectrometer, model 3375, in 10 ml of scintillation fluid containing toluene, Triton X-100 (Packard Instrument Company), and Liquliphor (New England Nuclear) (667:333:42, v/v). Counting efficiency under these conditions was 41% for nonaqueous samples and 34% for 1-ml aqueous samples. The radioactive steroid extracted from the macromolecular complex was identified by descending paper chromatography in ligrom-methanol-water (100:90:10, v/v) (25).

**Sucrose Gradient Centrifugation**—Linear gradients of 5 to 20% sucrose in various buffers were prepared with a Buchler triple outlet gradient mixer and Polystyral pump. Incubation of plasma or oviduct cytosol with the radioactive hormone for 20 to 45 min was found adequate, since binding also occurred during the first few hours of centrifugation. Samples were diluted 1:3 immediately before layering onto cold gradients. Centrifugation was performed at 1° in the Spinco model L2-65 ultracentrifuge using the Spinco SW-65 rotor at 60,000 rpm (average force 257,000 × g) or the Spinco SW-56 rotor at 55,000 rpm (average force 207,000 × g). Fractions were collected from the gradient bottom by means of the Buchler piercing unit and drop counter. Radioactivity was determined on 0.1 to 0.2 ml aliquots of undiluted fractions or on 1-ml aliquots of diluted fractions after measurement of absorbance in a Zeiss spectrophotometer. Quenching of radioactivity did not vary significantly with the concentration of sucrose in the fractions. Low recoveries of radioactivity in certain experiments were shown to be due to failure to recover highly aggregated forms of the labeled receptor complex from the bottom of the centrifuge tube and to adsorption of steroids to the Tygon tubing in the gradient fractionation setup. Sedimentation coefficients of steroid-binding components were estimated by comparison with those of ovalbumin, human CBG, and catalase.

**Heat and Enzymatic Release Studies**—The complex formed by incubation of [H]-progesterone (4.7 × 10⁻⁸ m) with oviduct cytosol from chicks on diethylstilbestrol was isolated by centrifugation for 21 hours at 257,000 × g in sucrose gradients containing 0.3 m KCl. Gradient fractions comprising the major peak of bound counts were pooled. Aliquots of 0.4 ml were incubated for 30 min at 0° with (a) no addition, (b) 0.5 mg of Pronase, (c) 0.5 mg bovine RNase, or (d) 0.5 mg of DNase plus 2 μl per ml of 1 m magnesium acetate; all enzymes were obtained from Worthington. Another aliquot was incubated 30 min at 37°. Treated samples were layered onto 4-ml columns of Sephadex G-50, eluted with 0.01 m Tris, 1.5 mm EDTA, pH 7.4, and collected into 1-ml fractions, all at 4°. The labeled steroid
which remained in a macromolecular complex was eluted immediately after the void volume, whereas the steroid released by the treatment was eluted in the total volume of the column. Recovery of radioactivity through the procedure was about 80%.

Polyacrylamide Gel Electrophoresis—Separation gels (4.2 × 0.6 cm) with total acrylamide concentrations of 4 to 11% were prepared from acrylamide monomer containing 2% of the cross-linking agent methylene bis-acrylamide. Photopolymerization (Buchler "Poly Prep ") was initiated by riboflavin with tetramethylethylene diamine as accelerator (27). Stacking gels (1.2 × 0.6 cm) containing a total acrylamide concentration of 3.125%, 20% methylene bis-acrylamide, were prepared, layered onto the separation gels, and photopolymerized similarly. The multiphasic buffer system was a modification to 0°C and pH 10.2 of the Tris system of Davis (28). Duplicate samples containing 50 to 100 µl of cytosol or 5 µl of plasma were incubated with labeled hormone, mixed with an equal volume of 50% sucrose containing bromphenol blue dye, and layered onto the stacking gels. Following electrophoresis at 2 ma per gel at 0°C (Buchler "Polyanalyt "), the separation gels were removed from the glass tubing, chilled to -20°C, and sectioned transversely into 1.1-mm slices with an "egg slicer" type of device (Earl Sandbek Specialized Medical Instrumentation, Baltimore, Maryland). The radioactive hormone in each slice was measured after extraction for 1 hour into a toluene-Liquifluor scintillation fluid (960:40, v/v). The molecular weight and net charge of each labeled complex was estimated from plots of the log of the mobility of the complex relative to the dye front versus acrylamide concentration in the gel (29). Under the conditions used, bromphenol blue had a relative mobility, Rf, of 1.0 so that the Rf of the standard and steroid-binding proteins could be calculated with reference to this dye (27).

Agarose Gel Filtration—All chromatographic procedures were performed at 1–4°C. Agarose beads for gel filtration (Bio-Gel A-0.5m from Bio-Rad, Richmond, California) were packed over a shallow base of fine glass beads in columns (1.27 × 110 cm) and washed extensively with 0.01 M Tris, 1.5 mM EDTA, pH 7.4, containing no salt or 0.3 M KCl. Samples of plasma (0.2 ml) or cytosol (0.4 to 2.0 ml) were generally placed on a column after incubation of cytosol with labeled hormone does not increase the fraction of radioactivity bound. In gradients containing 0.3 M KCl, the rate of sedimentation of the progesterone and cortisol bound by chick oviduct cytosol is comparable to that of human CBG (Fig. 1). A low concentration of steroid required (10⁻⁸ M), and a slow rate of release of the steroid at 1°C is inferred from the persistence of binding when the complex sediments away from the free steroid at the meniscus. The association reaction occurs either instantaneously or during the early hours of centrifugation; preliminary incubation of cytosol with labeled hormone does not increase the fraction of radioactivity bound. In gradients containing 0.3 M KCl, the rate of sedimentation of the progesterone and cortisol bound by chick oviduct cytosol is comparable to that of human CBG (Fig. 1). The reversibility of the steroid binding and the relative affinities of various steroids for the cytoplasmic components were established by a series of experiments like those shown in Fig. 2. A large sample of cytosol was incubated 2 hours with 3H-progesterone (2 to 5 × 10⁻⁸ M), then split into aliquots which were reincubated with the competing steroid (4 × 10⁻⁴ M) and centrifuged in 5 to 20% sucrose gradients containing 0.3 M KCl. The relative decrease in the peak of bound radioactivity suggests the following order of affinity under these conditions: progesterone > testosterone > 17α-ethynyl-19-nortestosterone > 20α-hydroxy-4-pregnen-3-one > 17β-estradiol > cortisol > estrone > androstenedione.

Centrifugation of oviduct cytosol incubated with increasing concentrations of 3H-progesterone demonstrated the saturation of a limited number of binding sites. Preliminary estimates of the binding constant and concentration of sites in the oviduct cytosol were obtained from the data in Fig. 3. Calculations were made according to the simple formulation

$$\frac{1}{(SP)} = \frac{k_d}{(P)} \frac{1}{(S)} + \frac{1}{(P)}$$

in which (SP) denotes the concentration of bound steroid, (S) is

1 The abbreviations used are: CBG, corticosteroid-binding globulin, also called transcortin; BSA, bovine serum albumin.
Fig. 2. Competition with $^3$H-progesterone binding to oviduct cytosol by cold progesterone and other steroids. Sucrose gradient centrifugation of each set of three samples was performed as in Fig. 1.

The free steroid concentration, $k_d$ is the dissociation constant, and $(P)$ is the total concentration of binding sites in the cytosol. This equation is rigorously applicable only to equilibria between a ligand and a single class of noninteracting binding sites. Since the separation of free from bound steroid favors dissociation of the complex, and since gradient centrifugation in 0.3 M KCl at available speeds does not resolve the several binding components revealed by other techniques, the following values serve only as first approximations:

$$k_d \approx 8.3 \times 10^{-9} \text{ M at 1-4^°}$$

$$P \approx 1.9 \times 10^{-4} \text{ M}$$

The assumption that the binding component is a protein with a molecular weight of 1.5 $\times$ 10$^5$ (see Table III) and a single site of interaction with steroid leads to a calculated weight concentration of 2.8 $\mu$g per ml of cytosol. The binding molecules thus represent about 0.02% of the total protein (15 mg per ml) in this cytosol preparation. This value for the relative concentration of the binding components is the same as that determined for the uterine estrogen receptors (6).

The concentration of KCl has been shown to influence drastically the molecular size of the estrogen-binding components of rat and rabbit uterus (30) and calf endometrium (31). The analogous sensitivity of the progesterone-binding components of chick oviduct cytosol is apparent in the gradient centrifugation results in Fig. 4. In the absence of salt, there is significant binding to macromolecules with sedimentation coefficients, $s_{20,w}$, of about 5 S and 8 S. In the short period of centrifugation used in the illustrated experiment, the smaller component in cytosol is not clearly distinguished from CBG ($s_{20,w}$ of human and presumably chick CBG = 3.79 S (32)). Its sedimentation coefficient was determined to be 5 S by longer centrifugation of samples to which crystalline ovalbumin ($s_{20,w} = 3.67$ S (33)) was added as an internal marker and was visualized as a peak of absorbance. The value of $s_{20,w}$ for the faster peak was determined by shorter centrifugation with catalase standards ($s_{20,w} = 11.2$ S (34)) run in parallel gradients not containing EDTA. The 8 S peak appears from its shape to encompass more than one component. Since there is no progesterone-binding component of comparable size in the plasma of the same diethylstilbestrol-treated chicks, the 8 S peak must represent one or more intracellular progesterone-binding components.

In the presence of 0.3 M KCl, the $^3$H-progesterone bound by oviduct cytosol sediments as a single peak at the same rate as CBG and as crystalline ovalbumin added as an internal marker (Fig. 4, upper right). $^3$H-Progesterone is not bound by ovalbumin itself in control experiments. The sedimentation coefficient of the radioactive complex in 0.3 M KCl is therefore given as 3.7 S, with the following reservations. (a) The 105,000 $\times$ g supernatant of cytoplasm is a crude preparation, in which the sedimentation rate of the progesterone complex may be altered by chemical or hydrodynamic interactions with other constituents of the mixture; (b) calculation of $s_{20,w}$ by comparison with other macromolecules requires that the unknown and standards have the same partial specific volume ($\bar{\varepsilon}$).

To ascertain whether $\varepsilon$ of the progesterone-binding components differs significantly from that of ovalbumin, centrifugation...
of labeled cytosol mixed with crystalline ovalbumin was performed in gradients of various densities. For example, 5 to 20% sucrose gradients were prepared in buffered solutions containing 25% (w/v) NaBr, to obtain gradients with densities (ρ) at 1° of 1.2024 to 1.2515 g per cm³ (35). These conditions were selected so that ovalbumin, having ϕ = 0.748 cm³ per g (36) or ρ = 1.336 g per cm³, would sediment slowly to the bottom of the tube, while even the most dense of lipoproteins (ρ = 1.21 g per cm³ (37)) would sediment through only the upper part of the gradient. In gradients containing 40% NaBr (ρ = 1.5134 to 1.3658 g per cm³) ovalbumin would sediment through part of the gradient while all lipoproteins would float.

The experimental results for three gradients of widely separated densities are shown in Fig. 5. The similar amount of retardation of the binding components and ovalbumin (peak of absorbance) indicates the similarity of their densities and the absence of significant lipid content in the binding components.

also be noted that the progesterone-binding components are neither precipitated nor denatured by these extremely high salt concentrations.

Other gradient centrifugation experiments showed that oviduct cytosol from chicks receiving diethylstilbestrol continuously and from chicks withdrawn from treatment for 10 days had similar patterns of progesterone binding; the concentration of sites in the cytosol from chicks off diethylstilbestrol has not been determined. The binding activity was also shown to be diminished but readily detectable in cytosol preparations stored frozen for 5 months.

Polyacrylamide Gel Electrophoresis—As in sucrose gradient centrifugation in the absence of KC1 (Fig. 4), two major peaks of bound 4-H-progesterone are revealed by electrophoresis of oviduct cytosol on polyacrylamide gels at pH 10.2 and 0° (Fig. 6). The major progesterone-binding molecules of cytosol (I and II) migrate more slowly under these conditions than the minor progesterone-binding component which also is the major cortisol-binding component in the cytosol (III). The latter peak is identical in relative mobility, Rf, with the progesterone- and cortisol-binding component of chick plasma, and presumably represents a small amount of CBG in the cytosol preparations (cf. Fig. 9). Unlabeled progesterone (10⁻⁷ M) completely abolished the binding of 10⁻⁹ M ²H-progesterone. The same concentration of unlabeled cortisol eliminated only Peak III from the progesterone-labeled electrophoretic pattern.

To distinguish between the contributions of net charge and molecular size to the electrophoretic mobility, experiments similar to those in Fig. 6 were carried out in gels of different total acrylamide concentration all cross-linked to the same extent (38). Fig. 7 shows the variation with total acrylamide concentration of log RF for the major progesterone-binding components of oviduct cytosol and plasma. From these results, estimates of the molecular weight and net charge of each labeled complex were obtained by applying the method and computer programs of Rodbard and Chrambach (27, 29).

The first step was to calculate the retardation coefficient (KR), which is the negative slope of the weighted least square linear
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Fig. 6. Resolution of the cytoplasmic progesterone-binding components from the cortisol-binding component (chick CBG) by disc gel electrophoresis. The major peaks of 3H-progesterone bound by cytosol (I and II) migrate more slowly than the minor progesterone-binding component but major cortisol-binding component in cytosol (III). Chick CBG has the same relative mobility as III under these conditions: 6% polyacrylamide gel; pH 10.2, Tris; 0°. Free steroid does not enter the gel.

Fig. 7. Variation of electrophoretic mobility with total acrylamide concentration in the gel. RF denotes mobility of bound steroid relative to the dye front (cf. Fig. 6). In this plot the slopes depend only on molecular size, whereas intercepts at 0% gel reflect the free electrophoretic mobility (27).

regression through the data for log RF versus total acrylamide concentration, from the results in Fig. 7 and analogous data for a series of protein standards run under the same conditions. Rodbard and Chrambach (29) have established theoretically and empirically that $K_R^{1/2}$ is a linear function of $R$, where $R$ is the radius of the equivalent unsolvated sphere, calculated from

$$R = \left(3 \theta \text{ mol wt} / 4 \pi N \right)^{1/2}$$

A standard curve for $K_R^{1/2}$ versus $R$ based on data for the proteins listed in Table I was then used to estimate $R$ and hence molecular weight for Components I and II and chick CBG. The result for CBG is consistent with the values obtained by Agarose gel filtration (see below).

The next step in the analysis of the data in Fig. 7 was to extrapolate the lines for log RF versus total acrylamide concentrations to 0% gel concentration to obtain the free solution mobility and hence the net charge on each component under the given conditions (27). The results summarized in Table I confirm the distinction between the cytoplasmic binding components and CBG with respect to both molecular weight and net charge to mass ratio under these conditions.

The observations that the charge to mass ratio for Components I and II is the same (within the experimental uncertainty) and that the binding components tend to aggregate under other conditions suggest that Component I is an aggregate of Component II. Given the computed values of molecular weight (Table I) and the knowledge that most regulatory proteins have the structures of symmetrical oligomers, we would tentatively propose that the correct molecular weight of Component II is about $9 \times 10^4$ and that Component I is a tetramer of II. Furthermore, comparison of these estimates with the sedimentation coefficients of the binding components in the absence of KCl (but at pH 7.4 rather than 10.2) suggests the identity of the S S component of the centrifugation experiments with the molecule of $9 \times 10^4$ daltons. The S peak is assumed to represent a dimer of the S S component, which itself forms a dimer of mol wt $\approx 36 \times 10^4$ under the conditions of the electrophoresis experiments. Further studies are in progress to verify the estimates of molecular weight under conditions of low ionic strength.

Agarose Gel Filtration—The binding of progesterone to macromolecular components of oviduct cytosol is sufficiently tight at low temperatures to withstand chromatographic separation of the complex from free steroid. To obtain the chromatogram in Fig. 8, oviduct cytosol from chicks off diethylstilbes-

| Standards      | $K_R^{1/2}$ | A  | $X \times 10^{-3}$ | Valence |
|----------------|-------------|----|-------------------|---------|
| Ovalbumin      | 0.2429      | 23.72 | 45.5             |         |
| BSA monomer    | 0.2607      | 26.99 | 67.0             |         |
| Pepsin dimer   | 0.2614      | 27.51 | 71.0             |         |
| Transferrin    | 0.2535      | 27.89 | 74.0             |         |
| BSA dimer      | 0.3102      | 34.00 | 134.0            |         |
| Phycoerythrin  | 0.3533      | 43.40 | 290.0            |         |
| Fibrinogen     | 0.4255      | 46.37 | 340.0            |         |
| Ferritin       | 0.3447      | 50.92 | 450.0            |         |
| Thyroglobulin  | 0.4680      | 58.14 | 670.0            |         |
| Binding components |       |    |                   |         |
| I              | 0.3898      | 47.18 | 357.0            | 20.5    |
| II             | 0.2784      | 31.05 | 102.0            | -8.0    |
| CBG (chick)    | 0.2433      | 25.08 | 60.0             | -7.0    |
| CBG (human)    | 0.2467      | 20.47 | 43.0             | -9.7    |

$^a$ $K_R$ (the retardation coefficient) is the negative slope of the line for log RF versus total acrylamide concentration (cf. Fig. 7).

$^b$ $R$ (the radius of the equivalent unsolvated sphere) = $\left(3 \theta \text{ mol wt} / 4 \pi N \right)^{1/2}$

$^c$ All values of mol wt are from Reference 27 except ovalbumin (39).

$^d$ Significant deviation from $K_R^{1/2}$ versus $R$ standard curve.
Gel filtration on Agarose A-0.5 m of oviduct cytosol incubated with \( ^{3}H \)-progesterone (4.7 \( \times \) \( 10^{-8} \) M) and eluted with buffered 0.3 M KCl at 1–4°C. The major peak of bound radioactivity \( (A) \), comprises more than one component and is eluted before most of the cytoplasmic proteins (see absorbance pattern). The control was labeled in vitro with \( ^{3}H \)-progesterone, layered onto a column (1.27 \( \times \) 110 cm) of Agarose A-0.5 m, and eluted with 0.01 M Tris, 1.5 mM EDTA, 0.3 M KCl, pH 7.4. The first peak of radioactivity and optical density represents materials excluded from the interstices of the gel (apparent molecular weight >5 \( \times \) \( 10^{5} \)). The major progesterone-binding activity \( (A) \), generally chromatographs as a double peak \( (\text{Components } A_{1} \text{ and } A_{2}) \). The relative amounts of \( A_{1} \) and \( A_{2} \) are constant for a given pool of cytosol but vary slightly among different preparations. The \( A \) components are eluted in a region of low optical density at 280 nm, indicative of a larger molecular size than most of the cytoplasmic constituents (see "Physical Parameters of the Binding Components in Presence of 0.3 M KCl").

The next peak of binding activity \( (B) \) is tentatively identified as chick CBG by the superposition of chromatographs of progesterone-labeled oviduct cytosol and chick plasma in Fig. 9. By contrast, the possibility that the \( A \) components are artifactual aggregates of CBG is negated by the complete absence of bound progesterone in the \( A \) region after filtration of labeled plasma or refiltration of the isolated \( B \) peak.

Treatment of the chicks with the synthetic estrogen diethylstilbestrol affects both the amount of \( ^{3}H \)-progesterone bound and the distribution of proteins in the oviduct cytosol. As shown in Fig. 10, the labeled macromolecular complex is just detectable in gel filtration Fractions 55 to 70 of cytosol from unstimulated chicks (no diethylstilbestrol), compared with extensive binding in these fractions of cytosol from chicks on diethylstilbestrol. This effect of continuous estrogen treatment may reflect an increased number of binding molecules, analogous to the increase of ovalbumin (absorbance peak in Fraction 82). Alternatively, diethylstilbestrol treatment may increase the affinity of these components for progesterone. Withdrawal of estrogen for 10 days before sacrifice (chicks off diethylstilbestrol) does not significantly change the pattern of progesterone binding (cf. Fig. 8) although the optical density pattern of the eluate reverts toward that of cytosol from unstimulated chicks. The concentration of sites in cytosol from chicks off diethylstilbestrol has not been determined.

If the progesterone-binding molecules of oviduct cytoplasm participate in the specific response of the organ to the hormone, the concentration of these components in nontarget organs is expected to be low or negligible. This prediction is borne out by the gel filtration patterns of progesterone-labeled lung and spleen cytosols from chicks on diethylstilbestrol (Fig. 11). In both organs the interaction of the hormone with materials which are excluded from the gel exceeds the binding in the region of the major oviduct components (cf. Fig. 8). The only significant binding activity in either nontarget organ, in Fractions 74 to 81...
of lung cytosol, is identical in elution position with chick CBG (cf. Fig. 9).

The change in molecular size of the binding components as a function of KCl concentration in centrifugation experiments (Fig. 4) was corroborated by the gel filtration experiments in Fig. 12. In the absence of KCl (upper left) the bulk of bound progesterone is eluted in a broad peak which trails from the excluded volume of the column (Fractions 51 to 62). The interconvertibility of the material in this peak with the forms of the binding components observed in 0.3 M KCl was shown by rechromatography of the peak fractions recovered in the absence of salt (arrows) on a column equilibrated with 0.3 M KCl. From the resultant elution pattern (upper right) it is inferred that the major components observed in the presence of 0.3 M KCl combine reversibly to a highly aggregated state when the salt is removed. To confirm the reversibility of this transition in both directions, the converse experiment was performed. All of the bound radioactivity from the A peak in 0.3 M KCl (lower left) was recovered in the excluded volume of the column without salt (lower right).

Other experiments were designed to determine whether the overlapping peaks (A1 and A2) observed in the presence of KCl represent interconvertible components which would re-equilibrate after isolation. From the chromatogram shown in Fig. 12 (lower left), the three fractions on each side of the arrows were rechromatographed on the same column. The resultant elution patterns (not illustrated) each contained a single peak of bound 

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### Chemical Identification of Bound Radioactivity
In earlier experiments on the incubation of chick oviduct minces with 

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**Fig. 11.** Agarose gel filtration of cytoplasmic supernatants of non-target organs from diethylstilbestrol (DES)-treated chicks after incubation with 1.2 x 10⁻⁸ M 

**Fig. 12.** Demonstration of the reversible interconversion of the A components, observed in 0.5 M KCl, with highly aggregated forms that are excluded or nearly excluded from Agarose A-0.5m in the absence of KCl. Fractions marked with arrows in elution patterns on left were pooled and rechromatographed in the opposite solvent, giving the results on right (cf. Fig. 4).
of the hormone, (d) inclusion of p-chloromercuribenzoate during incubation with the hormone, (c) mild heating of the isolated macromolecular hormone complex, and (d) enzymatic digestion of the complex.

Heating of the cytosol to 60° for 2 min prevented subsequent binding of ′H-progesterone except for limited interaction with aggregates excluded from Agarose A-0.5m in the presence of 0.3 M KCl.

Addition of p-chloromercuribenzoate to a final concentration of 1 mM in cytosol likewise eliminated all binding except barely detectable amounts to material in the excluded volume of the column. This result indicates not only the protein nature of the hormone-binding site, but also the involvement of sulfhydryl groups in either the interaction with the ligand or the maintenance of the active structure of the binding component.

The integrity of the macromolecular steroid complex isolated by sucrose gradient centrifugation and collection in the cold is demonstrable by gel filtration on Agarose (Fig. 13) or on Sephadex G-50. The pattern of elution from Sephadex G-50 (not illustrated) contains the bound hormone in the excluded volume and the free steroid in the total column volume. This sharp resolution of bound from free hormone by short Sephadex columns was used to measure the ′H-progesterone released from the isolated complex by mild heat, Pronase, and nucleases. The results in Table II indicate that proteins are essential for the binding of progesterone; the results do not exclude the presence in the macromolecule of small amounts of other constituents such as nucleic acids, lipids, or carbohydrates.

**Physical Parameters of Binding Components in Presence of 0.3 M KCl**—Gradient centrifugation of progesterone-labeled cytosol in the presence of 0.3 M KCl reveals one major peak of binding activity with a sedimentation coefficient of 3.7 S (Fig. 1). For globular proteins this value of $\xi_0$ corresponds to a molecular weight of 42,000 to 48,000 (34).

Molecular size may also be estimated from the Agarose gel filtration data. Distribution coefficients of the peaks of bound radioactivity, $K_D$ were calculated according to the standard formula

$$K_D = \frac{V_s - V_o}{V_t - V_o}$$

where $V_s$ is the elution volume of the component, $V_o$ is the void volume of the column, and $V_t$ is the total liquid volume, here approximated by the elution volume of free steroid. Apparent molecular weights of steroid-binding components were estimated by assuming a linear dependence of mol wt on $K_D^{1/2}$, using as standards, ovalbumin, bovine serum albumin monomer, and BSA dimer (30). Results for the major progesterone binding components (A1 and A2) and Component B (cf. Figs. 6, 12) are given in the last column of Table III. These values of apparent molecular weights correspond to true molecular weights only if the binding molecules have the same $\xi$, shape, and degree of solvation as the globular protein standards. Since the latter two factors are unknown for the cytosol components, the more valid parameter which may be determined from the chromatograms is the effective molecular radius $a$ (Stokes radii). For the binding components listed in Table III, Stokes radii were calculated according to the theoretical treatment of Ackers (40):

$$K_D = \left(1 - \frac{a^2}{r^2}\right) \left[1 - 2.104 \left(\frac{a}{r}\right) + 2.09 \left(\frac{a}{r}\right)^2 - 0.05 \left(\frac{a}{r}\right)^3\right]$$

where $\kappa$ is the Boltzmann constant, $T$ is absolute temperature, and $\eta$ is the solvent viscosity.

The apparent discrepancy between the slow sedimentation rate (3.7 S) and the large molecular size of the major binding components on gel filtration in 0.3 M KCl (a = 55 to 63 Å) could be reconciled by several hypotheses. (a) The binding components have unusually large $\xi$, characteristic of lipoproteins. (b) The sucrose in the centrifugation experiments alters the molecular size of the components. (c) The binding components are very asymmetric or have extraordinary solvation. (d) Distinct, unrelated components are revealed by the two techniques. (e) Different states of aggregation of the same components are being observed.

The first hypothesis is ruled out by the results of centrifugation experiments in high density gradients (Fig. 5). The alteration of the molecular size by sucrose was disproven by equilibrating the column and eluting with buffered 0.3 M KCl containing 5% sucrose. The amount and elution positions of bound ′H-progesterone were unaffected by the sucrose.

The third hypothesis was pursued by combining the results for the sedimentation coefficient ($s$) and the Stokes radius ($a$) to calculate the molecular weight ($M$) and the corresponding frictional ratio, $f/f_0$ (41, 42):

$$M = \frac{6\pi\eta N s a}{1 - \nu^2}$$

$$\frac{f}{f_0} = a^{10} \left[\frac{4\pi N}{3M(\theta + \delta/\rho)}\right]^{1/2}$$

where $N$ is Avogadro's number, $\rho$ is the solvent density in grams per cm$^3$, and $\delta$ denotes the solvation. When $\theta$ and $\delta$ were assigned values which are typical for proteins ($\theta = 0.754$ cm$^3$ per g, $\delta = 0.2$ g of solvent per g of protein (38, 42)), the estimates...
The recording of optical density in Fig. 13 shows that most of the protein in the 3.7 S peak is eluted from the column in Fractions 88 to 96, as expected for globular proteins with molecular weights of 42,000 to 48,000. By contrast, the macromolecular complex containing the radioactive steroid displays the characteristic elution pattern of the A components, corresponding to much larger molecules. This demonstration of the A peak in chromatograms of the isolated 3.7 S peak excludes the possibility that the components observed by the two techniques are unrelated.

The ensemble of results is compatible with the identity of the major components detected by centrifugation and gel filtration in the presence of KCl, if the components are rather asymmetric, or with the assumption that the A peak of the chromatograms represents specific aggregates of the 3.7 S components. Regardless of its molecular basis, this unique combination of a low sedimentation coefficient and large molecular size should greatly facilitate the isolation of the A components from other cytosol constituents.

DISCUSSION

The presence of specific progesterone-binding components in oviduct cytoplasm from estrogen-treated chicks has been demonstrated by ultracentrifugal, electrophoretic, and chromatographic techniques. These cytoplasmic components are readily distinguished from the progesterone-binding component in the plasma of the same chicks by all techniques utilized. The cytoplasmic components have significantly higher affinity for progesterone than for cortisol, with an estimated $k_d$ for progesterone of about $8 \times 10^{-16}$ M in 0.3 m KCl at 1°C. This value is remarkably close to that determined by the same technique for the interaction of estradiol with its uterine receptors in rat, $k_d \approx 7 \times 10^{-16}$ M (6). It is likely, however, that the initial estimate of $k_d$ for the progesterone-binding component will be revised by equilibrium studies on less crude preparations.

While several other workers have reported data which imply progesterone-binding to macromolecules in animal cells, the components described previously do not seem to fulfill the predictions for a specific target tissue progesterone "receptor". The molecule studied by Milgrom and Baulieu (16) was indis-
tistinguishable from rat plasma CBG by the criteria of relative affinity for cortisol and progesterone, sedimentation coefficient, electrophoretic mobility, distribution coefficient on Sephadex columns, or thermal lability. The pregnenolone- and progesterone-binding molecule described by Karsznia et al. (17) was isolated from the prostate gland, in which the progestins serve no known function, and differs from other reported receptors in its stability and enhanced affinity for steroids at 60°C. Lecymarie and Guzr guian (18) have achieved the chromatographic separation of a specific progesterone-binding component from all cortisol-binding activity in the corpus luteum of the pregnant cow. However, the function of the binding components in an organ which synthesizes and secretes the hormone is likely to differ from the "receptor" function in a target organ. The data reported here thus represent the initial isolation and partial characterization of specific target tissue receptors for progesterone which are not present in the plasma or nontarget tissues.

The progesterone-binding components of chick oviduct cytoplasm exhibit various states of aggregation or asymmetry as a function of the KCl concentration and the technique of isolation and detection. In the absence of KCl, the major components are characterized by sedimentation coefficients of about 5 S and 8 S, which for globular proteins correspond to molecular weights of 7.0 to 8.6 \times 10^4 and 1.5 to 1.8 \times 10^5, respectively, suggesting a monomer-dimer relationship (94). From the variation of electrophoretic mobility with acrylamide gel concentration and the extrapolated value for the free solution mobility of the major components, computer analysis gave molecular weights of 1.0 (or 0.9) \times 10^4 and 3.6 \times 10^4, respectively, and the same charge to mass ratio for both components, consistent with a monomer-tetramer relationship. Still higher states of aggregation are inferred from the elution pattern from Agarose A-0.5 m in solutions of low ionic strength. By contrast, in solutions containing 0.3 m KCl, the macromolecular progesterone complex appears to sediment as a single peak with $d_{20, w} \approx 3.7$ S, corresponding to molecular weight of 4.2 to 4.8 \times 10^4 for globular proteins, or about half the weight of the smaller component observed in the absence of KCl. Agarose gel filtration in the same solvent of the isolated 3.7 S peak partially resolves two major components of bound progesterone with Stokes radii of 55 and 63 A, respectively. As in the studies without KCl, the apparent size on gel filtration is larger than that expected from the centrifugation results. These observations may be reconciled by postulating that specific aggregates of the steroid-macromolecular complex are formed during gel filtration and gel electrophoresis or that the binding components are extremely asymmetric, with axial ratios in the range of 14 to 22. Further experiments are required to test the proposed relationships among the forms of the components detected in these preliminary studies.

The abilities of various steroids to compete with the binding of $\text{H}$-progesterone in the presence of KCl correlate well with their respective capacities to induce avian synthesis in vivo (measured as micrograms of avian per g of oviduct, 20 hours after injection of 5 mg of steroid (22)). The most active competitors of binding, 20α-hydroxy-4-pregnen-3-one, testosterone, and 17α-ethynyl-19-nortestosterone, are all active inducers. Conversely, cortisol and androstenedione, which are poor binding competitors, show no inductive activity. These results, coupled with the parallel effects of diethylstilbestrol treatment on progesterone-binding activity and on avian induction, suggest that the binding components play a functional role as the receptor in the induction process.

At present, we have no chemical data upon which to construct a solid hypothesis for the sequence of molecular events in steroid hormone action. An initial interaction of a steroid with a cytoplasmic receptor, followed by activation of the transcriptional apparatus to produce new RNA and finally new protein, is a popular concept (22, 45, 46). However, not only is there no consensus on whether the important regulatory step is at the level of transcription or translation (46), but there is no definitive evidence for a functional role for the target tissue receptors. Jensen et al. (47) have attributed the difference between the 5 S form of the estrogen receptor from uterine nuclei and the 4 S form of the cytoplasmic receptor in 0.3 m KCl to the addition of a nuclear constituent related to the regulatory function of the molecule. Raynaud-Jammet and Baulieu (48) have reported that incubation of endometrial nuclei with uterine cyto- plasm permitted an estrogen-mediated stimulation of CTP incorporation into nuclear RNA, possibly through a functional cytoplasmic estrogen receptor complex. The independent confirmation of these provocative experiments using the progesterone-binding components of oviduct cytoplasm and nuclei (49) may allow us to delineate a functional role for the receptor and define the biochemistry of the interaction of the hormone-receptor complex with the cell nucleus.

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