Progesterone Receptors of Chick Oviduct

STEROID-BINDING "SUBUNIT" FORMED WITH DIVALENT CATIONS*

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SUMMARY

In the presence of several divalent cations, progesterone receptors of estrogen-treated chick oviducts release a “subunit” characterized by a sedimentation coefficient of 2.6 S, Stokes radius of 21 Å, and apparent molecular weight of about 20,000. Charcoal-dextran assay of bound steroid reveals the same order of affinities for the “subunit” as for intact receptors: progesterone (K_d ~ 4 x 10^{-14} M) > deoxycorticosterone > testosterone > corticosterone > 17β-estradiol > cortisol. Among the cations tested, the order of effectiveness for “subunit” formation is Ca^{2+} > Mn^{2+} > Sr^{2+} > Ba^{2+}. Mg^{2+} is ineffective. “Subunit” can be obtained by CaCl_2 treatment of receptors partially purified by density gradient centrifugation (4 and 5 S components), isoelectric focusing, or polycation precipitation. “Subunit” is formed in the absence of steroid or of diisopropylfluorophosphate, a protease inhibitor. These observations and the results of preliminary studies of Caz+-binding are consistent with a direct interaction of Ca^{2+} and similar ions with a component of the receptor complex.

The term receptor is used in this report to denote a macromolecular component with which a steroid hormone interacts with high affinity and specificity as an early and indispensable event in the course of its action on the target cell. In the estrogen-primed chick oviduct, for example, the induction of avidin synthesis by progesterone is thought to be mediated by binding components or receptors that have been analyzed by several physico-chemical techniques (1). The objectives of the present research were to purify and characterize the steroid-binding moiety or “subunit” of the chick oviduct progesterone receptor, and to gain insight into the structure of the intact receptor complex and its interactions with divalent cations. In media of low ionic strength, many steroid-receptor complexes aggregate and resist fractionation by techniques such as gel filtration and gel electrophoresis (2). In concentrated solutions of monovalent salts, the complexes display anomalous molecular parameters, such as low sedimentation coefficients but large apparent radii on gel filtration (1). By contrast, preliminary characterization of progesterone receptors in low concentrations of calcium or manganous ions revealed a stable, compact structure (3).

An influence of Ca^{2+} and similar ions on receptors for non-progestational steroids has been recognized for several years. DeSombre et al. (4) reported in 1969 that if calf uterine cytosol is made 4 mM in CaCl_2, the estradiol-receptor complex precipitated by ammonium sulfate consists, in part, of a stabilized “subunit.” This unit has been characterized by an apparent molecular weight of 61,006, Stokes radius of 33 Å, and a heterogeneous distribution on isoelectric focusing (5). The irreversible conversion of estrogen receptors to this form was attributed to a macromolecular transforming factor, separable from the steroid-binding activity by fractional ammonium sulfate precipitation (6). Rochefort and Baulieu (7) interpreted related results in terms of a calcium activated proteolytic enzyme, although inhibitors of several known classes of proteases did not prevent receptor transformation. They noted the presence of “additional lighter (< 4 to 5 S) components” after exposure of calf uterine estrogen receptors to 1 mM CaCl_2. Similarly, Edelman and colleagues (8, 9) extracted a 3 S aldosterone receptor complex from rat kidney nuclei treated with 3 mM CsCl.

The preceding observations on estrogen and aldosterone receptors and our present findings on progesterone receptors support the concept that divalent cations act in vitro to alter the structure and activity of receptors for many, if not all, steroid hormones. The results in this report differ from those obtained with other systems insofar as (a) Ca^{2+}, Sr^{2+}, Ba^{2+}, and Mn^{2+} act either directly on progesterone receptors, or with the help of transforming factors that are inseparable from receptors under the conditions tested, and (b) the resultant steroid-binding unit (“subunit”) is smaller than the calcium-stabilized units previously described.

MATERIALS AND METHODS

Radioactive Materials and Analyses—[1,2-3H]Progesterone (90.3 Ci per mmole), [1,2,6,7-3H]Progesterone (81.1 Ci per mmole), [3H]toluene for instrument calibration, and [U-14C]Glyceraldehyde (7.4 mCi per mmole) were purchased from New England Nuclear. [1,2,6,7-3H]Progesterone (110 Ci per mmole) and aqueous 116CaCl_2 (initially 1.33 Ci per mmole) were obtained from Amersham-Searle. Radiochemical purity of the progesterone was checked...
intermittently by thin layer chromatography, using a Chroma-
gram chamber and Chromagram silica sheets (Eastman Kodak)
with benzene-ethyl acetate (60:10, v/v) as the solvent.

Radioactivity was measured in a Beckman model LS-250 liquid
scintillation counter. Aqueous samples of at most 1 ml, with
maximal ionic strength of 0.3, were counted with 10 ml of a solu-
tion prepared by mixing 9.6 g 2-(4'-butylphenyl)-5-(4'-biphenyl)-
1,4-tetrachloride (Butyl PPO), 1.5 g 1,4-bis(2-(4-toluol)-
4'-biphenyl) stilbene (PPPO), and 20 ml of a scintillator
BB8-3 (both from Beckman) and 1 liter of toluene. The percen-
tage counting efficiencies (100 X cpm per dpm) of all isotopes were
determined as a function of the external standard ratio. In sam-
ples containing a known number of disintegrations per min and
various amounts of chloroform, a quenching agent. Channels 1
and 5 of the counter were used for double label counting of [H
and 14C or [H and 45Ca, and channels 2, for single isotope experi-
ments.

The counting efficiencies of the least quenched samples in the
three channels, respectively, were 43, 0, and 44% for [H,
22, 71, and 96% for 14C, and 15, 80, and 96% for 45Ca.
The raw data for the standards and experimental samples were punched
onto paper tape by the counter and used as input to a Teletype
time-sharing computer terminal. The computer program fit the
theoretical and experimental data to the third order polynomial func-
tion of the external standard ratio. The background-corrected counts
in two channels and the corresponding efficiencies were used to
calculate the respective disintegrations per min in experimental
samples. The terminal output, corrected for variable sample
volume or uncounted samples, included graphs of the disintegra-
tions per min for each isotope and the isotope ratio as a function of
sample number.

Chemicals—Tris, N-tris(hydroxymethyl)methyl-2-amino-
esuanollic acid, ethylenediamine tetracetic acid disodium salt,
ethylene glycol bis(β-aminoethyl ether)-N,N'-tetraacetate, 
monooctylglycere, diethyllethylacetate, 0.8 N-HNO3.
triethylene uridine, p-nitroanilide HCI, diisopropylfluorophosphate, poly-(o-lysinle),
and calf thymus histones were purchased from Sigma. Protamine
sulfate (salmine) from Nutritional Biochemicals was more effec-
tive in precipitation of receptors than that obtained from other
sources. Suppliers of other proteins are listed in Table I. Am-
pholine anpholytes for isoelectric focusing were obtained from
LKB Instruments, ethylenediamine from Eastman Kodak, and
Ultrapure sucrose from Schwarz-Mann. Inorganic reagents were
obtained from J. T. Baker or from Fisher and were of analytical
grade.

Buffers—The following buffers were used at pH 7.4: 10 mM Tris,
15 mM EDTA (Tris-EDTA); 10 mM Tes,1 12 mM thioglycerol (Tes-
thioglycerol); 10 mM Tes, 12 mM thioglycerol, 0.4 M KCl (Tes
with 30% (w/v) glycerol); and 10 mM Tes, 12 mM thioglycerol, 0.4 M
KCl, 100 mM CaCl2, 100 mM MgCl2, 30% (w/v) glycerol (Tes/EGTA).

Tes, rather than Tris, was generally used as the buffer at physio-
logical pH, since the pK. is 7.5 at 20° and varies only slightly
with temperature (10).

Preparation of Cytosol—Female Rhode Island Red chicks (Hall Brothers Hatchery, Wall-
ingford, Conn.) were treated with diethyilthlestrol and cytosol
was prepared from excised oviducts as previously described (1).
The effects of diethyilthlestrol on the chik oviduct mimc those of
the natural compound 17β-estradiol (11). The homogenization
buffer contained 0.25 M sucrose in Tes-thioglycerol. Chelating
agents were omitted from the homogenization buffer, but were
included in incubation or fractionation media as indicated.

Isoelectric Focusing—To simplify the presentation of the precipitability of estradiol receptors by protamine sulfate. When
this procedure is applied to chick oviduct progesterone receptors,
the cationic composition of the extracting solution determines the
form of the receptor obtained. The recovery of bound radio-
activity is not altered by titration of the aqueous protamine sul-
fate from pH 2.4 to 7.4. Progesterone receptors with empty or
saturated steroid-binding sites are precipitable by protamine,
histones or poly(o-lysinle).

Progesterone receptor "subunit" was extracted from protamine-
precipitated cytosol with 0.1 M CaCl2. Slightly different proce-
dures were used to obtain (a) steroid-"subunit" complex or (b)
unlabeled "subunit" for steroid-binding studies. (a) Oviduct
cytosol (18 to 20 mg of protein per ml) labeled 3 hours at 4° with
4 X 10^-14 m [HIprogesterone was stirred in polyallomer or poly-
propylene test tubes on ice during dropwise addition of ½ volume of
protamine sulfate (7.5 mg per ml). Stirring was continued for
10 min before centrifugation at 500 X g for 10 min. The precipi-
itate was washed twice with a volume of Tes-thioglycerol buffer
equal to twice the initial cytosol volume and then extracted
with 2 M NaCl in Tes-thioglycerol, containing 0.1 M CaCl2,
(b) Unlabeled cytosol was precipitated with protamine sulfate and centrifuged as described above. The
pellet was washed twice with a volume of Tes-thioglycerol equal
to that of the initial cytosol and then extracted for 1 hour with 20
volumes of 0.1 M CaCl2 in Tes-thioglycerol, containing 5 mg of
carbonic anhydrase per ml which inactivated the

Protein and Enzyme Assays—Many components of the solutions
used in these studies interfere with protein determinations by
standard procedures (2, 13). Protein assays in the presence of
thioglycerol were performed either by the Lowry method af-
fer oviation of the sulfhydryl reagent (14), or by the method of
Warburg and Christian (15), or by the fluorescent assay (16)
using bovine serum albumin in the standard and 50 mM sodium
bromide, pH 8, as the buffer. The fluorescence was measured at an
excitation wavelength of 390 nm and emission wavelength of 480
nm on a Cary spectrophotometer. Colorless proteins in chro-
matographic effluents or density gradients were monitored by
optical density at 224, 231, 236, or 280 nm, depending on the
concentration. Myoglobin and cytochrome c were monitored at 409
and 412 nm when stained with Prussian blue. Protein phosphatase activity was assayed by the method of
Garen and Levinthal (17).

Gel Filtration—Theoretical and technical aspects of gel filtra-
tion of steroid receptors have been reviewed by Sherman (2).
All chromatographic data reported here were obtained at 1 to 4°
on the equipment described previously (1).

Protein-bound steroids or ions were separated from the free ligands by filtration
through small columns (3 to 10 ml) of Sephadex G-10 or G-15
(Pharmacia). Analytical gel filtration was performed on Sepha-
dex G-100 or agarose A-0.5m, 200 to 400 mesh (Bio-Rad) in glass
columns (110 X 1.27 cm). Elution buffers contained 12 mg thioglycerol (added within 2 days of use), 10 mM Tes, pH 7.4, up to
30% (w/v) glycerol (to stabilize receptors or "subunits") and
various salts or chelating agents (or both), depending on the
form of the receptor being studied.

Distribution coefficients, Kd, for steroid-binding components
and standard proteins were calculated from the elution volumes,
V, as usual (18). Thyroglobulin (1 mg) was added to indicate
the void volume, V0, when necessary. The internal liquid vol-
ume, Vv, was calculated from the difference between the slution
through small columns (3 to 10 ml) of Sephadex G-10 or G-15
(Pharmacia). Analytical gel filtration was performed on Sepha-
dex G-100 or agarose A-0.5m, 200 to 400 mesh (Bio-Rad) in glass
columns (110 X 1.27 cm). Elution buffers contained 12 mg thioglycerol (added within 2 days of use), 10 mM Tes, pH 7.4, up to
30% (w/v) glycerol (to stabilize receptors or "subunits") and
various salts or chelating agents (or both), depending on the
form of the receptor being studied.

Concentration—Samples of partially purified receptors were concentrated at 4° under 60 psi, in 10- or 50 ml Diafo cells
with UM-10 membranes (Amicon). Eight-fold concentration of a
column effluent in KCl-glycerol buffer was completed in about 4
hours. Progesterone-labeled receptor preparations were treated with dextran-
coated charcoal to remove unbound and weakly bound steroid
molecules. This technique, first applied to steroids by Nugent
and Mayes (35) in 1966, has produced highly variable results in
extensive studies of intracellular steroid-binding proteins. In
this work, procedures for the removal of charcoal-coated steroids were added simultaneously to samples of crude or
partially purified receptors in polypropylene (Falcon) test tubes.
After incubation, a small aliquot was counted, ½ volume of
charcoal-dextran suspension was added, mixed on a Vortex,
shaken for a specified period, and the mixture was centrifuged
20 min at 1000 X g. The residual radioactivity in an aliquot of
the supernatant fluid was corrected for the radioactivity in a similarly
treated buffer or protein-containing control. The total steroid
concentration was computed from the radioactivity in the un-

1 The abbreviations used are: Tes, N-tris(hydroxymethyl)-
methyl-2-aminoethanesulfonic acid; EGTA, ethyleneglycol
bis(β-aminoethyl ether)-N,N'-tetraacetate.
The ionic strength of the charcoal-dextran suspension should be considered in studies, the final concentrations of charcoal and dextran in the range of 1:10 to 1:1 (e.g. Dextran T-40, Pharmacia) in the range of 1:10 to 1:100 has little influence on the results. In these studies, the final concentrations of charcoal and dextran in the treated samples were 0.5% and 0.05% (w/v), respectively. (c) It is important to wash the charcoal-dextran suspension to ensure that it is free of contaminants, such as fresh thioglycerol in the sample. (d) Receptor stability is enhanced by low concentrations of reducing agents such as polyethylene glycol or dextran.

The effects of several experimental variables on the apparent binding are summarized below. (a) Acid-washed charcoal (Norit A, Pfanstiehl) must be exhaustively washed or titrated to neutrality, since oviduct progesterone receptors and presumably others, are unstable in acidic solutions. (b) The ratio of charcoal to dextran (e.g. Dextran T-40, Pharmacia) in the range of 1:10 to 1:100 has little influence on the results. In these studies, the final concentrations of charcoal and dextran in the treated samples were 0.5% and 0.05% (w/v), respectively. (c) Theionic strength of the charcoal-dextran suspension should be equal or exceed that of the sample to avoid aggregation of receptor co-sedimentation with the charcoal during centrifugation. (d) Receptor stability is enhanced by low concentrations of reducing agents such as fresh thioglycerol in the sample. (e) Use of polypropylene rather than glass test tubes reduces but does not eliminate adsorptive losses of [3H]progesterone during incubation. (f) The amount of [3H]progesterone not removed by charcoal-dextran treatment of controls varied between 0.6 and 1.4% of the total added, depending on the pH, salt and protein concentrations, and on the particular batch of charcoal. (g) Since the protein content of a treated sample influences the recovery of steroid-binding sites, the concentration of bound steroid from the sample, the concentration of bound steroid from the treated sample were 0.5% and 0.05% (w/v), respectively. (c) It is important to wash the charcoal-dextran suspension to ensure that it is free of contaminants, such as fresh thioglycerol in the sample. (d) Receptor stability is enhanced by low concentrations of reducing agents such as polyethylene glycol or dextran.

The concentration of bound [3H]progesterone, [P*], was evaluated by charcoal-dextran treatment. The percentage of tritiated steroid bound in the presence of a competitor, relative to that bound in its absence, Y, was plotted as a function of log [C], from which the value of log [C]/[P*] corresponding to Y = 50% was determined. Computer simulation of the curves for Y versus log [C]/[P*] was performed as follows. The concentrations of bound [3H]progesterone and bound competitor, [C], were expressed as functions of the free concentrations of both steroids, [P*] and [C], the respective dissociation constants, Kp and Kc, and the concentration of the competitor could, therefore, be approximated by

\[ [P^*] = \frac{K_p C}{K_p + [P^*] + K_p [C]} \]  

where the total concentrations of the competitor and unlabeled progesterone were those corresponding to Y = 50% (39). In

<table>
<thead>
<tr>
<th>Protein</th>
<th>Source</th>
<th>Sedimentation Coefficient</th>
<th>Molecular Weight</th>
<th>Stokes Radius</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome C</td>
<td>Equine heart, Sigma</td>
<td>3.53</td>
<td>43,000</td>
<td>28.0</td>
</tr>
<tr>
<td>Ribonuclease A</td>
<td>Bovine pancreas, Sigma</td>
<td>4.4</td>
<td>67,000</td>
<td>36.3</td>
</tr>
<tr>
<td>Myoglobin</td>
<td>Equine heart, Sigma</td>
<td>4.9</td>
<td>73,200</td>
<td>36.6</td>
</tr>
<tr>
<td>Chymotrypsinogen A</td>
<td>Bovine pancreas, Sigma</td>
<td>6.3</td>
<td>134,000</td>
<td>43.5</td>
</tr>
</tbody>
</table>

Parameter values for standard proteins for density gradient and gel filtration analyses are shown in Table I.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Source</th>
<th>Molecular Weight</th>
<th>Stokes Radius</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome C</td>
<td>Equine heart</td>
<td>11,700</td>
<td>17.4</td>
</tr>
<tr>
<td>Ribonuclease A</td>
<td>Bovine pancreas</td>
<td>17,200</td>
<td>20.2</td>
</tr>
<tr>
<td>Myoglobin</td>
<td>Equine heart</td>
<td>25,700</td>
<td>26.0</td>
</tr>
<tr>
<td>Chymotrypsinogen A</td>
<td>Bovine pancreas</td>
<td>43,000</td>
<td>28.0</td>
</tr>
<tr>
<td>Albumin (monomer)</td>
<td>Bovine serum</td>
<td>67,000</td>
<td>36.3</td>
</tr>
<tr>
<td>Albumin (dimer)</td>
<td>Bovine serum</td>
<td>73,200</td>
<td>36.6</td>
</tr>
</tbody>
</table>

Experiments—Samples of dilute cytosol or CaCl₂ extracts of progesterone-precipitated cytosol supplemented with ovalbumin were incubated with a given total [3H]progesterone concentration, [P*], plus various total concentrations of competing unlabeled steroids, [C], including progesterone. The concentration of bound [3H]progesterone, [P*], was evaluated by charcoal-dextran treatment. The percentage of tritiated steroid bound in the presence of a competitor, relative to that bound in its absence, Y, was plotted as a function of log [C]/[P*] corresponding to Y = 50% was determined. Computer simulation of the curves for Y versus log [C]/[P*] was performed as follows. The concentrations of bound [3H]progesterone and bound competitor, [C], were expressed as functions of the free concentrations of both steroids, [P*] and [C], the respective dissociation constants, Kp and Kc, and the concentration of the competitor could, therefore, be approximated by

\[ [P^*] = \frac{K_p C}{K_p + [P^*] + K_p [C]} \]  

where the total concentrations of the competitor and unlabeled progesterone were those corresponding to Y = 50% (39). In
Equation 1, $[P,]*$ was replaced by $[P,] - [P,]$, where $[P,]$ was experimentally known. The resultant quadratic equation in $[P,]$ was solved for the positive root, for several assumed values of the free competitor concentration, $[C]$. The corresponding values of $[P,]$ and $[C]$ from Equations 1 and 2 were used to calculate $Y$ and $log [C]/[P,*]$.  

Density Gradient Centrifugation—Technical details and special problems encountered with steroid receptors have been discussed by Toft and Sherman (13). This report includes results of centrifugation in Spinco SW 56 and SW 40 rotors at 1° in linear gradients of 5 to 25% sucrose, 10 to 30% sucrose, and 8 or 10 to 35% glycerol. Gradients were collected into 4- or 5-drop fractions (SW 56) or into 10- or 11-drop fractions (SW 40). For labeled samples, radioactivity was counted in aliquots of diluted fractions, after optical detection of proteins (Zeiss spectrophotometer). Radioactivity on the bottom and sides of the centrifuge tube was also evaluated. The apparent distance migrated by a macromolecular species through a gradient was corrected for the thickness of the initial sample layer (13). A linear relationship between the corrected migration distance and sedimentation coefficient (40) was established for each gradient system utilizing the standards listed in Table I.

Conductivity—The conductance of electrolyte solutions containing various concentrations of sucrose or glycerol was determined in a Yellow Springs Instruments model 31 conductivity bridge. In the cell used, the measured conductance (mho) gave the conductivity or specific conductance (mho cm⁻¹). Sample temperature was maintained at 22 ± 1°.

Isoelectric Focusing—An LKB gradient mixing device was used to prepare continuous gradients of 0 to 47% sucrose or 20 to 70% glycerol containing 1% Ampholine (all w/v), pH 3 to 10, in a 110 ml LKB column. The anode and cathode solutions were 1.5% phosphoric acid in 55% sucrose and 1.8% ethylenediamine (all w/v), respectively. Aqueous ethylene glycol (50%, v/v) was maintained at 1° and circulated through the jacketed compartments of the column by a Perma pump. In most experiments, the ampholytes were focused 24 hours at 500 to 800 volts (Buehler power supply) before introduction of a sample. Excess unbound radioactivity was removed from samples prior to isoelectric focusing. Free [H]progesterone was eliminated by density gradient centrifugation or by treatment with dextran-coated charcoal. Free [H]progesterone was removed by centrifugation after addition of sodium oxalate or by chromatography on a 3.5-ml column of Chelex-100, 100 to 200 mesh (Bio-Rad). With the current turned off, a 2-ml portion of the neutral region of the prefocused gradient was withdrawn through polyethylene tubing into a disposable syringe, mixed with the sample (of correct density), and reinjected at the same level in the column. The voltage was reapplied and slowly increased to 800 volts. When the sample had been focused for 22 to 24 hours, the column contents were fractionated in the cold. The pH of the fractions at 1° was determined on a Beckman Expandomatic pH meter, restandardized at every pH unit. Radioactivity and optical density were measured on aliquots of the fractions, before and after centrifugation, and of extracts of the resultant pellets.

RESULTS

Gel Filtration: “Subunit” Formation by CaCl₂—The complexes formed by [H]progesterone with chick oviduct cytosol have been characterized previously by filtration on agarose A-0.5m in 10 mM Tris, 1.5 mM EDTA, pH 7.4 (Tris-EDTA) containing or lacking 0.3 M KCl (1). The steroid-receptor complexes, as well as the major oviduct protein ovalbumin, are excluded from this gel in eluents of low ionic strength but are included in the presence of 0.3 M or higher concentrations of monovalent salts. Fig. 1 shows the effect of CaCl₂ on unfraccionated or protamine-prefocused progesterone receptors as observed by filtration on agarose A-0.5m in the presence of 0.4 M KCl. Oviduct cytosol was incubated 3 hours at 4° with $4.4 \times 10^{-5}$ M [H]progesterone and freed of excess steroid by treatment with charcoal-dextran containing 0.4 M KCl. The sample in Fig. 1, center, was incubated 1 hour with 0.1 M CaCl₂ before charcoal-dextran treatment. In the untreated sample (top), most of the bound progesterone is eluted as a double peak between the void volume (Fraction 39) and the ovalbumin, as previously observed in Tris-EDTA buffer containing 0.3 M KCl (1). The same receptor species are observed after precipitation of labeled cytosol either by protamine sulfate or by isoelectric focusing and extraction of the precipitate with buffered 0.4 M KCl (see Fig. 6B). The minor binding component in the untreated sample (Fraction 69) is present in variable amounts in different preparations, and may correspond to the form eluted immediately after ovalbumin in the calcium-treated sample. Free steroid (Fractions 100 to 120) is eluted after the total column volume, indicated by trace of [Ca²⁺]. Ovalbumin (OVA), cytochrome c (CYT) and myoglobin (MYO) were monitored by optical density at 236 or 409 nm.

![Fig. 1](http://www.jbc.org/)

Fig. 1. Agarose A-0.5m filtration of progesterone receptors in oviduct cytosol that was untreated (top), incubated 1 hour with 0.1 M CaCl₂ (center), or precipitated by protamine sulfate and extracted with 0.1 M CaCl₂ (bottom). Eluent was 0.4 M KCl, 30% glycerol, 12 mM thioglycollate, 10 mM Tce (pH 7.4) containing 50% v/v and 10 mM EDTA. Major components labeled by [H]progesterone in the untreated sample (Fractions 48 through 57) are referred to as “KC₁ units” in text. Complex at fraction 84 in protamine extract is termed “subunit.” Free steroid (Fractions 100 to 120) is eluted after the total column volume, indicated by trace of [Ca²⁺]. Ovalbumin (OVA), cytochrome c (CYT) and myoglobin (MYO) were monitored by optical density at 236 or 409 nm.
Gel filtration in KCl-EGTA-glycerol following incubation of labeled cytosol for 1 hour with 5 mM (not shown) or 0.1 M CaCl₂ (Fig. 1, center) reveals at least two steroid receptor complexes with apparent molecular radii between those of cytochrome c and ovalbumin. When the column was equilibrated and eluted with buffered 0.1 M CaCl₂ (not shown), the major peak of bound radioactivity corresponded to the second eluted species in the calcium-treated sample.

For the experiment in Fig. 1, bottom, unlabeled cytosol was precipitated with protamine sulfate, the precipitate was washed with Tes-thioglycerol and extracted with 0.1 M CaCl₂. The extract was incubated 3 hours at 4°C with 4 × 10⁻¹⁰ M [³H]progesterone and treated with dextran-coated charcoal containing 10 mg of bovine serum albumin per ml. Gel filtration of the resultant sample revealed a single, symmetrical peak of tritiated steroid, one or two fractions ahead of the myoglobin marker.

Evidence that this radioactivity corresponds to a macromolecular complex was obtained in two ways. Peak fractions from the agarose column were rechromatographed on a 3.5-ml column of Sephadex G-15 with the same buffer. About 44% of the radioactivity placed on the second column was recovered in the void volume, indicated by the optical absorption peak. An independent estimate of residual steroid binding was obtained by treating alternate fractions from the agarose column with dextran-coated charcoal in the same buffer containing 10 mg of bovine serum albumin per ml. The elution patterns of the bound and total radioactivity in this region coincided, and the bound steroid accounted for 62% of the total, by this criterion. Thus, about half the radioactivity eluted at Fraction 84 is bound to a macromolecular component that is referred to as a "subunit" of the receptor throughout this report. Since the protamine extracts showed no detectable activity toward α-N-benzoyl-γ-arginine-p-nitroanilide, it is inferred that "subunit" formation is not due to a trypsin-like protease (42, 43).

Binding of Progesterone and Other Steroids to Dilute Cytosol and "Subunit"—The affinity and specificity of steroid binding to different receptor forms were evaluated by incubation with [³H]progesterone (plus competing unlabeled steroids) and adsorption of unbound steroids to dextran-coated charcoal. Preliminary experiments established that (a) for both untreated and calcium-treated receptors, incubation for 3 hours at 4°C was sufficient for maximal binding, and (b) extraction of protamine-precipitated cytosol with 20 times the initial cytosol volume of buffered 0.1 M CaCl₂ gave a convenient range of values of bound/free progesterone. The steroid-binding properties of these "subunit" preparations were compared with those of ovuidt extract diluted 1:20 with Tes-thioglycerol containing the same additives, except the CaCl₂. The concentrations of endogenous proteins in the 1:20 protamine extracts and cytosol were 0.1 and 1.0 mg per ml, respectively (15, 16).

Under all conditions tested, graphs of bound/free versus bound steroid, Scatchard plots (44), were convex with respect to the abscissa. Data for progesterone binding in the absence of competitors were analyzed in terms of the apparent concentration of binding sites, Kᵦ, estimated by extrapolation of the Scatchard plot, and the free steroid concentration corresponding to half-saturation, Kᵦₜₚ, estimated from the graph of bound versus free steroid. The relative affinities of several other steroids were evaluated from the binding of a constant, nearly saturating concentration of [³H]progesterone in the presence of increasing concentrations of competing steroids.

Fig. 2 illustrates the effect of supplementing the protein content of the protamine extract or the charcoal-dextran suspension (or both) on the detectable binding of [³H]progesterone to the "subunit." The graph of bound versus free steroid in the absence of added ovalbumin is sigmoidal, and approaches a maximum value that is less than one-fourth that observed when the charcoal-dextran suspension contained 20 mg of ovalbumin per ml. This sigmoidal shape, or the corresponding arch in a Scatchard plot, has been reported for steroid binding to receptors from various target organs (5, 45-47) and has often been attributed to cooperative binding. The remaining data in Fig. 2, however, show that addition of protein to either the extracting solution or the charcoal-dextran suspension diminishes the apparent cooperativity, while increasing the maximal binding. These results underlie any detailed interpretation of either the cooperativity or the value of Kᵦ. Instead, they point out the susceptibility of data obtained by this technique to artifacts, and the necessity to make comparisons between two binding species under controlled conditions of protein concentration.

Fig. 3 compares the Scatchard plots for progesterone binding to dilute cytosol and to "subunit" when both the diluent and the extracting solution contained 5 mg of ovalbumin per ml, and the charcoal-dextran suspensions contained or lacked 20 mg of ovalbumin per ml. Under each condition of protein concentration, approximately the same amount of free progesterone was required for half-saturation of the untreated or calcium-treated receptors. These results for Kᵦₜₚ, 4.0 to 5.6 × 10⁻¹⁰ M, are in reasonable agreement with the dissociation constant previously reported for oviduct receptors in KCl-containing density gradients, Kᵦ = 8 × 10⁻¹⁰ M (1). The most important observation for this study is that the calcium-induced conversion of receptors to "subunits" does not diminish their affinity for progesterone. About 40% of the binding sites detected in dilute cytosol were recovered in the protamine extract. This suggests that the "subunit" makes a major contribution to the progesterone-binding activity of the cytosol, but does not prove that every receptor molecule contains or is capable of forming this unit.

The abilities of intact receptors and "subunits" to discriminate among different steroids were compared in the next set of experiments (Fig. 4, top). Among the steroids tested, the relative affinities of both the intact receptor and "subunit" were progesterone > deoxycorticosterone > testosterone > corticosterone > 17β-estradiol > cortisol. For the computer simulation of the competitive steroid-binding data (Fig. 4, bottom), the dissociation constants of progesterone from each receptor form were approximated by the corresponding values of Kᵦₜₚ and the values of Kᵦ for the other steroids were calculated as described under "Materials and Methods." The strong resemblance among the four sets of curves in Fig. 4 attests to the compatibility of these estimates of Kᵦ with the experimental data. In conclusion, the unit obtained by CaCl₂ extraction of protamine-precipitate cytosol contains all the determinants for specific, high affinity interaction with progesterone.

Density Gradient Centrifugation—The apparent sedimentation coefficients, sₑ₂₀,₀, of progesterone-binding components of chick oviduct cytosol were previously reported to be about 5 and 8 S in gradients of low ionic strength, and 3.7 S in gradients containing 0.3 M KCl (1). In more recent experiments in low salt gradients, the estimated sₑ₂₀,₀ of the larger receptor species has been revised downward to about 7 S, and the 4 and 5 S receptor species have been resolved from each other (see Fig. 9). Furthermore, use of the value of 3.53 S (28) rather than 3.67 S (32) for ovalbumin has led to a lower estimate of sₑ₂₀,₀ for the receptors in KCl-containing gradients, in which they coexisted with the standard.

Fig. 5 compares the effects of CaCl₂ treatment of whole cytosol...
FIG. 2 (top). Alteration of apparent binding of \([3H]\)progesterone by addition of ovalbumin to "subunit" preparations or to charcoal dextran suspensions (or both) used to remove free steroid. Cytosol proteins precipitated by protamine sulfate were extracted with 0.1 M CaCl₂, TES-thioglycerol, 5 mg ovalbumin per ml. After incubation for 3 hours at 4°C with different amounts of \([3H]\)progesterone, aliquots were counted and the remainder was agitated 20 min with 2.5% Norit A, 0.25% Dextran T-40 in 0.1 M CaCl₂, TES-thioglycerol, 20 mg ovalbumin per ml, and centrifuged. Bound steroid was calculated from supernatant radioactivity, free steroid from the total minus bound. The presence or absence of ovalbumin in the respective solutions is indicated on the right of each binding curve.

FIG. 3 (bottom). Scatchard plots (44) of \([3H]\)progesterone binding to dilute oviduct cytosol and to 0.1 M CaCl₂ extract of protamine-precipitated cytosol ("subunit"), both supplemented with 5 mg of ovalbumin per ml. Samples labeled as in Fig. 2 were treated with charcoal-dextran suspensions in TES-thioglycerol containing no addition (○), 0.1 M CaCl₂ (□), 20 mg ovalbumin per ml (●), or 0.1 M CaCl₂ and 20 mg ovalbumin per ml (■). Free steroid concentrations corresponding to half-saturation (\(K_d\)) were interpolated from graphs of [Bound] versus [Free] for the same data. The abscissa intercepts of data for dilute cytosol and for protamine extracts treated with charcoal-dextran containing ovalbumin (filled symbols) correspond to 1.35 and 5.0 pmole of steroid-binding sites per mg of endogenous protein, respectively.

![Graph](image)

and of protamine-precipitated cytosol on the \([3H]\)progesterone distribution in glycerol gradients containing 0.4 M KCl. Centrifugation after calcium treatment (left) partially resolves at least two labeled complexes. The larger (3.4 S) component sediments at about the same rate in these gradients as do untreated or protamine-precipitated, KCl-extracted receptors (not shown). The more slowly sedimenting complex in the calcium-treated sample (2.5 to 2.7 S) is the only form obtained when protamine-precipitated cytosol is extracted with 0.1 M CaCl₂ (Fig. 5, right). In 38 gradients containing myoglobin or ovalbumin (or both) as internal markers, this component was found to have an average \(S_{20,w}\) of 2.58 ± 0.11 S. The sedimentation coefficient and the yield of this receptor form were unaltered by the presence in both the cytosol and the extracting solution of 5 mM disopropylfluorophosphate, a potent inhibitor of serine-active proteases (43). Similar results were obtained when protamine-precipitated, la-
beled cytosol was extracted with 0.1 M BaCl₂ or SrCl₂ instead of CaCl₂. The same concentration of MnCl₂ released the 2.6 S unit in much lower yield, while MgCl₂, like KCl, solubilized only the 3.5 S complex. CaCl₂ did not alter the sedimentation behavior of corticosteroid-binding globulin, a progesterone-binding constituent of chick serum and a potential contaminant of oviduct cytosol preparations.

“Subunit” and “KCl Units” from Isoelectrically Focused Receptors—The experiments summarized in this section showed that (a) progesterone-receptor complexes are coprecipitated with other proteins when cytosol is focused after injection into a preformed pH gradient, (b) either the “subunit” or the “KCl units” can be extracted from the resultant precipitate, depending on the ionic composition of the medium, (c) formation of “subunit” from receptors in this partially purified state requires lower concentrations of CaCl₂ (0.1 to 1 mM) than in unfractionated or protamine-precipitated cytosol (10 mM to 0.1 mM), and (d) protamine (or another polyamine) is not essential for “subunit” formation, although it is useful in preliminary purification.

Oviduct cytosol (0.4 or 0.6 ml) labeled with 4 x 10⁻⁸ M [³H]-progesterone was injected into the neutral region of a glycerol-stabilized, prefocused pH 3 to 10 Ampholine gradient and focused 22 or 24 hours at 800 volts. The column contents were collected into polypropylene test tubes, and the radioactivity in an aliquot of each fraction was measured before and after centrifugation. The pattern of [³H]-steroid at the electrical steady state contained a major peak at pH 4.83 ± 0.16 (8 experiments) and a diffuse distribution of unbound radioactivity in the neutral pH region where the sample was injected (Fig. 6A). Of the radioactivity associated with the major peak, 70 ± 5% was sedimented by low speed centrifugation, and about 90% of the latter could be resolubilized in 30 min by 0.4 M KCl in Tes-thioglycerol. Omission of the KCl in some experiments reduced the recovery of radioactivity from the pellet by about 90%.

The molecular sizes of the steroid-receptor complexes were examined by agarose gel filtration after extraction of the pellets with buffers containing either 20 mM EGTA or 1 mM CaCl₂ (Fig. 6B).
When the extracting buffer contained the chelating agent, with or without KCl, the predominant receptor forms were the "KCl units." By contrast, inclusion of 1 mM CaCl₂ in extraction media containing or lacking KCl resulted in nearly complete conversion to the "subunit." Gel filtration of extracts containing only 0.1 mM CaCl₂ revealed a mixture of the "subunit" and larger forms. When the extraction medium contained 10 mM or 0.1 M CaCl₂, no receptor-bound steroid was detectable.

The preceding focusing experiments were performed after injecting labeled cytosol into a preformed pH gradient, as described under "Materials and Methods." Under these conditions, the radioactive complex at about pH 4.8 coincided with the optical density peak (see Fig. 10). It was therefore possible that the apparent isoelectric point, pI, represented that of an aggregate of receptor with other constituents of the crude preparation. When progesterone-receptor complexes (5 S components) were isolated with other constituents of the crude preparation. When the extraction medium contained 10 mM or 0.1 M CaCl₂, no receptor-bound steroid was detectable.

In preliminary research, an estimate of 20.7 Å for the "subunit" radius was obtained by filtration of labeled, unfractionated, protamine-precipitated or isoelectrically focused oviduct cytosol to a sufficient concentration of CaCl₂ produces a steroid-binding component with a molecular radius about the same as that of myoglobin and about half that of the complexes obtained with KCl.

Gel filtration data for receptors are frequently analyzed in terms of molecular weight rather than radius. This practice is based on assumptions about the density, shape, and solvation of receptors that have been critically reviewed (2). When the distribution coefficients in Fig. 7A are expressed as a function of M (Fig. 7B), the estimate and standard deviation for the "subunit" are 18,300 ± 1,500 compared with a range of 79,000 to 138,000 for the monovalent salt forms.

The apparent identity of the 2.6 S complex revealed by density gradient centrifugation with the "subunit" of the gel filtration patterns (Table II) permitted the combined analysis of the parameters obtained by these techniques (2). The results for Rs, 20.5 ± 0.5 Å, and s₂₀,ω, 2.58 ± 0.11 S, were used together with an estimate of the partial specific volume, 0.734 cm³ g⁻¹, as for bovine serum albumin (49), to calculate an apparent M. The proximity of the resultant value, 22,500 ± 1,500, to that obtained by gel filtration alone, 18,300 ± 1,500, suggests that the "subunit" is similar in physical chemistry to the globular proteins used to calibrate the column.

### Table II

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (ml)</th>
<th>Protein (mg/ml)</th>
<th>Subunit (µg)</th>
<th>Recovery (%)</th>
<th>M × 10⁴ 'Subunit' per mg protein</th>
<th>Purification (fold)</th>
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<tr>
<td>Oviducts of DES-treated chicks (14 g)</td>
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<td>24.6</td>
<td>100</td>
<td>0.0345</td>
<td>1</td>
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<tr>
<td>Cytosol, TES-thioglycerol</td>
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<td>3.6</td>
<td>18.8</td>
<td>77</td>
<td>0.287</td>
<td>8</td>
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<tr>
<td>First CaCl₂ extract of protamine pellet</td>
<td>18.5</td>
<td>1.4</td>
<td>2.4</td>
<td>10</td>
<td>0.095</td>
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<tr>
<td>Second CaCl₂ extract of protamine pellet</td>
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<td>0.029</td>
<td>4.2</td>
<td>17</td>
<td>7.45</td>
<td>216</td>
</tr>
</tbody>
</table>

* Fluorescein assay (16).

* Bound [³H]progesterone in labeled cytosol was determined by charcoal-dextran assay. All radioactivity recovered in other steps was assumed to be bound. Mass of "subunit" was calculated assuming 1 µmole of bound [³H]progesterone/21,000 µg of "subunit."

* Pooled gradient peaks were resaturated with an additional 5 × 10⁻⁴ M [³H]progesterone before concentration in a Diaflo apparatus.
effects of calcium and other metal ions have also been studied by centrifugation of receptors through gradients containing only the divalent metal chloride or other salt. These studies showed that Ca$^{2+}$, Sr$^{2+}$, Ba$^{2+}$ and Mn$^{2+}$, but not Mg$^{2+}$, convert a significant fraction of the receptor molecules to forms sedimenting more slowly than 3 S and (b) that KCl is not essential for "subunit" formation. A comparison of the minimum concentrations of the different cations that yield the smaller receptor forms (first 3.5 S then 2.6 S) indicated that Ca$^{2+}$ was the most effective of the cations tested. By this criterion, Mn$^{2+}$ appeared to be more potent than Sr$^{2+}$ or Ba$^{2+}$, although Mn$^{2+}$ was less effective in extracting the "subunit" from protamine-precipitated cytosol.

Exposure of progesterone receptors to Zn$^{2+}$ caused them to aggregate and sediment rapidly to the bottom of the centrifuge tube. LaCl$_3$ was similar in effect to ZnCl$_2$, although La$^{3+}$ mimics Sr$^{2+}$ and similar ions specifically release the "subunit" from receptors precipitated by polyamines or Zn$^{2+}$, but not Mg$^{2+}$, Ca$^{2+}$, Sr$^{2+}$, or Ba$^{2+}$, except for incompletely dissociated salts, e.g., calcium acetate.

The inclusion of different salts in density gradients has the advantage of continuous equilibration of the receptor or steroid-receptor complex with the cation of interest. The interpretation of data obtained under these conditions, however, is complicated by the changing properties of the medium surrounding the macromolecules as they sediment through the gradient. First, as predicted from the effects of sucrose and glycerol on the dielectric constant of aqueous solutions (53), the conductance of the electrolyte solutions used in these experiments decreases from the top to the bottom of the centrifuge tube. For example, the conductances at 22°C of 10 mM or 0.1 M solutions of CaCl$_2$, BaCl$_2$, and SrCl$_2$ are decreased by 50% by the presence of 30% (w/v) glycerol. Second, sucrose and glycerol stabilize receptors, making them less susceptible to the effects of metal ions. This was demonstrated by incubation of progesterone-labeled cytosol for 1 hour at 4°C with various concentrations of CaCl$_2$, ± glycerol, followed by centrifugation through KCl-containing gradients. At each CaCl$_2$ concentration, more "subunit" was obtained in the absence than in the presence of glycerol. Moreover, in comparisons between solutions of different glycerol and CaCl$_2$ concentrations but the same conductivity, the one having lower calcium and lower glycerol content converted more of the labeled complexes to the 2.6 S form. The latter results showed that "subunit" formation is not simply a function of conductivity, but represents a specific action of the divalent cation that is opposed by glycerol-induced stabilization of intact receptors.

**pH Dependence of "Subunit" Formation—**Metal ions and protons compete for many of the same reactive groups on proteins, each altering the apparent dissociation constant of the other (54, 55). The amount of receptor "subunit" formed by suboptimal concentrations of calcium ions was therefore expected to vary with pH. The data in Fig. 8 were obtained on aliquots of labeled cytosol incubated with 10 mM Tes or Tris to pH 6.4, 7.4, 8.4, or 10.2. Each sample was then centrifuged through a KCl-containing gradient with myoglobin as an internal marker. The results in Fig. 8 indicate an increasing conversion to the 2.6 S "subunit" with increasing pH from 6.4 to 8.4. No further change in sedimentation behavior was evident up to pH 10.2, above which the recovery of bound radioactivity declined. In control samples, similarly centrifuged at pH 7.4 after incubation in 0.4 M KCl solutions of each pH, the sedimentation coefficients of the labeled complexes were all 3.5 S. An effect of pH on the formation of "KCl units" would be expected only at a much lower KCl concentration.

The observed pH dependence of "subunit" formation may indicate a direct interaction of calcium ions with a group having a pK$_a$ of about 7 to 8, such as an imidazole or a-amino group (56). Alternatively, divalent cations may compete with charged amino groups for the same carboxylate ions (57). The observation that Ca$^{2+}$ and similar ions specifically release the "subunit" from receptors precipitated by polyamines is compatible with the latter interpretation.

**Conversion of 4 and 5 S to 2.6 S Receptor Forms—**The objective of the following experiments was to determine whether calcium and similar ions act directly on progesterone receptors or require specific proteases (58) or other transforming factors (6). Fig. 9 demonstrates the formation of the 2.6 S "subunit" by addition of CaCl$_2$ to individual fractions collected after centrifugation of labeled cytosol through a low salt gradient. These results suggest that if a separate transforming factor is required, it must cosegregate with both the 4 and 5 S forms of the labeled receptor during the first centrifugation step.

Unlike the 4 and 5 S receptor forms isolated from low-salt gradients, the 3.5 S units from KCl-containing gradients resist conversion to the "subunit." Addition of a wide range of CaCl$_2$ concentrations to 3.5 S complexes resulted in release of most of the bound steroid, whether or not the receptor-containing fraction...
Fraction Number

FIG. 9. Formation of "subunit" by addition of CaCl₂ to 4 and 5 S receptors from low-salt gradient. Oviduct cytosol labeled with $2 \times 10^{-7}$ M [³H]progesterone was centrifuged 61 hours at 199,000 × g through an 8 to 35% glycerol gradient in Tes-thioglycerol. Optical density at 236 nm (to locate ovalbumin) and total radioactivity in odd-numbered fractions were determined. Even-numbered fractions were treated with charcoal-dextran to evaluate tightly bound steroid (left). Two fractions each from the 4 and 5 S regions of an identical gradient were made 5 mM in CaCl₂ for 2 hours and recentrifuged 61 hours at 199,000 × g through glycerol gradients in 0.4 M KC1 and Tes-thioglycerol. The resultant distributions of total radioactivity from calcium-treated 4 S (center) and 5 S (right) complexes indicate conversion to "subunit" form in both cases.

FIG. 8. Effect of pH during exposure to CaCl₂ on the sedimentation profile of progesterone receptors at pH 7.4. Samples of labeled cytosol were mixed with 3 volumes of 13.3 mM CaCl₂ in Tes-thioglycerol (top), or in 13.3 mM Tris-thioglycerol (bottom), of the appropriate pH to obtain a final CaCl₂ concentration of 10 mM and the pH indicated in each panel. After 1 hour, each sample was centrifuged through an 8 to 35% glycerol gradient in 0.4 M KC1, 10 mM EGTA, and Tes-thioglycerol. Sedimentation coefficients of labeled complexes were evaluated by interpolation between positions of endogenous ovalbumin (3.5 S) and added myoglobin (2.0 S, arrow). Cytosol incubated with 0.4 M KC1 at each pH and similarly centrifuged contained only 3.5 S complexes.

was combined with other regions of the gradient. The "KC1 units" fractionated by agarose gel filtration likewise produced no detectable "subunit" and released all the bound steroid when treated with CaCl₂. In separate experiments, addition of 0.4 M KC1 to cytosol before 10 mM or higher concentrations of CaCl₂ did not prevent "subunit" formation. The preceding results suggest that the action of calcium and similar ions on progesterone receptors requires a configuration, constituent, or contaminant of the receptor complex that is present in the 4 S, 5 S, and protamine-precipitated forms, but is altered or eliminated during fractionation in the presence of KC1. It is also evident that "subunits," once formed by exposure to calcium ions, do not revert to "KC1 units" or larger receptor forms, despite prolonged equilibration with solutions containing chelating agents.

"Calcium Binding Studies"—Column isoelectric focusing was utilized to test the hypothesis that calcium ions interact directly with progesterone receptors in media of low ionic strength. A sample of oviduct cytosol was labeled 1 hour with $5 \times 10^{-7}$ M $^{45}$CaCl₂, then 1 hour with $4 \times 10^{-9}$ M [³H]progesterone. It was freed of excess radioactivity, injected into a sucrose-stabilized, prefocused pH 3 to 10 Ampholine gradient, focused 24 hours at 800 volts, and analyzed as described under "Materials and Methods." The resultant distributions of [³H]progesterone, $^{45}$Ca⁺, and optical density are shown as functions of the steady state pH in Fig. 10. Bound $^{45}$Ca⁺ coincides with the optical density peak and with tritiated complexes focused at about pH 4.7. Separate experiments showed that (a) ovalbumin, which has nearly the same pH as untreated receptors, binds no detectable progesterone or calcium ions under these conditions, and (b) the calcium binding observed in Fig. 10 is not attributable to contamination of cytosol by serum.
component of chick oviduct cytosol that binds steroids with the same specificity and affinity as intact progesterone receptors and is formed by brief exposure of partially purified receptors to 1 nm CaCl$_2$ at 0°. It is tempting to speculate that this unit comprises the steroid-binding subunit of a multi-functional receptor complex. Reference to it as a “subunit” (with quotation marks), however, emphasizes the lack of evidence for its existence in vivo as a discrete polypeptide chain.

Most of the data reported here are consistent with a cation-induced change in receptor conformation, leading to disaggregation into subunits. Many precedents can be cited for the action of divalent cations as specific modifiers of protein isomerization and polymerization (62–69). Two negative results, however, are difficult to reconcile with this interpretation. First, removal of divalent cations by chelating agents does not result in reassociation of the 2.6 S units into the faster sedimenting receptor forms. Second, we have been unable to prepare “subunit” (2.6 S) from chromatographically or ultracentrifugally purified “KC1 units” (3.5 S), although both the 2.6 and 3.5 S forms are apparently derived from the same low-salt receptor species. When “KC1 units” are exposed to CaCl$_2$, all the bound steroid is released, perhaps because an endogenous inhibitor of calcium-activated proteases was removed during the fractionation of these receptor forms.

It remains possible that the progesterone-binding “subunit” is formed by divalent cation-stimulated cleavage of a specific, susceptible peptide bond. This event could result either from a cation-induced conformational change in the receptor, exposing the susceptible bond to the action of an unaltered protease, or from cation stimulation of a protease to act on an unaltered receptor. The latter mechanism has been invoked in several independent studies of calcium effects on mammalian estradiol receptors (6, 7, 58). The data reported here permit the following characterization of the responsible enzyme in oviduct cytosol. (a) It is neither a serine-active protease nor a trypsin-like protease (43). (b) The putative enzyme is precipitable by protamine sulfate and extractable by CaCl$_2$. (c) It is coprecipitated with the receptor during isoelectric focusing in preformed pH 2.5 gradients. (d) It displays a heterogeneous pattern (4 to 5 S) on centrifugation through low salt gradients. A plausible explanation for this coincidence of properties with those of the receptor is that the calcium-sensitive protein is itself a constituent of the untreated receptor complex. This interpretation is strengthened by the superposition of calcium- and progesterone-binding activities in whole cytosol fractionated by isoelectric focusing or gel filtration.

The order of effectiveness of Ca$^{2+}$ > Sr$^{2+}$ > Ba$^{2+}$ > Mg$^{2+}$ in promoting “subunit” formation parallels the relative affinities of these ions for the calcium-binding protein of hen uterus (70). The similar isoelectric points of the calcium-binding protein and the progesterone receptor, and the similar molecular weights of the calcium-binding protein and the receptor “subunit” (70), provide further impetus for future analysis of the relationship between these proteins.

The failure of either Mg$^{2+}$ or La$^{3+}$ to convert receptors to the “subunit” has implications both for the method of subcellular fractionation of steroid target organs and for the mechanism of the cation effect. First, these observations would recommend the use of Mg$^{2+}$ rather than Ca$^{2+}$ to stabilize nuclei during tissue homogenization, prior to studies of steroid receptor structure. Second, Mg$^{2+}$ may be incapable of forming the “subunit” because its ionic radius is too small to accommodate bulky coordinating groups of the receptor or transforming factor (71, 72). Ionic
radius is not the sole determinant of "subunit" induction, however, since Na⁺ and Ca²⁺ cannot replace Ca²⁺, despite their similar sizes (73).

The 2.6 S progesterone-receptor complex of chick oviduct cytosol may be analogous to a 3 S form of aldosterone receptors extracted from rat kidney nuclei in the presence of Ca²⁺ (9). The "subunit" described here also resembles a 2.4 S form of rat uterine estradiol receptor obtained after exposure to high concentrations of β-mercaptoethanol (7). These similarities suggest that the general mechanism for regulatory interactions between steroids and divalent cations in organs responsive to both classes of ligands.

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Progesterone Receptors of Chick Oviduct: STEROID-BINDING "SUBUNIT" FORMED WITH DIVALENT CATIONS
Merry R. Sherman, Sui Bi P. Atienza, Janet R. Shansky and Linda M. Hoffman


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