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Steroid–Receptor Quantitation and Characterization by Electrophoresis in Highly Cross-Linked Polyacrylamide Gels†

Lorraine K. Miller,‡ Soledad C. Diaz, and Merry R. Sherman*

ABSTRACT: Conditions for discontinuous polyacrylamide gel electrophoresis have been defined in which progesterone receptors of chick oviduct cytosol and a variety of steroid-binding proteins from other sources are stable and amenable to quantitative analysis. The essential modifications from standard procedures include the use of (1) separation gels in which the cross-linking agent/acrylamide monomer = 15:85, (2) glycerol (10% v/v) in all phases of the Tris-glycine-HCl buffer system (pH 10.2 in the separation phase during electrophoresis at 0°), and (3) a layer of a charged reducing agent, thioglycolate, beneath the sample layer. Electrophoresis of untreated oviduct cytosol labeled with [^{3}H]progesterone ± competing steroids revealed a heterodisperse slow peak and a sharp fast peak. Both peaks displayed the steroid-binding specificity and saturability that are characteristic of intracellular receptors. Recovery of steroid from both the slow and fast components increased linearly with sample load up to 60 μl of cytosol (1.2 mg of protein)/gel (6 mm diameter). The specific progesterone binding detected by this technique was comparable to that detected by charcoal-dextran treatment or ion exchange filtration. Relative electrophoretic mobilities (R_f) of globular protein standards and steroid–protein complexes in cytosol and chick serum were measured in separation gels with total gel concentrations (T) systematically varied from 5 to 15% (w/v). Data were processed by computer programs to obtain weighted linear regressions of log R_f on T (Ferguson plots) and the joint 95% confidence limits of the slopes ($-K_R$) and intercepts of these plots. Molecular radii ($\bar{R}$) of the binding components and apparent molecular weights (M) were calculated from the linear correlation of $\bar{R}$ with $K_R^{1/2}$ for the standards. The value of M ~158,000 obtained for the cytosol fast component was independent of the length of the separation gel, the presence of a stacking gel or prior exposure of the cytosol to KCl. It was higher than expected from the sedimentation coefficient of 4.2 S in the same pH 10.2 buffer. Electrophoresis in 170-mm separation gels without stacking gels revealed that KCl extracts of protamine-precipitated cytosol contain a different receptor form, of lower net negative charge than the cytosol fast form. The results demonstrate the utility of electrophoresis in highly cross-linked gels of several concentrations to discriminate between various receptor forms and steroid-binding components of serum. This method may lead to overestimates of M for highly asymmetric receptor forms.

Attempts to purify steroid hormone receptors and to investigate their modes of action are complicated by the structural similarities of the receptors for different steroids, their tendencies to aggregate or form subunits, and the lability of the steroid–receptor complexes. In theory, receptor analysis should be facilitated by the technique of quantitative polyacrylamide gel electrophoresis, in which mobility is measured as a function of total gel concentration. This technique, developed by Chrambach and Rodbard (1971), permits rapid fractionation and characterization of large numbers of samples on the basis of both net charge and size. In practice, gel electrophoresis has been of limited utility in research on steroid–receptors, primarily because of the restricted mobility of large macromolecules, e.g., 8 S–10 S, in

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Table I: Published and Calculated Parameters of Standard Proteins.

Protein	Source: Natural	Source: Commercial	Molecular Weight	Partial Specific Volume ($cm^3\ g^{-1}$)	Calculated Radius[a] (A)	Stokes Radius[b] (A)
Myoglobin	Equine heart	Schwarz/Mann	17,200[c]	0.741[d,e]	17.2	20.2[f]
Ovalbumin	Hen egg	Sigma	43,000[g]	0.748[h]	23.4	28.0[i]
Hemoglobin	Human blood	Sigma	64,500[j]	0.749[e]	26.7	35.7[k]
Albumin	Human serum	Sigma				
Monomer			66,000[l]	0.733[m]	26.8	35.1[m]
Dimer			132,000		33.7	43.8[n]
Albumin	Bovine serum	Sigma				
Monomer			67,000[o,p]	0.734[h]	26.9	36.3[o]
Dimer			134,000		33.9	43.5[o]
Trimer			201,000		38.8	50.4[n]
Transferrin	Human serum	Sigma, Pentex	74,000[q]	0.725[m]	27.8	36.6[q]
Ferritin	Equine spleen	Schwarz/Mann	443,000[r]	0.731[s]	50.4	61.4[t]
Thyroglobulin	Bovine thyroid	Sigma	669,000[u]	0.713[u]	57.4	86.1[u]

[a] Equation 3. [b] Calculated from $D_{20,w}{}^0$ and eq 4. [c] Weber and Osborn (1969). [d] Theorell (1934). [e] Behlke and Wandt (1973). [f] Riveros-Moreno and Wittenberg (1972). [g] Castellino and Barker (1968). [h] Dayhoff et al. (1952). [i] Champagne (1950). [j] Braunitzer et al. (1964). [k] Rossi Fanelli et al. (1964). [l] Heimburger et al. (1964). [m] Oncley et al. (1947). [n] Estimated from $\bar{R}$ and the mean $R_S/\bar{R} = 1.30$ for the other standards. [o] Loeb and Scheraga (1956). [p] Squire et al. (1968). [q] Roberts et al. (1966). [r] Crichton et al. (1973). [s] Bryce and Crichton (1971). [t] $D_{20,w}{}^0$ from M, $\bar{v}$, and $s = 17.12$ S (Crichton et al., 1973). [u] Edelhoch (1960).

the small pores of conventional polyacrylamide gels (Sherman et al., 1970). This report describes a procedure for electrophoresis of steroid-receptors in gels with large pores, with a buffer system and sample underlayer designed to enhance receptor stability.

The characteristics of a polyacrylamide gel network—fiber length and pore size distribution—can be controlled by separately varying the total acrylamide concentration[1] (T) and the percentage of the cross-linking agent (C), e.g., N,N'-methylenebisacrylamide (Bis):[2]

$$T = \left(\frac{\text{grams of monomer + Bis}}{\text{ml of solution}}\right) 100$$

$$C = \left(\frac{\text{Bis}}{\text{monomer + Bis}}\right) 100$$

Ferguson (1964) showed that the logarithm of the absolute or relative mobility (R_f) of a protein was a linear function of T:

$$\log R_f = \log Y_o - K_R T \qquad (1)$$

in which the negative slope, i.e., the retardation coefficient (K_R), is a function primarily of molecular size, and the ordinate intercept ($\log Y_o$) is a function primarily of net charge/surface area. Rodbard et al. (1972) showed that stable gels with increased effective pore sizes, and hence higher protein mobilities, were obtained by increasing C.

In the present study, progesterone-binding components of chick oviduct cytosol and serum were electrophoresed in 15% C gels of 5–15% T. The data were analyzed according to eq 1 by the procedures of Rodbard and Chrambach (1974). The results obtained by electrophoresis and other physical-chemical techniques have been applied to an analysis of the relationships among various forms of the progesterone receptor and its steroid-binding "subunit" (Sherman et al., 1974a,b, 1975).

Materials and Methods

Materials. [1,2,6,7-^{3}H]Progesterone (110 Ci/mmol) was obtained from Amersham-Searle, [1,2,6,7-^{3}H]hydrocortisone (82.7 Ci/mmol; cortisol) and [^{3}H]toluene for instrument calibration were from New England Nuclear, and L-[^{14}C]valine (260 Ci/mol) was from Schwarz/Mann. Acrylamide, N,N'-methylenebisacrylamide (Bis), N,N,N',N'-tetramethylethylenediamine (TMED), ammonium persulfate, and agarose (Bio-Gel A 0.5m, 200–400 mesh) were purchased from Bio-Rad Laboratories, riboflavine and 3′,3″,5′,5″-tetrabromophenolsulfonephthalein (Bromophenol Blue) were from Eastman Kodak, trizma base, N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (Tes), diethylstilbestrol (DES), thioglycolic acid, and monothioglycerol were from Sigma, protamine sulfate was from Nutritional Biochemicals, Amido Black (Aniline Blue Black) was from Canalco, and 2-(4′-t-butylphenyl)-5-(4′-biphenylyl)-1,3,4-oxadiazole (butyl-PBD) and Bio-Solv BBS-3 were from Beckman. All other chemicals were of analytical reagent grade and were obtained from Fisher or Mallinckrodt. The sources and the macromolecular parameters of the standard proteins used in this study are listed in Table I.

Preparation, Labeling and Preliminary Fractionation of Steroid-Binding Components. Cytoplasmic supernatant fraction (cytosol) in 10 mM Tes–12 mM thioglycerol (pH 7.4) (Tes–thioglycerol)[3] containing 0.25 M sucrose was prepared from oviducts of DES-treated chicks as described by Sherman et al. (1970). All procedures were carried out at 0–4°. Unless otherwise indicated, cytosol was labeled for 3 hr with 4×10^{-8} M [^{3}H]progesterone. Under these conditions, virtually all of the high affinity binding sites were

[1] Total acrylamide denotes the sum of the acrylamide monomer and the cross-linking agent, N,N'-methylenebisacrylamide. In the original definition of the parameter T, Hjertén (1962) referred the weight of the dry ingredients to the volume of solvent rather than solution.

[2] Abbreviations used are: Bis, N,N'-methylenebisacrylamide; Tes, N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; TMED, N,N,N',N'-tetramethylethylenediamine; DES, diethylstilbestrol; butyl-PBD, 2-(4′-*tert*-butylphenyl)-5-(4′-biphenylyl)-1,3,4-oxadiazole.

[3] Buffers: Tes–thioglycerol, 10 mM Tes–12 mM thioglycerol (pH 7.4 at 4°); TKO, Tes–thioglycerol containing 0.5 M KCl and 5 mg of ovalbumin/ml; separation phase buffer, 0.368 M Tris–48.3 mM glycine (pH 10.2 at 0°).

occupied and about half of the steroid in the incubation mixture remained free, as measured by charcoal-dextran and ion exchange filtration assays (see below). Blood was collected from chicks by cardiac puncture at the time of sacrifice. The serum was centrifuged for 1 hr at 105,000*g* and labeled for 1 hr with 5×10^{-9} *M* [^{3}H]cortisol. Progesterone–receptor complexes were precipitated from oviduct cytosol by addition of protamine sulfate to a final concentration of 0.8 mg/ml. The "protamine extract", was obtained by stirring the precipitates for 1 hr in Tes–thioglycerol containing 0.5 *M* KCl and 5 mg of ovalbumin/ml (TKO buffer) brought up to the original volume of cytosol.

Radioassays. Radioactivity was measured at room temperature in a Beckman LS-250 scintillation spectrometer. The counting efficiency for tritium was evaluated as a function of the external standard ratio (Sherman et al., 1974a). Aqueous samples (e.g., eluates of ion exchange filters, fractions from columns, or density gradients) were counted at a volume ratio of 1:15 (sample/counting fluid), in a solution prepared by mixing 9.6 g of butyl-PBD, 200 ml of Bio-Solv BBS-3, and 1 l. of toluene. Ion-exchange filters were counted in 10 ml of the same medium with a counting efficiency of 38%. Wet slices of polyacrylamide gels were extracted for 3 hr at room temperature into 5 ml of toluene containing 8 g of butyl-PBD/l. and counted with an efficiency of 51%.

Charcoal-Dextran and Ion Exchange Filtration Assays of Steroid Binding. Charcoal-dextran assays of bound steroid were performed as described by Sherman et al. (1974a). Briefly, 100-μl samples were shaken 20 min with 25 μl of 2.5% charcoal, 0.25% Dextran T-40, and 2% ovalbumin in Tes–thioglycerol. After centrifugation for 15 min at 1000*g*, an aliquot of the supernatant fluid was counted. Ion-exchange filtration was performed by the method of Santi et al. (1973) with several modifications. DEAE-coated filters (Whatman DE-81) were used in a Hoefer Model FH 204 manifold. Samples (100 μl) were adsorbed to dry filters for 8 min and washed five times with 1 ml of Tes–thioglycerol in 1-min intervals. Half the filters were counted to assess the total adsorbed steroid. The remaining filters were extracted twice for 8 min with 1 ml of TKO buffer. Radioactivity was measured on filters and aliquots of each extract. Corrections were made for differences in counting efficiency under various conditions and for the efficiency of extracting [^{3}H]progesterone from the filters, which was 87%.

Electrophoresis: Preparation of Buffers and Gels. Electrophoresis was performed in a Buchler Polyanalyst apparatus with an extended lower buffer chamber. The lower electrode (anode) buffer was magnetically stirred and was maintained at 0 ± 1.2° by circulation of aqueous ethylene glycol (50%, v/v) through the jacket of the chamber (Forma 2095 bath and circulator). The upper buffer compartment was surrounded by crushed ice in sealed plastic bags. Gels were formed in Pyrex tubes (6 mm i.d., 8 mm o.d.) to facilitate heat transfer. Each gel was supported by a single thickness of washed dialysis tubing held in place by a rubber O-ring.

The buffer system, consisting of an electrode buffer, a stacking gel buffer, and a separation gel buffer, was a modification of the Tris-glycine-HCl system of Davis (1964). Buffers were prepared at 25° and cooled to 0° prior to use, resulting in an increase in pH. Gels and buffers routinely contained glycerol (10%, v/v), which stabilizes steroid–protein complexes during electrophoresis (Ritzén et al., 1971) and other fractionation procedures (Feil et al., 1972; Sherman et al., 1974a). As suggested by Singer and Norris (1973), the concentrations of the electrode buffer components were increased above those used by Davis (1964) to 10 m*M* Tris–77 m*M* glycine (pH 8.3 at 25°). This decreased the voltage required to maintain the desired current of 2 mA/gel in the presence of 10% glycerol (Buchler Model 3-1014 A power supply).

Separation gels of two lengths, 110 and 170 mm, and six total acrylamide concentrations, 5, 7, 9, 11, 13, and 15%, were used. In preliminary experiments, the cross-linking agent Bis comprised 2, 10, or 15% of the total acrylamide. Except for the results in Figure 1a, all data reported here were obtained with 15% *C* separation gels. As *T* was varied from 5 to 15%, the amounts of the catalysts required to polymerize 10 ml of gel varied from 9.0 to 1.8 mg of ammonium persulfate and from 20 to 50 μl of TMED. Separation gels were stored up to 1 week at 4° in contact with buffer of the same composition as the gel: 0.38 *M* Tris, 0.06 *N* HCl, and 10% glycerol (pH 8.9 at 25°). More prolonged storage of the gels resulted in diminished mobilities of the steroid-binding proteins and standards, presumably due to progressive solvation of the polyacrylamide.

Stacking gels of 3% *T*, 20% *C* containing 10% v/v glycerol, 0.06 *M* Tris, and 0.06 *N* HCl (pH 6.7 at 25°) were prepared within 1 hr of use. Polymerization was catalyzed by 1.5 mg of ammonium persulfate, 30 μl of TMED, and 50 μg of riboflavine per 10 ml of gel and a 10-min exposure to fluorescent light. Just prior to electrophoresis, the top gel surface was rinsed with electrode buffer and the portion of the Pyrex tube above the gel was filled with electrode buffer containing 5 μg of Bromophenol Blue/ml.

The "stacking" or concentration of each protein into a thin zone at the electrophoretic boundary in the upper gel was tested as described by Rodbard and Chrambach (1971). The receptors in the protamine extract, which were not stacked under these conditions, were analyzed and compared with untreated receptors and standard proteins in separation gels without stacking gels.

During electrophoresis, buffer constituents from the stacking gel and electrode solutions migrate into the separation gel, creating an operative buffer that is different from the initial separation gel buffer. The composition of this operative "separation phase buffer" was computed with the program of Jovin (1973): 0.368 *M* Tris–48.3 m*M* glycine (pH 9.4 at 25°). This buffer was used to simulate the electrophoretic conditions during density gradient centrifugation and gel filtration analyses of the receptors (see below).

Electrophoresis: Sample Analysis. Brewer (1967) and Dirksen and Chrambach (1972) had shown that thioglycolate protects enzymes against inactivation by uncharged persulfate reaction products. Our preliminary experiments revealed similar effects on progesterone receptors. Accordingly, 5 μmol of Tris-thioglycolate (at the pH of the upper gel) in 75 μl of 50% glycerol was layered onto each gel. Labeled samples and/or standard proteins (10 mg/ml), adjusted to 20% glycerol and the ionic composition of the upper gel, were then layered over the thioglycolate. Most labeled samples contained bovine serum albumin, detectable as the Bromophenol Blue complex, and myoglobin as internal standards. Exogenous albumin was omitted from receptor quantitation experiments since it binds steroids. The upper buffer reservoir was filled with cold electrode buffer, surrounded by crushed ice, and a current of 2 mA/gel was applied for about 4 hr (110-mm gels) or about 6 hr (170-mm gels).

Each steroid-binding component and standard protein was analyzed in a minimum of three sets of gels with the full range of T. Comparisons of various binding components or electrophoretic conditions (see Figures 1 and 5) were performed simultaneously in gels prepared at the same time. Gels containing only standard proteins were stained for 15 min with Amido Black, 5 mg/ml in 7% (v/v) acetic acid (Davis, 1964) containing 10% glycerol, and destained by diffusion overnight at 25° in 7% acetic acid and 10% glycerol, which was circulated through a charcoal filter. Gels cross-linked with a high percentage of Bis are opaque and unsuitable for spectrophotometric scanning. Therefore, each gel on a piece of Parafilm was aligned on graph paper above a fluorescent light, and the positions of the stained protein bands, the ends of the gel, and the chloride-thioglycolate boundary (see below) were marked on the graph paper.

Gels containing labeled samples were frozen and stored at −80°. After partial thawing, they were cut into 1.2-mm slices with a modified egg-slicer type device (Chrambach, 1966) built by Nils Jernberg of the Rockefeller University Instrument Shop. The reproducibility of gel slicing, efficiency of extraction of free steroid from the gels, and counting efficiency were evaluated in 15% C, 7% T separation gels uniformly labeled with 1.7×10^{-9} or 4.0×10^{-8} M [^{3}H]progesterone. Optimal extraction time, 3 hr at room temperature, was established by recounting the same slices after various periods of extraction into counting fluid (see Radioassays). The same time was required for maximal recovery of [^{3}H]progesterone from steroid–receptor complexes that were electrophoresed into the gels as for extraction of free steroid added before polymerization. The extraction efficiency was not improved by addition of a detergent, Bio-Solv BBS-3, to the scintillation fluid and was decreased by drying the gel slices at 120° for 24 or 48 hr. The average recovery of [^{3}H]progesterone placed in the acrylamide before polymerization was 75%. Reproducibility of the cpm/slice in slices from the central portion of the gel was ±2% (s.d.) of the average cpm/slice. At the ends of the gel, the cpm/slice were consistently lower, presumably as a result of compression of the gel during slicing.

Evaluation of Relative Mobility. Since the major component of the tracking dye Bromophenol Blue is significantly retarded in separation gels of high T, protein mobilities were calculated relative to the chloride–thioglycolate boundary. This boundary, detected by immersing the bottom of the gels into ice-cold 0.2 M $AgNO_3$ (Rodbard and Chrambach, 1971), preceded the leading Bromophenol band by a distance proportional to the amount of thioglycolate, and independent of the volumes of the thioglycolate or sample layers. With 5 μmol of thioglycolate, the R_f of the leading Bromophenol band was 0.82 relative to the chloride–thioglycolate boundary.

Computer programs kindly provided by David Rodbard were used to calculate the weighted linear regression of log R_f on T according to eq 1 and the 95% confidence limits for a single observation, for the line, the slope, $-K_R$, and the intercept, log Y_0. Data for the standard proteins were used to compute the unweighted linear regression of the equivalent molecular radius, $\bar{R}$, on $K_R^{1/2}$.

$$\bar{R} = c(K_R)^{1/2} - r \tag{2}$$

where

$$\bar{R} = \left(\frac{3\,M\bar{v}}{4\pi N}\right)^{1/3} \tag{3}$$

c is an empirical constant, r is the acrylamide fiber radius, M is the molecular weight, $\bar{v}$ is the partial specific volume, and N is Avogadro's number. The molecular weights of the steroid-binding components were calculated from eq 3, with a value of $\bar{v} = 0.74$ cm^3 g^{-1} for the receptors, based on studies in density gradients containing NaBr (Sherman et al., 1970), and $\bar{v} = 0.695$ cm^3 g^{-1} for chick corticosteroid binding globulin, by analogy with the corresponding rabbit protein (Chader and Westphal, 1968).

Since the preceding method neglects the potential effects of unusual asymmetry or solvation on electrophoretic retardation, an alternative analysis of the data was performed. The Stokes radius, R_S, of each standard (in angstroms) was calculated from the diffusion coefficient, $D_{20,w}{}^0$:

$$R_S = \frac{2.143 \times 10^{-5}}{D_{20,w}{}^0} \tag{4}$$

Estimates of R_S for human serum albumin dimer and bovine serum albumin trimer were calculated from the corresponding $\bar{R}$ and the average ratio of $R_S/\bar{R}$ for the other standards in Table I. The value of R_S for each binding component was calculated from the linear regression of R_S on $K_R^{1/2}$ for the standards and was combined with the sedimentation coefficient and an estimate of $\bar{v}$ to calculate M, as described by Sherman (1975, eq 10).

Density Gradient Centrifugation. Gradients of 10–35% (w/v) glycerol in the operative buffer in the separation phase during electrophoresis were centrifuged in an SW-56 rotor and L2-65 B ultracentrifuge (Beckman) for 22 hr at 308,000g. Sedimentation coefficients were evaluated by the method of Martin and Ames (1961) with ovalbumin, 3.53 S (Castellino and Barker, 1968), or hemoglobin, 4.42 S (Schumaker and Schachman, 1957), as an internal standard. There was no evidence of dissociation of hemoglobin in gradients containing the separation phase buffer, despite the reported lability of the tetrameric form at high pH (Rossi Fanelli et al., 1964).

Solutions of 15 and 41% (w/v) glycerol in Tes–thioglycerol were determined to have the same densities as 10 and 35% (w/v) glycerol in the separation phase buffer. Sedimentation coefficients in linear 15–41% glycerol gradients in Tes–thioglycerol were evaluated by comparison with myoglobin, 2.0 S (Rossi Fanelli et al., 1958), in addition to the standards listed above.

Gel Filtration. A column of Agarose A 0.5m (1.27 × 43 cm) was packed in water then equilibrated with the separation phase buffer containing 10% (v/v) glycerol. Satisfactory resolution was not obtained when the gel was equilibrated with this buffer before the column was packed. The void volume was marked by the elution of Blue Dextran 2000 (Pharmacia) and the total liquid volume by [^{14}C]valine. We attempted to calibrate the column for M and R_S as described by Sherman et al. (1974a). Of the proteins tested, thyroglobulin, lactic dehydrogenase, aldolase, hemoglobin, pepsin, and cytochrome c were not eluted at the expected volume in this medium. This anomalous behavior may have resulted from dissociation into subunits or from interactions with the agarose under the combined influences of dilution, low ionic strength, and high pH. Therefore, gel filtration in this buffer was used only for qualitative characterization of the steroid-binding components.

Results

Effect of Cross-Linking on Mobility of Progesterone-Binding Components. To demonstrate that free progester-

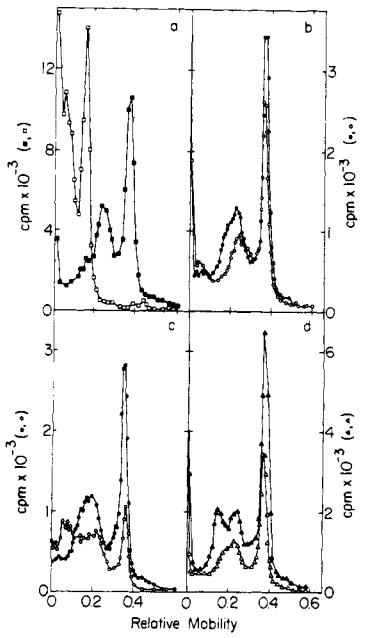

FIGURE 1: Resolution and recovery of progesterone-binding components by gel electrophoresis under various conditions. The results of simultaneous analyses of 25 μl of [^{3}H]progesterone-labeled cytosol in 7% *T* separation gels are compared in each panel. (a) The cross-linking agent Bis comprised 2% (□) or 15% (■) of the total concentration of Bis + acrylamide monomer in 3-ml separation gels. Stacking gels (0.5 ml) contained 20% *C* and 3% *T*. (b) Stacking gels were omitted (○) or included (●) above 5-ml separation gels. (c) Glycerol (10%, v/v) was omitted (○) or included (●) in the separation and stacking gels and electrode buffer. (d) 3-ml (▲) or 5-ml (△) separation gels were used with stacking gels.

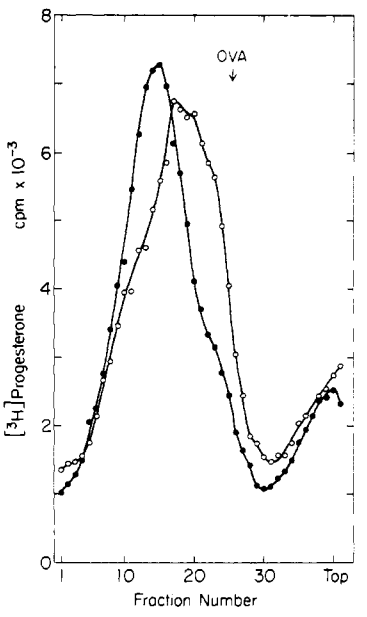

FIGURE 2: Ultracentrifugal analyses of progesterone-binding components in Tes-thioglycerol and in the operative separation phase buffer of the electrophoretic system. [^{3}H]Progesterone-labeled cytosol (0.1 ml) was centrifuged for 22 hr at 308,000*g* through gradients of 10–35% (w/v) glycerol in 0.368 *M* Tris–48.3 m*M* glycine (pH 10.2 at 0°) (○), or gradients of 15–41% (w/v) glycerol in Tes-thioglycerol (pH 7.4) (●), which have the same densities. Ovalbumin (OVA), $s_{20,w}$ = 3.53 S, was an internal marker.

one does not migrate into the gels, samples containing [^{3}H]progesterone and ovalbumin, which does not bind progesterone, were electrophoresed as described above. No radioactivity was detected in the separation gel, whether or not a stacking gel was included. The two peaks of radioactivity detected after electrophoresis of [^{3}H]progesterone-labeled oviduct cytosol, therefore, represent macromolecular complexes containing the labeled steroid. Figure 1a illustrates the higher mobilities of both peaks in highly cross-linked gels (i.e., 15% *C* vs. 2% *C*). The slow peak moved only a short distance into 2% *C*, 7% *T* gels and was completely excluded from 2% *C*, 11% *T* gels (not shown). In contrast, 15% cross-linking permitted measurement of the mobility of the slow peak in gels of 5–11% *T* and of the fast peak in gels of 5 to >15% *T*.

The labeled complex with higher mobility is referred to as the "cytosol fast" component in this report. The appearance of the peak with lower mobility varies with gel length and sample load and among different cytosol preparations (see Figures 1 and 4). The shape and width of this peak are indicative of heterodispersity of the "cytosol slow" components, possibly due to aggregation. Treatment of cytosol with 0.5 *M* KCl for 2 hr decreased the slow peak but had no significant effect on the mobility or yield of the fast component (see Figure 7, bottom).

Optimization of Electrophoretic Conditions. In extensive analyses of [^{3}H]progesterone-labeled cytosol, the following conditions enhanced the recovery and/or the resolution of the steroid-containing complexes at 0°: (1) 3-ml (110 mm) separation gels, 15% *C*; (2) 0.5-ml stacking gels; (3) inclusion of 10% glycerol, v/v, in all phases of the Tris-glycine-HCl buffer system; and (4) a layer containing 5 μmol of Tris-thioglycolate beneath the sample layer.

Omission of the stacking gel (Figure 1b) or increase of the length of the separation gel from 110 to 170 mm (Figure 1d) decreased the recovery of both the fast and slow components, with a greater loss of the slower species. Therefore, 110-mm separation gels with stacking gels were used routinely except for analyses of complexes with very low net negative charge, e.g., those in the protamine extract. Both glycerol (Figure 1c) and thioglycolate increased the recovery of the fast component and diminished the apparent electrophoretic heterogeneity of the slow peak. The beneficial effects of glycerol were evident at all gel concentrations tested, while those of thioglycolate were consistently observed only in gels containing >7% *T*.

Density Gradient Centrifugation. It was plausible that the high pH during electrophoresis might destabilize the receptor or the steroid–receptor complex. Therefore, labeled cytosol was analyzed by the more conventional techniques of density gradient centrifugation and gel filtration in the Tris-glycine buffer generated in the separation phase during electrophoresis.

The recovery of bound [^{3}H]progesterone after centrifugation through glycerol gradients in the Tris-glycine buffer was similar to that obtained in gradients of the same density range in Tes-thioglycerol (Figure 2). This result demonstrated that the steroid–receptor bonds were as stable at pH 10.2 as at pH 7.4 for 22 hr, more than three times the duration of electrophoresis in long gels. The nearly symmetrical distribution of labeled complexes in gradients containing Tes-thioglycerol covered a range of sedimentation coefficients of about 4.6 S–7 S, with the highest radioactivity at 5.8 S. In gradients containing the separation phase buffer, there was a decrease in the proportion of 6S–7S complexes and an increase in the 4S components. The ultracentrifugal

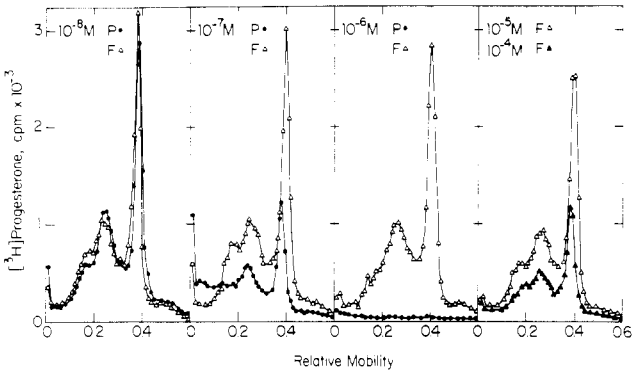

FIGURE 3: Steroid-binding specificity and saturability of receptor forms analyzed by electrophoresis. Chick oviduct cytosol containing about 2.6×10^{-8} *M* progesterone binding sites was labeled simultaneously with 10^{-8} *M* [^{3}H]progesterone and the indicated concentrations of nonradioactive progesterone (P) or cortisol (F) and incubated for 3 hr at 4°. Electrophoresis was performed on duplicate 40-μl aliquots on 3-ml separation gels (15% *C*, 7% *T*) with 0.5-ml stacking gels (20% *C*, 3% *T*) in the complete buffer system with a thioglycolate underlayer.

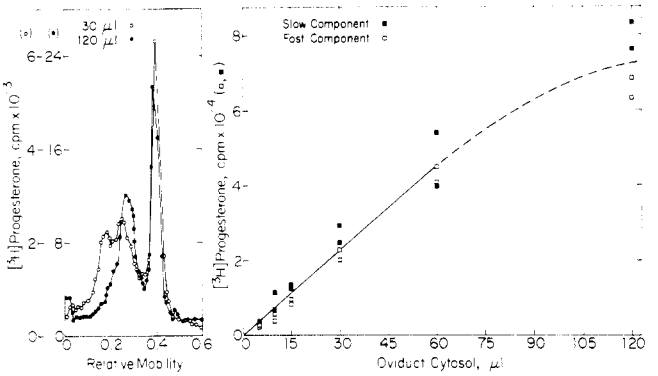

FIGURE 4: Effects of protein load on the quantitation of progesterone receptors by electrophoresis and the distribution between slow and fast forms. Aliquots of [^{3}H]progesterone-labeled cytosol containing ~20 mg of protein/ml were analyzed on duplicate 3-ml separation gels (15% *C*, 7% *T*) with 0.5-ml stacking gels. (Left) Representative gel patterns show that although the shape of the slow peak (R_f = 0.11–0.34) changes with sample volume, the slow and fast peaks each contain about half the total bound steroid, over a fourfold range of cytosol volumes (note the fourfold difference in the scale of the ordinates). (Right) The total extractable radioactivity in the slow (■) and fast (□) peaks increases linearly with cytosol volume, up to 60 μl.

resolution between different cytosol components (Figure 2) was clearly inferior to the electrophoretic resolution (Figure 1).

Steroid-Binding Specificity and Saturability of Electrophoretic Components. The progesterone-binding components revealed by electrophoresis of chick oviduct cytosol exhibited the steroid-binding specificity and limited binding capacity characteristic of intracellular receptors, as opposed to corticosteroid-binding globulin from chick serum. The presence of competing steroids at 10^{-8} *M* did not alter the pattern of bound [^{3}H]progesterone from that of cytosol incubated without a competing steroid in parallel experiments (Figure 3, left). This result was consistent with the high concentration of binding sites in the cytosol (2.6×10^{-8} *M*) compared to the total steroid concentration in these incubations (2.0×10^{-8} *M*). The [^{3}H]progesterone bound to both slow and fast electrophoretic components was significantly reduced by 10^{-7} *M* progesterone and was completely eliminated by 10^{-6} *M* progesterone. In contrast, concentrations of cortisol three to four orders of magnitude higher were required to depress [^{3}H]progesterone binding to the same extent (compare 10^{-7} *M* progesterone with 10^{-4} *M* cortisol). These results were consistent with the respective dissociation constants of progesterone (~4.0×10^{-10} *M*) and cortisol (2.5×10^{-6} *M*) estimated by charcoal-dextran assay of dilute unfractionated cytosol (Sherman et al., 1974a).

Quantitation of Steroid–Receptor Complexes by Electrophoresis. Duplicate samples containing 5–120 μl of [^{3}H]progesterone-labeled cytosol were electrophoresed in 110-mm separation gels with stacking gels. As shown in Figure 4, the [^{3}H]progesterone was distributed approximately equally between the slow and fast peaks, regardless of sample load. Furthermore, the amount of radioactivity associated with each electrophoretic form increased linearly with cytosol volume, up to 60 μl (about 1.2 mg of protein). While the mobility and shape of the fast peak were unaffected by increasing sample load, the slow peak became less heterodisperse, with an upward shift in the average mobility.

Comparison of Electrophoretic, Ion-Exchange Filtration, and Charcoal-Dextran Assays of Specific Steroid Binding. Cytosol was labeled for 3 hr with 4.2×10^{-8} *M* [^{3}H]progesterone ± 4×10^{-6} *M* unlabeled progesterone and aliquots were analyzed by three techniques. Six 40-μl aliquots were electrophoresed in 110-mm separation gels (15% *C*, 7% *T*) with stacking gels. The resultant distributions of [^{3}H]progesterone in samples incubated without competitor were divided into four regions: the top of the separation gel, R_f = 0–0.04, presumably containing receptor aggregates; the slow components, R_f = 0.04–0.28; the fast peak, R_f = 0.28–0.38; and the region ahead of the fast peak, R_f = 0.38–1.0. Plots of the radioactivity in the last region (on an expanded scale of cpm/slice) revealed a small peak of labeled steroid at the R_f of chick serum albumin. Gel patterns from samples incubated with excess unlabeled progesterone were divided into the same ranges of R_f, although the only consistently detectable peak of radioactivity corresponded to chick serum albumin.

Six 100-μl aliquots of each incubation were treated with dextran-coated charcoal. Twelve 100-μl aliquots of each incubation were adsorbed to ion-exchange filters, six of which were eluted twice with 1 ml of TKO buffer. The results of separate experiments, including charcoal-dextran treatment and analytical gel filtration, supported the assumption that the [^{3}H]progesterone in the filter eluate was bound to receptors (M. R. Sherman, F. B. Tuazon, S. C. Diaz, and L. K. Miller, in preparation).

The dissociation constant and concentration of high affinity progesterone binding sites in diluted oviduct cytosol were previously evaluated by the charcoal-dextran assay (Sherman et al., 1974a). Based on these results, the high affinity sites in undiluted cytosol were saturated at 4.2×10^{-8} *M* [^{3}H]progesterone. Therefore, the binding of [^{3}H]progesterone in the presence of a 95-fold excess of unlabeled progesterone is attributable to high capacity, low affinity binding sites in the cytosol. Such sites are often referred to as "nonspecific", although the steroid-binding specificities are rarely evaluated. By convention, the difference between the [^{3}H]progesterone bound in the absence and presence of the excess unlabeled steroid is referred to as the "specific" binding. The results of all three procedures, expressed as the percentage of the total [^{3}H]progesterone in each sample, are summarized in Table II.

The amounts of specific binding determined by electro-

Table II: Evaluation of the Percentage of Specifically Bound [^{3}H]Progesterone by Three Methods.

Method, Status	[^{3}H]Progesterone	[^{3}H]P + Unlabeled P	Specific Binding
Charcoal-dextran treatment			
Supernatant	59.0 ± 0.6	5.6 ± 0.1	53.4 ± 0.6
Ion exchange filtration			
On filter before elution	52.3 ± 2.2	6.0 ± 0.2	46.3 ± 2.2
On filter after elution	2.7 ± 0.1	1.2 ± 0.1	1.5 ± 0.2
Total KCl eluate	48.6 ± 0.9	7.6 ± 0.4	41.0 ± 1.0
Electrophoresis			
Slow component(s)	23.5 ± 0.6	1.5 ± 0.1	22.0 ± 0.6
Fast component	19.9 ± 1.0	0.2 ± 0.01	19.7 ± 1.0
Slow plus fast	43.4 ± 1.4	1.7 ± 0.1	41.7 ± 1.4
Total on gel	51.7 ± 1.5	4.0 ± 0.1	47.7 ± 1.5

[a] Aliquots of cytosol incubated with 4.2×10^{-8} *M* [^{3}H]progesterone ([^{3}H]P) ± 4×10^{-6} *M* unlabeled progesterone (P) were treated with dextran-coated charcoal or fractionated by ion-exchange filtration or electrophoresis in 7% *T* gels. The [^{3}H]progesterone recovered in the various fractions is expressed as a percentage of the total [^{3}H]progesterone in the sample, ± S.E.M. in six determinations. Specific binding denotes the difference between the quantity bound in the absence and presence of excess unlabeled progesterone.

phoresis and ion-exchange filtration were in good agreement. The total percentage of [^{3}H]progesterone specifically bound, calculated from the radioactivity on the filter before elution, was the same as that calculated from the total recovery from the gels (about 47%). There was similar agreement between the percentage of [^{3}H]progesterone associated with well-defined electrophoretic components (slow and fast) and that extractable from the filters by high ionic strength buffer (about 41%). The charcoal-dextran assay indicated a slightly higher percentage of specific binding (about 53%) than either the filter method or the gels. The preceding results justify the use of electrophoresis for the quantitative evaluation of receptor content in unknown specimens.

Dependence of Mobility on Gel Concentration. Figure 5 shows the effects of total acrylamide concentration on the relative mobilities of progesterone receptors in untreated and protamine-precipitated cytosol and of chick serum corticosteroid-binding globulin. The decrease in the R_f of each labeled complex with increasing T illustrates the principle expressed in eq 1, on which the analysis of size and net charge by gel electrophoresis is based. The superposition of patterns for different steroid-binding components illustrates an important consequence of the dependence of R_f on T: in a given buffer system, the resolution between different pairs of components may be optimized in gels of different T (see Rodbard et al., 1974).

When [^{3}H]progesterone-labeled cytosol was compared with [^{3}H]cortisol-labeled chick serum in short separation gels with stacking gels, the resolution between the labeled complexes in cytosol and serum was improved with increasing gel concentration (Figure 5, top). Therefore, gels of 13 and 15% T were used to determine whether a complex with the R_f of corticosteroid-binding globulin was present in cytosol labeled with progesterone or with 1.7×10^{-7} *M* [^{3}H]cortisol. No such peak was evident, although the complex was readily detected in chick serum under these conditions. On the other hand, the receptors in 0.5 *M* KCl extracts of protamine-precipitated cytosol were clearly distinguishable from the heterodisperse slow components of untreated cytosol only in the most dilute gels (5% T, Figure 5, bottom).

Ferguson Plots and Confidence Limits of the Slopes and Intercepts. Replicate gel patterns, like those in Figure 5, were used to prepare Ferguson plots (lot R_f vs. T) for the

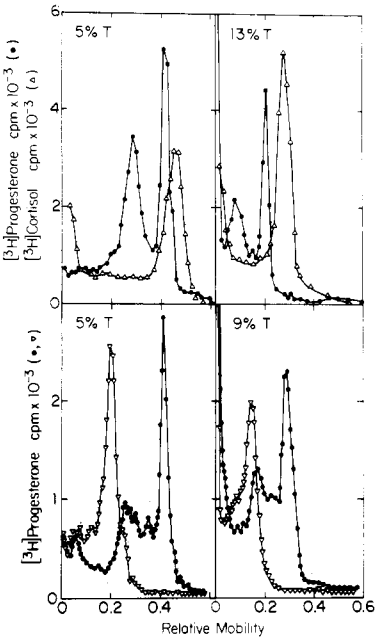

FIGURE 5: Effects of total acrylamide concentration (T) on the mobilities of steroid-binding components and the resolution between them. (Top) Samples of 25 μl of [^{3}H]progesterone-labeled cytosol (●) or [^{3}H]cortisol-labeled chick serum (△) were analyzed on 3-ml (110 mm) separation gels (15% C) with 0.5-ml stacking gels (20% C, 3% T). (Bottom) Samples of 25 μl of [^{3}H]progesterone-labeled cytosol (●) or 50 μl of labeled cytosol that was precipitated with protamine sulfate and extracted with the same volume of 0.5 *M* KCl (protamine extract, ▽) were electrophoresed on 5-ml (170 mm) separation gels without stacking gels.

various steroid-binding components. Figure 6 (top) shows the results in short separation gels with stacking gels for the complexes in untreated cytosol and in chick serum. The similar ordinate intercepts of these plots (log Y_0) indicated similar ratios of net charge/surface area at the pH of the separation phase (Rodbard and Chrambach, 1971). The slopes of the lines ($-K_R$), however, differed markedly, indicating the following sequence of molecular sizes: corticosteroid-binding globulin < cytosol fast < cytosol slow. In the more concentrated gels, the resolution of the serum component from the cytosol fast component was improved, but the R_f of the cytosol slow form was too low for reliable determi-

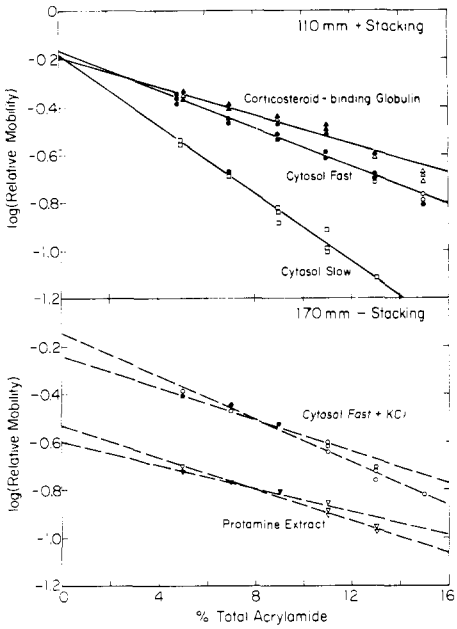

FIGURE 6: Ferguson plots of electrophoretic data for cytosol receptors and corticosteroid-binding globulin. (Top) Relative mobilities (R_f's) of the labeled complexes in cytosol (○,□) or in serum (△) were determined in 110-mm separation gels of 5–15% T with stacking gels (see Figure 5, top). Solid lines show the weighted linear regressions of log R_f on T. (Bottom) Samples containing 50 μl of protamine extract (▽, see Figure 5, bottom) or 30 μl of labeled cytosol that was mixed with an equal volume of 1 M KCl for 2 hr (○) were electrophoresed on 170-mm separation gels of 5–15% T without stacking gels. Dashed lines show the upper and lower joint 95% confidence limits of the slopes and intercepts of these plots. Filled symbols indicate the superposition of data points in replicate analyses.

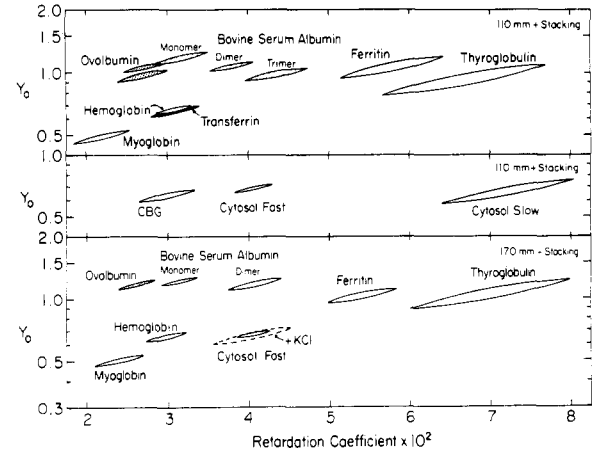

FIGURE 7: Joint 95% confidence limits of the slopes (retardation coefficient, K_R) and intercepts (log Y_o) of log R_f vs. T. Results in the center panel were computed from the data in Figure 6 for two forms of the progesterone receptor and for chick serum corticosteroid-binding globulin (CBG) in 3-ml separation gels with stacking gels. The top panel shows results for standard proteins in the same system. The bottom panel shows analogous results in 5-ml separation gels without stacking gels.

nation. Further studies of this form, therefore, were limited to gels of <13% T.

The ordinate intercept of the Ferguson plot for the protamine extract is lower than that of the fast form in KCl-treated cytosol (Figure 6, bottom). These results indicate that the complex in the protamine extract has a lower net negative charge than the cytosol fast form, and that the difference was not caused by the KCl in the extraction medium.

As emphasized by Rodbard and Chrambach (1974), errors in K_R and Y_o are highly correlated, e.g., an erroneously high value of R_f at a low T yields spuriously high values for both K_R and Y_o. For this reason, the joint confidence limits of the slopes and intercepts were determined as illustrated in Figure 6 (bottom). The lack of overlap of the 95% confidence limits of the data for receptors in KCl-treated cytosol and in KCl extracts of protamine-precipitated cytosol demonstrated the nonidentity of these components with $p < 0.0025$.

A graph of the joint confidence limits of log Y_o and K_R forms an ellipse characteristic of the protein in that electrophoretic system (Figure 7). In this graph, vertical displacement indicates a difference in the net charge/surface area, e.g., between two forms of ovalbumin containing different amounts of phosphate (Perlmann, 1952) or carbohydrate (Cunningham et al., 1965). Horizontal displacement reflects a difference in molecular size, e.g., between bovine serum albumin monomer, dimer, and trimer. The size of an ellipse indicates the uncertainty in the determination of the Ferguson plot parameters and generally decreases with replicate analyses. The wide confidence limits for K_R of the cytosol slow component, despite triplicate analyses, reflect the variability and heterodispersity of the slow peak in the individual gel patterns. The overlap of the ellipses for the fast component in untreated and KCl-treated cytosol (Figure 7, bottom) indicates the similarity of size and charge of these receptor forms but does not prove their identity. Coincidental overlap or superposition of the ellipses for different proteins may occur, as illustrated for hemoglobin and transferrin (Figure 7, top).

Results are shown in Figure 7 for several standard proteins and for the cytosol fast receptor form both in short separation gels with stacking gels (top and center) and in long separation gels without stacking gels (bottom). Since the resolution of the bands in the latter system was inferior to that in the former, the minor component in ovalbumin was not detectable by visual inspection, and the band identified as bovine serum albumin dimer in the long gels may actually represent a mixture of the dimer and trimer. Despite these uncertainties, the 95% confidence limits for K_R of each protein (e.g., the cytosol fast component) overlap in the short and long gels.

Calculation of Molecular Radii and Apparent Molecular Weights. The values of $\bar{R}$ for the standard proteins (Table I) were analyzed as linear functions of the values of $K_R^{1/2}$ obtained in short separation gels with stacking gels or in long separation gels. The two values of K_R found for each protein studied under both conditions showed no systematic variation and the unweighted linear regressions for the standards were virtually identical:

$$\bar{R} = 360.8K_R^{1/2} - 36.84 \quad \text{(short gels)}$$

$$\bar{R} = 362.9K_R^{1/2} - 37.12 \quad \text{(long gels)}$$

$$\bar{R} = 361.7K_R^{1/2} - 36.95 \quad \text{(combined data)}$$

The 95% confidence limits for a single determination were wider in the long gels, however, since fewer standards were used and some of the protein bands were more diffuse under those conditions.

The midpoint of the ellipse for each steroid-binding component in Figure 7 gives the best estimate of K_R. The values of $\bar{R}$ determined from this point and from the 95% confidence limits of the standard curve are listed in Table III.

Table III: Estimates of Molecular Size of Steroid-Binding Components by Electrophoresis at pH 10.2.

Component[a]	Gel System[b]	R_S, A (Range)[c]	$\bar{R}$, A (Range)[d]	$M \times 10^{-3}$ (Range)[e]
Chick CBG	110+	32.4 (23.0–41.7)	25.7 (22.4–28.9)	61 (41–88)
Cytosol slow	110+	83.6 (72.4–94.8)	60.2 (56.3–64.1)	744 (609–899)
Cytosol fast	110+	47.3 (38.1–56.5)	35.7 (32.5–38.9)	156 (117–201)
	170–	48.0 (37.3–58.7)	36.1 (29.7–42.4)	160 (89–261)
Cytosol fast + KCl	170–	47.7 (37.0–58.4)	35.9 (29.5–42.2)	157 (87–257)

[a] Corticosteroid-binding globulin (CBG) was studied in [^{3}H]cortisol-labeled chick serum. Cytosol slow and fast components denote the major peaks in unfractionated [^{3}H]progesterone-labeled oviduct cytosol. The fast form was also studied after exposure to cytosol to 0.5 *M* KCl for 2 hr. [b] Electrophoresis was performed in 110-mm separation gels, 15% *C*, 5–15% *T*, with stacking gels (110+) or in 170-mm separation gels without stacking gels (170–). [c] The Stokes radius, R_S, and range were calculated from the unweighted linear regressions of R_S for the standards (Table I) on $K_R^{1/2}$ in each gel system and the 95% confidence limits for a single determination. [d] The equivalent molecular radius, $\bar{R}$, and range were calculated from the unweighted linear regressions of $\bar{R}$ on $K_R^{1/2}$ for the standards in each gel system and the 95% confidence limits for a single determination. [e] Molecular weights were calculated from the $\bar{R}$'s and eq 3.

The values and confidence limits of the Stokes radii, R_S, were determined from analogous linear regressions of R_S on $K_R^{1/2}$. The results for $\bar{R}$ were combined with estimates of the partial specific volume of the steroid-binding components to calculate *M* by rearrangements of eq 3.

The apparent *M* of chick corticosteroid-binding globulin evaluated by electrophoresis in 15% *C* gels is similar to the values obtained in 2% *C* gels and by gel filtration (Sherman et al., 1970). The two estimates of *M* for the fast electrophoretic component of untreated cytosol are not significantly different from each other or from that obtained after treatment of cytosol with KCl (~158,000). Estimates of $\bar{R}$ and *M* of the cytosol slow components are included in Table III for comparison only. It is clear from the individual gel patterns that the cytosol slow peak encompasses several partially resolved receptor forms or aggregates (see Figures 1 and 3–5). Estimates of molecular size based on such patterns are highly unreliable. Furthermore, the value of K_R for this composite peak exceeds that of the largest standard, thyroglobulin.

The analysis of electrophoretic data in terms of the equivalent molecular radius, $\bar{R}$ is based on the assumption that the asymmetry of the unknown proteins is comparable to that of the standards. If some forms of the progesterone receptor are much more asymmetric than the globular standards in this buffer system, as they appear to be in the presence of KCl (Sherman et al., 1970), then the values of *M* in Table III may be overestimates. The magnitude of the potential discrepancy is illustrated by the following calculation.

Among the proteins used as standards in the present study, the total frictional ratio, $R_S/\bar{R}$, varied only slightly: $R_S/\bar{R} = 1.30 \pm 0.04$ (S.E.M.) (see Table I). Thus, the Stokes radii of the steroid-binding components calculated from the linear regressions of R_S on $K_R^{1/2}$ for the standards were about 1.3 times the respective values of $\bar{R}$ (Table III). R_S for each component was combined with its sedimentation coefficient, $s_{20,w}$, and an estimate of $\bar{v}$ to obtain an estimate of *M* that involved no assumptions about solvation or asymmetry (Siegel and Monty, 1966; Sherman, 1975). In the case of the cytosol fast component, the values of R_S = 47.7 Å, $s_{20,w}$ = 4.2 S (see below), and $\bar{v}$ = 0.74 cm^3 g^{-1} gave a molecular weight of 87,000, which is only 55% of that calculated from $\bar{R}$.

Relationships between Receptor Forms Detected by Electrophoresis, Gel Filtration, and Ultracentrifugation. Labeled cytosol was filtered at 4° through Agarose A 0.5m equilibrated either with the separation phase buffer [0.368 *M* Tris and 48.3 m*M* glycine (pH 10.05 at 4°)] containing 10% (v/v) glycerol or with 0.4 *M* KCl, Tes–thioglycerol, and 10% (w/v) glycerol (pH 7.4 at 4°). Selected fractions from the column were analyzed by electrophoresis or by density gradient centrifugation. The gel filtration pattern in the separation phase buffer contained a large peak at the void volume (I, Figure 8, top) and a single included peak (II). The elution pattern for this preparation of cytosol in high salt neutral buffer contained very little [^{3}H]progesterone at the void volume, but a partially resolved double peak in fractions corresponding to pools II and III in Figure 8, top (cf. Sherman et al., 1974a, Figure 1, top).

Aliquots of the column eluates were electrophoresed in 15% *C* separation gels of several concentrations. As shown for the 7% *T* gels in Figure 8, pool I from gel filtration in the separation phase buffer contained mainly the slow electrophoretic components. The gel patterns from pools II and III showed that the included peak in the column equilibrated with the separation phase buffer contained a single major receptor form, the cytosol fast component. In contrast, electrophoretic analyses of the left and right sides of the composite peak eluted from the column in the presence of KCl revealed the cytosol fast form and a discrete component of lower mobility, respectively. The characterization of the latter receptor form will be included in a separate report (M. R. Sherman et al., in preparation).

Aliquots of pooled fractions from the gel filtration columns were centrifuged through glycerol gradients in the separation phase buffer with hemoglobin or ovalbumin as an internal standard. The labeled complexes in pools II (not shown) and III sedimented as a sharp peak at 4.2 S. Pool I contained a heterodisperse distribution of more rapidly sedimenting components.

In summary, the progesterone receptor form with high electrophoretic mobility (the cytosol fast component) is detectable as a discrete complex by gel filtration and by density gradient centrifugation at pH 10.2. Quantitative electrophoretic data indicate a molecular radius much larger than that of serum albumin, despite the similar sedimentation coefficients. The exclusion of the slow electrophoretic components from agarose supports the inference from the electrophoretic patterns of a highly aggregated state.

Discussion

Tissue extracts often contain several receptors and plasma proteins of similar sizes and overlapping steroid-binding specificities. Clear discrimination among these components is essential to basic and clinical investigations of steroid action. Examples include studies of the sites (target tissue and/or peripheral) and mechanisms of interaction between

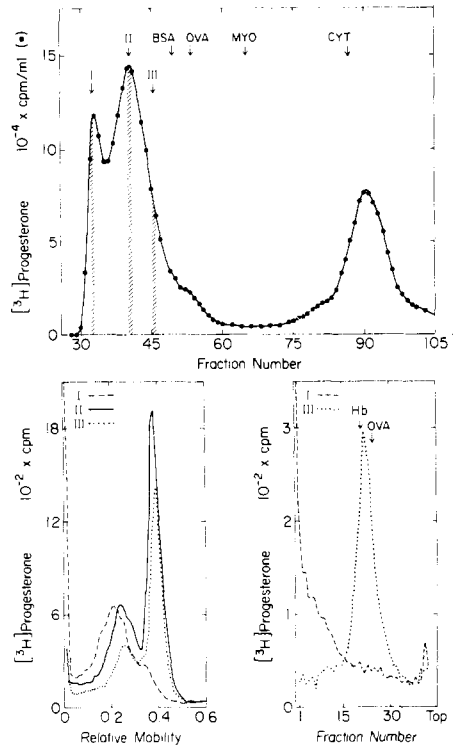

FIGURE 8: Electrophoresis and ultracentrifugation of receptors fractionated by gel filtration. (Top) [^{3}H]Progesterone-labeled cytosol (0.4 ml) was filtered on Agarose A 0.5m (1.27 × 43 cm) in the separation phase buffer (0.368 *M* Tris and 48.3 m*M* glycine) containing 10% (v/v) glycerol. Aliquots (0.1 ml) were counted and the remainder of fractions indicated by shading formed pools I, II, and III for further analyses. Standard proteins bovine serum albumin (BSA), ovalbumin (OVA), myoglobin (MYO), and cytochrome *c* (CYT) were detected optically. (Bottom left) Aliquots (0.15 ml) of pools I (- - -), II (—), and III (···) were electrophoresed on 3-ml separation gels of 15% *C* and 7% *T* with stacking gels. (Bottom right) Aliquots (0.15 ml) of pools I and III were centrifuged for 22 hr at 308,000*g* in 10–35% (w/v) glycerol gradients in the separation phase buffer with hemoglobin (Hb) or OVA as an internal standard.

physiologically antagonistic steroids, e.g., between estrogens and androgens in mammary and prostatic cancers. The applicability of quantitative polyacrylamide gel electrophoresis to research in this field was demonstrated previously (Sherman et al., 1970) and has been enhanced by the modifications described in the present report.

The increased mobilities attained by the use of highly cross-linked gels (Figure 1a) permit evaluation of the quantity (Figure 4, Table II) and steroid-binding specificity (Figure 3) of each receptor form or distinct component in a mixture. The systematic variation of total gel concentration provides optimal resolution between different components in a given buffer system (Figures 5 and 6) and an estimate of the molecular size of each (Table III). Analysis of the joint 95% confidence limits of the parameters of charge and size (Figure 7) permits the sensitive discrimination between different steroid-binding components, e.g., corticosteroid-binding globulin vs. intracellular progesterone receptors, or between different forms of the same receptor.

The data in Figures 6 and 7 indicate that KCl has no effect or fully reversible effects on the fast electrophoretic component of oviduct cytosol, but that KCl extracts of protamine-precipitated cytosol contain a complex with lower net negative charge. The relationship between the receptor forms identified by electrophoresis of untreated and protamine-precipitated cytosol and those fractionated by ion exchange chromatography will be discussed in a separate report (M. R. Sherman et al., in preparation). The value of *M* calculated from the equivalent molecular radius of the high mobility cytosol component (~158,000) is significantly higher than that calculated from the Stokes radius (48 Å) and the sedimentation coefficient (4.2 S). Resolution of this apparent discrepancy may require an investigation of the role of molecular asymmetry in the electrophoretic retardation of well-characterized fibrous proteins.

Steroid-binding specificities and capacities have been evaluated most often by the charcoal-dextran technique (Nugent and Mayes, 1966; Korenman, 1968) or by gel filtration on short columns of low porosity media, e.g., Sephadex G-25 (Puca and Bresciani, 1968). Analysis of mixtures of binding components by these techniques is facilitated by the use of excess unlabeled competitors for selected classes of sites (Feldman et al., 1973). For molecular size determinations, density gradient centrifugation has been most widely used despite the poor resolution, the limited number of samples that can be processed simultaneously, the duration of ultracentrifugation, and fraction collection and the expense of the equipment. Filtration on long columns of porous gels, e.g., Sephadex G-200 or agarose, provides adequate fractionation on the basis of size, but requires relatively large sample volumes and as much time as density gradient analysis, and is not suitable for the simultaneous processing of multiple samples.

Several laboratories have utilized polyacrylamide gel electrophoresis at a single gel concentration to identify steroid–receptors in cytosol or to distinguish them from steroid-binding proteins of serum (Ritzén et al., 1971; Mainwaring and Irving, 1973). The advantages of systematically varying the pore size, however, have been demonstrated by Corvol et al. (1971) and by the data in Figures 5 and 6. Ritzén et al. (1974) incorporated radioactive steroids into the acrylamide to permit quantitation of binding proteins with rapid rates of association and dissociation, but they recognized that this procedure is inapplicable to most steroid–receptor complexes, which dissociate very slowly.

Agar gel electrophoresis has been used to fractionate steroid-binding components of target organs and serum (Wagner, 1970; Wagner et al., 1972). A major drawback of this technique is that free steroid, carried into the gel by electroendosmosis, may obscure the pattern of bound steroid. Furthermore, the resolution among the binding components studied by Wagner et al. (1972) and in other applications of agar gels (Jungblut et al., 1971; Krieg et al., 1975) is inferior to that attainable in polyacrylamide gels.

Hansson et al. (1974) utilized 3.25% acrylamide gels containing 0.5% agarose (Dingman and Peacock, 1968) to distinguish testicular and epididymal receptors from an extracellular androgen-binding protein. If such composite gels were used for systematic electrophoresis, a variation in the chemical nature of the gel might accompany the variation in pore size. Studies such as those in Figure 8 have revealed that many proteins, including some receptors, undergo interactions with agarose in media of low ionic strength and high pH. Agar gels are notoriously subject to electroendosmotic effects and agarose is not free of the responsible charged groups (Ackers, 1970; Lăăs, 1972; Ghosh and Moss, 1974).

In conclusion, the procedure for quantitative polyacrylamide gel electrophoresis described in this report provides a convenient method for fractionating and characterizing steroid–receptors. Potential applications and extensions of this technique include the isolation of steroid-free receptors and

steroid–receptor complexes on a preparative scale and studies of the relationship between cytoplasmic and nuclear receptors and the mechanism of receptor activation (Jensen and DeSombre, 1973).

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