# Multiple Forms of Oviduct Progesterone Receptors Analyzed by Ion Exchange Filtration and Gel Electrophoresis†

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ABSTRACT: Resolution of the multiple forms of steroid receptors in small samples has been improved by two new techniques: preparative ion exchange filtration and electrophoresis in highly cross-linked polyacrylamide gels of varied concentration. These techniques were used in conjunction with protamine precipitation, gel filtration, and density gradient centrifugation to separate five forms of the progesterone receptor of chick oviduct cytosol. These complexes, numbered I to V in order of elution from agarose gel columns, have been characterized with respect to apparent molecular weight, shape, and relative net charge. Form I, which is eluted in the void volume after gel filtration of cytosol in hypotonic media, is heterodisperse with respect to sedimentation coefficient and electrophoretic mobility ($R_f$). Form I is converted to form III by KCl. Form II has the highest axial ratio and the highest $R_f$ at pH 10.2. This 4.2S complex can be extracted from DEAE filters, but not from protamine-precipitated cytosol, by 0.3 to 0.5 M KCl. Form III is slightly smaller (3.9S) and less asymmetric than form II. It is released from DEAE filters and protamine-precipitated cytosol by 0.15 M KCl and displays increased $R_f$ upon purification. Forms II and III correspond to the B and A components described by W. T. Schrader and B. W. O'Malley ((1972), *J. Biol. Chem. 247,* 51). Form IV may result from the proteolytic cleavage of forms II and/or III. Form V is a globular polypeptide obtained in the presence of certain divalent cations. This complex has been named the "mero-receptor" since it is the smallest part or fragment of the receptor that contains the steroid-binding site.

The progesterone-binding activity of chick oviduct cytoplasmic extract (cytosol) has been fractionated into several distinct components by a variety of techniques. Agarose gel filtration of [$^3$H]progesterone-labeled cytosol in the presence of 0.3 M KCl revealed a partially resolved double peak of bound steroid (Sherman et al., 1970). These complexes were resolved into components A and B by sequential elution from DEAE-cellulose[1] columns with KCl (Schrader and O'Malley, 1972) and have reportedly been purified to homogeneity (Schrader et al., 1974, 1975b; Kuhn et al., 1975). Electrophoresis of unfractionated cytosol in highly cross-linked polyacrylamide gels at pH 10.2 resolved a high mobility complex, that may be the B component of Schrader and O'Malley (1972), from a heterodisperse slow peak (Miller et al., 1975). Electrophoresis of KCl extracts of protamine-precipitated cytosol revealed a discrete low mobility complex that may correspond to the A component of Schrader and O'Malley (1972). Finally, Sherman et al. (1974) obtained a receptor form with a molecular weight of about 20 000 by extracting receptors precipitated by protamine sulfate or by isoelectric focusing with $CaCl_2$. As the latter form retained all of the steroid-binding properties of the untreated complex, it was identified as the steroid-binding "subunit" or fragment of the receptor and is now designated the "mero-receptor".[2]

The objective of the present study was to clarify the relationships among the various receptor forms fractionated by ion exchange chromatography, extraction from protamine-precipitated cytosol with KCl or $CaCl_2$, density gradient centrifugation, and electrophoresis at pH 10.2. For the ion exchange studies, we adapted the technique of receptor assay by adsorption to DEAE-coated filters (Santi et al., 1973; Baxter et al., 1975) to a small-scale preparative method in which receptor forms are sequentially eluted from the filters by buffers of increasing salt concentration (Sherman and Miller, 1975).

## Materials and Methods

All procedures were carried out at 0 to 4 °C with the materials and equipment described by Sherman et al. (1974) or Miller et al. (1975), unless otherwise indicated.

*Cytosol and Protamine Extracts.* Rhode Island Red chicks were injected subcutaneously daily with 5 mg of diethylstilbestrol in sesame oil. After 15 days, the oviducts were removed and homogenized with a Polytron PT20 (Brinkmann) in 5 ml/g of 10 mM Tes,[1] 12 mM thioglycerol, pH 7.4 (Tes–thioglycerol), containing 0.25 M sucrose. The cytosol obtained by centrifugation for 1 h at 105 000*g* contained about 18 mg of protein/ml, by the method of Waddell (1956) with bovine serum albumin as the standard. The cytosol was incubated for 3 h with $4 \times 10^{-8}$ M [1,2,6,7-$^3$H]progesterone (105 Ci/mmol, New England Nuclear) and stored at −80 °C for up to 6 months. More than 90% of the bound progesterone, as measured by charcoal–dextran assay (see below), was precipitated from the cytosol by dropwise addition of protamine sulfate (7.5 mg/ml) to a final concentration of 0.8 mg/ml. The pellets were collected by centrifugation for 15 min at 2500*g*, washed twice with Tes–thioglycerol, and extracted for 1 h into Tes–thioglycerol containing 0.15 or 0.5 M KCl ± 5 mg

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[1] Abbreviations used are: Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; DEAE, diethylaminoethyl; Bis, *N,N'*-methylenebisacrylamide.

[2] The term *mero,* from the Greek for part or fraction, is used here to denote the smallest form of the receptor that contains the steroid-binding site (Sherman et al., 1974).

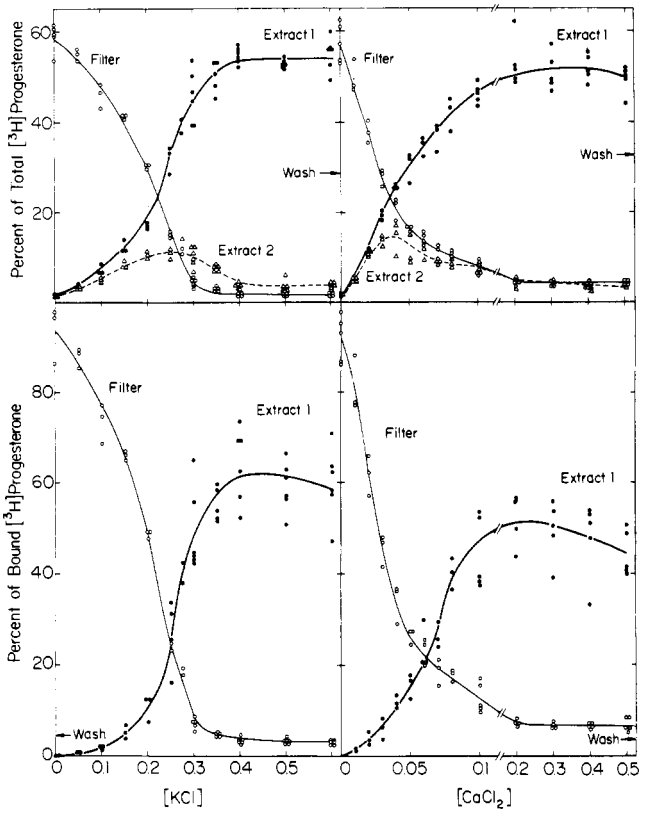


FIGURE 1: Salt-dependent elution of total and bound [$^3$H]progesterone from ion exchange filters. Labeled cytosol (100 $\mu$l) was adsorbed to DE-81 filters which were washed to remove free steroid and extracted twice with 1 ml of Tes-thioglycerol containing 5 mg of ovalbumin/ml and the indicated molar salt concentration. Aliquots of the cytosol, wash, and extracts were treated with charcoal-dextran. (Top) Total cpm in the pooled washes (Wash), the first (●) and second (△) extracts and on the filter (○) are expressed relative to the total cpm in the sample. (Bottom) The cpm on the filter and the charcoal-resistant cpm in the washes and first extract are expressed relative to the charcoal-resistant cpm in the sample.

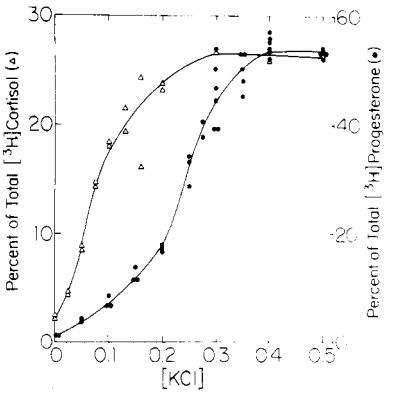


FIGURE 2: Elution of corticosteroid-binding globulin and progesterone receptors from ion exchange filters. Aliquots (100 $\mu$l) of diluted chick serum labeled with $1.5 \times 10^{-8}$ M [$^3$H]cortisol (△) or oviduct cytosol labeled with $4.5 \times 10^{-8}$ M [$^3$H]progesterone (●) were adsorbed to separate filters. Prior to extraction, the washed filters retained 27 and 59% of the labeled steroids, respectively. Each symbol represents the percent of the total steroid in the sample recovered in the first 1 ml of extract with Tes-thioglycerol containing 5 mg of ovalbumin/ml and the indicated KCl concentration.

of ovalbumin/ml, brought up to the initial volume of cytosol. For preparation of the labeled mero-receptor, the KCl was replaced by 0.02 to 0.1 M $CaCl_2$ (Sherman et al., 1974).

*Charcoal-Dextran Treatment.* Labeled cytosol was shaken for 20 min with one-fourth the volume of 2.5% Norit A, 0.25% Dextran T-40 in Tes-thioglycerol, centrifuged 15 min at 1000*g*, and an aliquot of the supernatant fluid was assayed for tritium content. The charcoal-dextran suspensions were supplemented with 2% ovalbumin and either 0.5 M KCl or 0.1 M $CaCl_2$ when used to assay bound steroid in DEAE filter extracts (see below).

*Preparative Ion Exchange Filtration.* Chick serum diluted with 3 volumes of Tes-thioglycerol was labeled with [$^3$H]cortisol and oviduct cytosol with [$^3$H]progesterone. Aliquots (100 $\mu$l) were adsorbed to DEAE-coated filters (Whatman DE-81) in a manifold as described by Miller et al. (1975). In preliminary experiments, adsorption of steroid-receptor complexes to a single filter was proportional to the cytosol load, up to at least four 100-$\mu$l aliquots, with five 1-ml washes after each addition. To reduce the background of free steroid on the filters to 1.5% of the total or less, filters used to isolate [$^3$H]progesterone complexes were washed 5 times, and filters used for [$^3$H]cortisol complexes were washed 12 times with 1 ml of Tes-thioglycerol. For the experiments shown in Figures 1 and 2, replicate filters were extracted twice for 8 min with 1 ml of Tes-thioglycerol containing 5 mg of ovalbumin/ml and various concentrations of KCl or $CaCl_2$; a portion of each extract was treated with charcoal-dextran. The results for the total steroid in the filter extracts were expressed as the percent of the total steroid in the sample. The results for the charcoal-resistant or bound steroid in the extracts were expressed as the percent of the total bound steroid in the sample. As less than 2% of the free steroid remained on the washed filter, all of the radioactivity on the filter was assumed to be protein bound. Sequential extractions of complexes from DEAE filters with solutions of increasing KCl concentration were analyzed by electrophoresis, gel filtration, and density gradient centrifugation.

*Electrophoresis.* Polyacrylamide gel electrophoresis was performed in the Tris-glycine-HCl buffer system of Davis (1964), as modified by Miller et al. (1975), in cylindrical 3-ml (110 × 6 mm) separation gels without stacking gels. The total concentration ($T$) of acrylamide monomer plus the cross-linking agent *N,N'*-methylenebisacrylamide (Bis) was varied from 5 to 11% (w/v). The degree of cross-linking ($C$) was 15%, i.e Bis/monomer = 15:85. All buffers and contained gels 10% (v/v) glycerol and the samples were underlayered with 5 $\mu$mol of Tris-thioglycolate. The chloride-thioglycolate boundary, detected by immersing the gel in $AgNO_3$, was the reference for the calculation of relative mobility ($R_f$) (Rodbard and Chrambach, 1971). The composition of the operative buffer in the separation gel during electrophoresis was computed to be 0.368 M Tris, 48.3 mM glycine, pH 10.2 at 0 °C using the program of Jovin (1973). This Tris-glycine buffer is referred to as the separation phase buffer.

*Density Gradient Centrifugation.* Samples were centrifuged at 0 °C through 10 to 35% (w/v) glycerol gradients for 45 h at 227 000*g* (48 000 rpm in a Spinco SW-56 rotor). The linear dependence of migration distance on sedimentation coefficient under these conditions was verified with horse heart cytochrome *c*, 1.95S (Stellwagen, 1968), ovalbumin, 3.53S (Castellino and Barker, 1968), and human hemoglobin, 4.42S (Schumaker and Schachman, 1957), as standards (Martin and Ames, 1961). The significance of differences between the sedimentation coefficients of the various receptor forms was evaluated by the Student's *t* Test.

*Gel Filtration.* One column of Agarose A 0.5m (42 × 1.27 cm) was equilibrated with the separation phase buffer containing 10% (v/v) glycerol and another (87 × 1.27 cm) with Tes–thioglycerol containing 0.4 M KCl and 10% (w/v) glycerol (high salt buffer). The total liquid volume ($V_t$) was marked by L-[$^{14}$C]valine (260 mCi/mmol, Schwarz/Mann) or by [$^{14}$C]urea (55 mCi/mmol, Schwarz/Mann). Blue Dextran 2000 (Pharmacia) marked the void volume ($V_0$) in experiments with standard proteins but was omitted in the presence of receptors. The distribution coefficient ($K_D$) of each binding component and standard was calculated from its elution volume ($V_e$) as usual: $K_D = (V_e - V_0)/(V_t - V_0)$ (Gelotte, 1960).

Since few standard proteins of high molecular weight are stable in the high salt buffer, covalently linked polymers of bovine serum albumin were prepared by a modification of the method of Wolf et al. (1970). Two microliters of diethyl oxydiformate (Eastman) were added to 1 ml of bovine serum albumin (10 mg/ml) at room temperature, mixed vigorously on a Vortex, dialyzed overnight at 4 °C against the high salt buffer, and centrifuged 15 min at 2500*g*. Under these conditions, a satisfactory yield of the dimer, but not of the higher polymers, of albumin was obtained. Stokes radii ($R_s$) and apparent molecular weights ($M$) of the smaller receptor forms were calculated by interpolation of linear correlations of log $R_S$ with $K_D$ and $M^{1/2}$ with $K_D^{1/3}$ for myoglobin, ovalbumin, human serum albumin, and bovine serum albumin monomer and dimer in the high salt buffer (Sherman, 1975). The molecular parameters of the largest receptor form, that was eluted before the albumin dimer, were estimated from short extrapolations of the standard curves. Elution volumes from the column equilibrated with the separation phase buffer were not proportional to molecular radius or weight, presumably because of charge-dependent interactions with the agarose (Miller et al., 1975).

## Results and Discussion

*Nomenclature.* To facilitate the description of the various forms of the chick oviduct progesterone receptor, we have numbered them I to V in order of decreasing molecular size as measured by gel filtration. Thus, form I is a highly aggregated complex, detectable in the void volume of a column of Agarose A 0.5m when crude cytosol is filtered in buffers of low ionic strength. Forms II and III are the major complexes detectable in untreated cytosol in buffers containing high concentrations of monovalent cations (e.g., 0.4 M $K^+$). As shown below, forms II and III correspond to components B and A of Schrader and O'Malley (1972), respectively. Forms III and V are the major complexes in KCl and $CaCl_2$ extracts of protamine-precipitated cytosol, respectively; IV is a minor constituent of both. Form V is the mero-receptor obtained by treatment with certain divalent cations, e.g., $Ca^{2+}$ (Sherman et al., 1974).

*Elution of Steroid-Binding Components from Ion Exchange Filters.* Aliquots of cytosol incubated with 4.2 × $10^{-8}$ M [$^3$H]progesterone ± 4 × $10^{-6}$ M unlabeled progesterone were treated with charcoal–dextran or adsorbed to DE-81 filters, washed, and extracted as described in Materials and Methods. The charcoal-resistant steroid in samples labeled in the absence and presence of competitor indicated the total and nonspecific binding, respectively, and the difference was identified as the amount specifically bound. As shown by Miller et al. (1975, Table II), both the total bound and specifically bound progesterone were retained by the washed filters in good yield (about 87%).

Figure 1 (top) shows the progressive elution of [$^3$H]progesterone from the filters by solutions of increasing KCl or $CaCl_2$ concentration. The yield of steroid in the extract varied with salt concentration and was maximal at 0.4 to 0.6 M KCl or 0.2 to 0.5 M $CaCl_2$. At lower salt concentrations, the yield was increased by a second extraction. The maximum recovery of [$^3$H]progesterone in the combined extracts was comparable to the amount initially retained by the filters. Since half-maximal recovery of [$^3$H]progesterone in the combined extracts was obtained with about 0.2 M KCl or 0.025 M $CaCl_2$, elution was not simply a function of ionic strength. Of the free steroid in the labeled cytosol, i.e., that removed by charcoal–dextran treatment of parallel samples, more than 80% was recovered in the pooled filter washes. The average recovery of total steroid in these experiments was 88%.

At each salt concentration, the yield of steroid–receptor complexes, assayed by the charcoal–dextran method, was proportionate to the total steroid in the extract (cf. Figure 1, top and bottom). A comparison of the amount of bound [$^3$H]progesterone in the high salt extracts with the bound steroid in the samples indicated that about 60% of the complexes were recovered from the filters with KCl, and about 50% with $CaCl_2$. These yields were sufficient to permit physical chemical analyses of the receptors in the extracts.

Ion exchange filters have been used recently for the quantitation of individual steroid-binding components (Santi et al., 1973; Baxter et al., 1975; Rosner, 1975). To evaluate the capacity of DE-81 filters to fractionate mixtures, we compared the elution profiles of [$^3$H]progesterone–cytosol complexes and [$^3$H]cortisol–serum complexes (presumably labeled corticosteroid-binding globulin), as a function of KCl concentration. The results of this experiment (Figure 2) indicated the feasibility of differential elution of the constituents of a mixture of steroid-binding proteins. For example, about 80% of the serum complexes, but only 20% of the [$^3$H]progesterone–receptor complexes, were eluted by 0.15 M KCl.

Schrader and O'Malley (1972) adsorbed partially purified oviduct [$^3$H]progesterone–receptor complexes to DEAE-cellulose columns and sequentially eluted two components with 0.15 and 0.3 M KCl, labeled A and B, respectively. The experiments described below were performed to test the applicability of DEAE filters to the rapid fractionation of different forms of the progesterone receptor in small samples. The complexes eluted from the filters by various concentrations of KCl were compared with those characterized previously in this laboratory, i.e., complexes I, II, III, and V (Sherman et al., 1974; Miller et al., 1975).

*Gel Filtration in High Salt Buffer.* A column of Agarose A 0.5m equilibrated with Tes–thioglycerol containing 0.4 M KCl and 10% (w/v) glycerol (high salt buffer) was used both analytically and preparatively. As shown in Figure 3a, and in earlier experiments with buffers of slightly different composition (Sherman et al., 1970; Sherman et al., 1974), most of the bound [$^3$H]progesterone in unfractionated cytosol was eluted as a partially resolved double peak (fractions 49–60). The separate components within this double peak were identified by electrophoretic and ultracentrifugal analyses of the indicated pooled fractions (II and III). Unlike unfractionated cytosol, sequential 0.15 M KCl (low salt) and 0.5 M KCl (high salt) extracts of cytosol adsorbed to DEAE filters each contained a single major receptor form (Figure 3b). From their elution volumes, these complexes

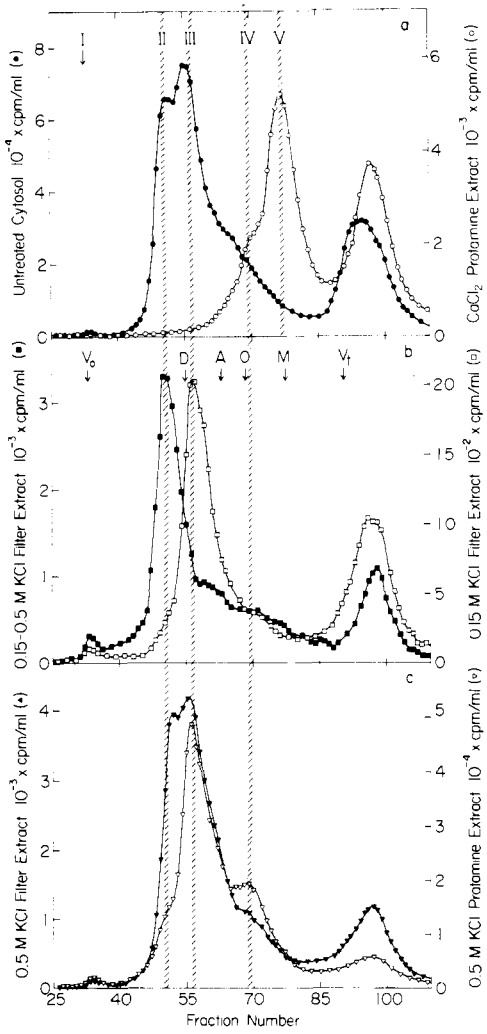


FIGURE 3: Resolution of several forms of the progesterone receptor by gel filtration. An Agarose A 0.5m column (1.27 × 88 cm) equilibrated with 0.4 M KCl, 10% (w/v) glycerol in Tes-thioglycerol was used at 4 °C. (a) Radioactivity was measured in aliquots of eluate from 0.8 ml of oviduct cytosol labeled with $4.9 \times 10^{-8}$ M [$^3$H]progesterone (●). The expected elution volume of receptor aggregates is indicated by I. Pools II and III (shaded) were analyzed by electrophoresis, ultracentrifugation, and gel filtration in Tris-glycine buffer (cf. Figures 4, 5, and 6). A 20 mM $CaCl_2$ extract of protamine-precipitated cytosol (0.37 ml; ○) contained the mero-receptor (V) and a larger complex (IV). (b) DEAE-coated filters, to which 0.1 or 0.12 ml of labeled cytosol was adsorbed, were washed with Tes-thioglycerol, extracted first with 0.5 ml, then with 1 ml of 0.15 M KCl in Tes-thioglycerol containing 5 mg of ovalbumin/ml and then with 0.5 ml of 0.5 M KCl in Tes-thioglycerol, 5 mg of ovalbumin/ml. The samples were 0.8 ml of the first 0.15 M KCl extract (□) and 0.8 ml of the subsequent 0.5 M KCl extract (■). The void volume ($V_0$) and total liquid volume ($V_t$) were marked by dextran blue and [$^{14}$C]valine, respectively. Standard proteins bovine serum albumin dimer (D) and monomer (A), ovalbumin (O), and myoglobin (M) were detected optically. (c) Labeled receptors were extracted from protamine precipitated cytosol or from DEAE filters with 0.5 M KCl in Tes-thioglycerol, 5 mg of ovalbumin/ml, without prior extraction with 0.15 M KCl. The samples were 0.55 ml of the filter extract (▼; 0.5 ml of extract/0.1 ml of cytosol) and 0.9 ml of the protamine extract (▽; 1 ml of extract/ml of cytosol). Pools III and IV of the protamine extract were analyzed by electrophoresis and ultracentrifugation (cf. Figure 6 and Tables I and III).

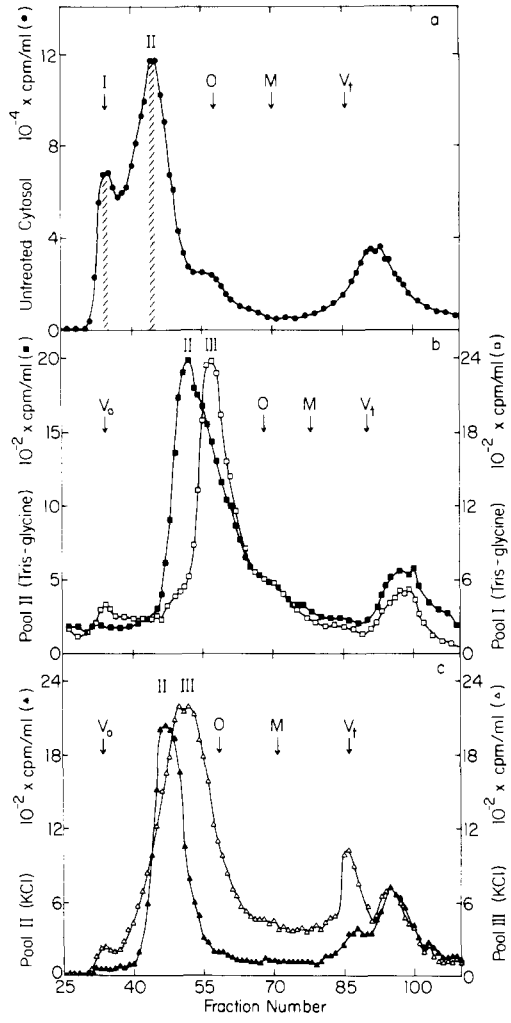


FIGURE 4: Relationships between receptor forms fractionated by gel filtration in different buffers. (a) Untreated cytosol (0.6 ml) was filtered on a 55-ml column of Agarose A 0.5m in the separation phase buffer of the electrophoretic system (0.368 M Tris, 48.3 mM glycine) containing 10% (v/v) glycerol. (b) The excluded peak (I; 0.95 ml) from the preparation shown in (a) and the included peak (II; 0.4 ml) from an analogous experiment were rechromatographed separately on the 110-ml agarose column equilibrated with high salt buffer [0.4 M KCl, 10% (w/v) glycerol in Tes-thioglycerol (pH 7.4)]. (c) Pools II (0.8 ml) and III (1 ml) from filtration of whole cytosol in high salt buffer (cf. Figure 3a) were rechromatographed separately in the Tris-glycine buffer. The apparent conversion of I to III on transfer from Tris-glycine to high salt buffer (a and b) was not reversible under these conditions. The void volume ($V_0$), total liquid volume ($V_t$) and elution positions of ovalbumin (O) and myoglobin (M) were different in the column equilibrated with Tris-glycine buffer (a and c) from those in the column equilibrated with high salt buffer (b).

appeared to be the same as the smaller and larger complexes, i.e., III and II, in unfractionated cytosol. In a previous study, the agarose gel filtration patterns of forms II (B) and III (A) obtained from DEAE columns and of unfractionated [$^3$H]progesterone receptors were indistinguishable (Schrader and O'Malley, 1972; Figure 2). The present results indicate clear resolution of forms II and III on agarose (Figure 3b).

Figure 3c shows the elution profiles for receptors extracted by 0.5 M KCl from protamine-precipitated cytosol and from DEAE filters (without prior extraction with 0.15 M KCl). The high salt filter extract apparently contained both forms II and III that were also detected in whole cytosol (cf. Figures 3a and 3c). Gel filtration of the high salt protamine extract, like the low salt filter extract, revealed only form III and a minor constituent, form IV, that co-chromatographed with ovalbumin.

Protamine-precipitated cytosol was extracted with 0.15 M KCl or with various concentrations of $CaCl_2$ and analyzed on Agarose A 0.5m. The yield of [$^3$H]progesterone in

Table I: Physical–Chemical Parameters of the Partially Purified Receptor Forms and of the Standards used for Gel Filtration Analyses.

| Protein[a] | $s_{20,w}$ ± SEM ($n$)[b] (S) | $K_D$ ± SEM ($n$)[c] | $R_S$ ± SEM[d] (Å) | ($M \times 10^{-3}$) ± SEM[e] from $R_S \times s_{20,w}$ | ($M \times 10^{-3}$) ± SEM[f] from $M^{1/2}$ vs. $K_D^{1/3}$ |
|---|---|---|---|---|---|
| II (B) | 4.23 ± 0.03 (4) | 0.317 ± 0.007 (14) | 50.5 ± 1.1 | 93 ± 2 | 162 ± 4 |
| III (A) | 3.89 ± 0.04 (4) | 0.400 ± 0.003 (18) | 43.0 ± 0.8 | 73 ± 1 | 115 ± 3 |
| IV | 3.63 ± 0.05 (4) | 0.636 ± 0.004 (11) | 27.4 ± 0.5 | 43 ± 1 | 39 ± 2 |
| V (mero-receptor) | 2.94 ± 0.01 (3)<br>2.58 ± 0.02 (39)[g] | 0.778 ± 0.004 (5) | 20.8 ± 0.6 | 27 ± 1<br>23 ± 1 | 17 ± 1 |
| Bovine serum albumin | | | | | |
| Dimer | | 0.371 ± 0.009 (4) | 43.5[h] | | 134[h,i] |
| Monomer | | 0.518 ± 0.011 (4) | 36.3[h] | | 67[h,i] |
| Human serum albumin | | 0.510 ± 0.011 (4) | 35.1[j] | | 66[k] |
| Ovalbumin | | 0.621 ± 0.009 (25) | 28.0[l] | | 43[m] |
| Myoglobin | | 0.785 ± 0.008 (39) | 20.2[n] | | 17[o] |

[a] Alternate nomenclature for receptor forms is shown in brackets. [b] Samples were centrifuged for 45 h at 227 000$g$ through 10 to 35% glycerol gradients in Tris–glycine buffer, pH 10.2, with hemoglobin (4.42S) as an internal standard. The standard error of the mean (SEM) is shown for the indicated number of determinations ($n$). In two experiments, receptor form II was obtained as the 0.15 to 0.5 M KCl extract of DEAE filters. All other samples for ultracentrifugation were obtained by agarose filtration in high salt buffer (see below) of the following preparations: II, cytosol; III, cytosol or 0.5 M KCl protamine extract; IV, 0.1 M $CaCl_2$ protamine extract or 0.5 M KCl protamine extract; V, 0.02 or 0.1 M $CaCl_2$ protamine extract. Neither preparation of V was completely free of IV (cf. Figure 3a). [c] Distribution coefficients ($K_D$) were calculated as described in Materials and Methods from elution volumes on Agarose A 0.5m in high salt buffer [0.4 M KCl, 10% (w/v) glycerol, Tes–thioglycerol (pH 7.4)]. [d] Stokes radii ($R_S$) of receptors were calculated from the linear correlation of log $R_S$ vs. $K_D$ for the five standards. The values of SEM include the uncertainty of the standard curve as well as the variability between experiments. [e] Molecular weights ($M$) of the receptors were calculated from $R_S$ and $s_{20,w}$ (eq 1). The formula for the SEM of a product was from Kendall and Stuart (1969). [f] Values of $M$ for receptors were calculated from linear correlation of $M^{1/2}$ vs. $K_D^{1/3}$ for the five standards. [g] In analyses of form V in salt-containing gradients, the mean $s_{20,w}$ ± the standard deviation was 2.58 ± 0.11S (Sherman et al., 1974). [h] Loeb and Scheraga (1956). [i] Squire et al. (1968). [j] Oncley et al. (1947). [k] Heimburger et al. (1964). [l] Champagne (1950). [m] Castellino and Barker (1968). [n] Riveros-Moreno and Wittenberg (1972). [o] Weber and Osborn (1969).

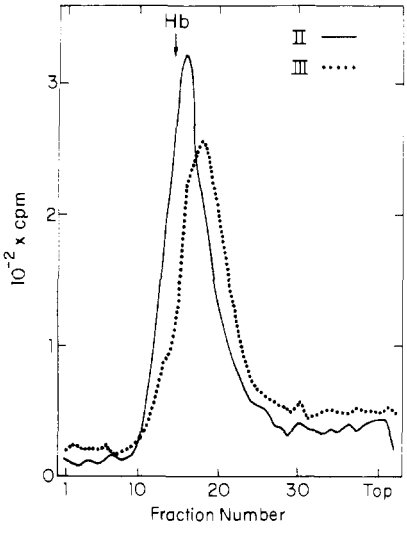


FIGURE 5: Density gradient centrifugation of receptor forms fractionated by gel filtration in high salt buffer. Aliquots of pools II (—) and III (···) from filtration of cytosol on agarose in 0.4 M KCl, 10% (w/v) glycerol, Tes-thioglycerol (Figure 3a) were centrifuged for 45 h at 227 000$g$ through 10 to 35% (w/v) glycerol gradients in Tris-glycine buffer, pH 10.2, with hemoglobin (Hb) as an internal standard.

the 0.15 M KCl extract of the protamine precipitate was only 30% of that in 0.5 M KCl extract, but the gel filtration pattern was essentially the same (cf. Figure 3c). In contrast, the bound [$^3$H]progesterone in the $CaCl_2$ extracts was associated primarily with complex V, that co-chromatographed with myoglobin (cf. Figure 3a and Sherman et al., 1974).

*Relationship of Forms I and III.* In conjunction with electrophoretic studies, cytosol receptors were fractionated by filtration on agarose in the separation phase buffer [0.368 M Tris, 48.3 mM glycine (pH 10.05 at 4 °C)] containing 10% (v/v) glycerol. Under these conditions, the double included peak of bound [$^3$H]progesterone seen in the high salt buffer (II and III, Figure 3a) was replaced by a peak at the void volume (I, Figure 4a) and a single included peak, labeled II. To determine the relationship between the complexes resolved by agarose in the two buffers, we applied pooled fractions from one column as the sample on the second column. As shown in Figures 4a and 4b, nearly all of the bound steroid in the excluded peak in Tris-glycine buffer was converted to form III in the presence of 0.4 M KCl. In the reverse experiment, there was no significant conversion of the complex in pool III from the high salt eluate (Figure 3a) to a form excluded from the gel in Tris-glycine buffer. Instead, most of the bound steroid was eluted in fractions 48 to 57 (Figure 4c). Similar elution patterns were obtained in the Tris-glycine buffer for pool II from the high salt column (Figure 4c) and for the 0.15 M KCl extracts of [$^3$H]progesterone complexes from DEAE filters.

We infer from these experiments that I is an aggregate of form III and other constituents of the cytosol, and that form III is liberated from this aggregate by ion exchange filtration or gel filtration in high salt buffer. These other constituents may be basic proteins or nucleic acids to which III (A) is known to bind (Schrader and O'Malley, 1972).

*Density Gradient Centrifugation.* It was of interest to determine if the receptor forms fractionated by ion exchange filtration and gel filtration were distinguishable by density gradient centrifugation. Miller et al. (1975) showed that forms I and II obtained by gel filtration in Tris-glycine buffer sedimented as a heterodisperse population (>8S) and as a distinct 4.2S peak, respectively. In the present study, forms II–V prepared by ion exchange filtration, protamine precipitation, and/or gel filtration were compared during prolonged centrifugation with a closely sedimenting internal standard, to increase the precision of the method. The results in Figure 5 and Table I show small but significant ($p < 0.01$) differences in the sedimentation coefficients of these components. Nevertheless, a mixture of forms II and III would have sedimented as a single "4S"

Table II: Friction Factors and Axial Ratios of Receptor Forms.

| Receptor Form[a] | $(f/f_0)_{shape}$[b] | Axial Ratio[c] Prolate | Oblate |
|---|---|---|---|
| II (B) | 1.55 | 10 | 12 |
| III (A) | 1.43 | 8 | 10 |
| IV | 1.09 | 3 | 3 |
| V (Mero-receptor) | 0.96 | 1 | 1 |
| V[d] | 1.02 | 1 | 1 |

[a] Alternate nomenclature is shown in brackets. [b] Calculated from eq 2 with values of $R_S$ and M (from $R_S$ and $s_{20,w}$) from Table I. [c] For each value of $(f/f_0)_{shape}$, the corresponding axial ratios for prolate and oblate ellipsoids of revolution were obtained from Schachman (1959). [d] Values based on $s_{20,w}$ = 2.58S (Sherman et al., 1974).

peak under these conditions. The preparations of V (the mero-receptor) used in these studies contained the larger form, IV (see Figure 3a). This, and/or the change from a neutral high salt buffer to an alkaline buffer of low ionic strength, may explain the discrepancy between the value of 2.94 ± 0.01S (Table I) and that reported previously for the mero-receptor, 2.58 ± 0.02S (Sherman et al., 1974).

*Stokes Radii, Molecular Weights, and Axial Ratios of Receptor Forms II–V.* The distribution coefficient ($K_D$) on gel filtration provides a more reliable estimate of the Stokes radius ($R_S$) than of the molecular weight ($M$) of the receptors (Sherman, 1975). The results for both parameters of receptor forms II–V are shown in Table I. The value of $R_S$ = 50.5 Å for form II is in good agreement with the value of 47.7 Å calculated from the electrophoretic retardation coefficient ($K_R$) of the fast component (Miller et al., 1975). The $R_S$ of 20.8 Å for form V is the same as that obtained by Sherman et al. (1974) in more extensive gel filtration analyses. The apparent $M$ of 162 000 for form II, calculated from the correlation of $M^{1/2}$ with $K_D{}^{1/3}$, is close to that obtained from a correlation of equivalent molecular radius (a function of $M^{1/3}$) with $K_R{}^{1/2}$ (Miller et al., 1975). Since both of these correlations are based on the assumption that the unknown and standard proteins have similar axial ratios, they may result in overestimates of $M$ for highly asymmetric proteins.

An alternative estimate of $M$, which avoids this assumption, is obtained by combining $R_S$ and the sedimentation coefficient ($s$) with an estimate of the partial specific volume ($\bar{v}$):

$$M = \left[\frac{6\pi\eta N}{1 - \bar{v}\rho}\right] sR_S = 435sR_S \qquad (1)$$

where the solvent viscosity ($\eta$) is 0.01 poise, the solvent density ($\rho$) is 1.00 g cm$^{-3}$, $\bar{v}$ is assumed to be 0.74 cm$^3$ g$^{-1}$, $N$ is Avogadro's number, $R_S$ is in angstroms and $s$, in svedberg units ($10^{-13}$ s), refers to 20 °C and water ($s_{20,w}$). In the case of the mero-receptor, the estimate of $M$ obtained from $M^{1/2}$ vs. $K_D{}^{1/3}$ is presumably more reliable than that calculated from $R_S$ and $s$ since this receptor form was incompletely resolved from larger contaminants by ultracentrifugation. The agreement between the two estimates of $M$ for form IV in Table I indicates that this complex is similar in shape to the globular standards. In contrast, the size and the direction of the discrepancy between the two estimates of $M$ for forms II and III are consistent with a high degree of asymmetry (see below). Schrader et al. (1974) obtained molecular weights of 117 000 and 110 000 for forms II and III, respectively, from the electrophoretic mobilities of the highly purified components in the presence of a detergent, sodium dodecyl sulfate. Their results, which are intermediate between our two estimates based on hydrodynamic parameters, involve the assumption that the receptors and standard proteins bind the detergent in proportion to $M$.

The degree of asymmetry of receptor forms II–V was evaluated in terms of $(f/f_0)_{shape}$, the frictional ratio due to shape, defined by:

$$\left(\frac{f}{f_0}\right)_{shape} = R_S\left[\frac{4\pi N}{3M(\bar{v} + \delta/\rho)}\right]^{1/3} = 1.390 \times 10^8 \left(\frac{R_S}{M^{1/3}}\right) \qquad (2)$$

where it was assumed that $\bar{v}$ = 0.74 cm$^3$g$^{-1}$, the degree of solvation $\delta$ = 0.2 g per g of protein, and $R_S$ was in angstroms (Sherman et al., 1970). Table II shows the results for $(f/f_0)_{shape}$ and the corresponding axial ratios (Schachman, 1959). These results confirm that forms IV and V are globular, in contrast to the highly asymmetric forms II and III. The dramatic fall in axial ratio (from about ten to one) when the larger units are converted to the mero-receptor may indicate the cleavage of an asymmetric portion of the molecule from a globular region containing the steroid-binding site. In this respect, the receptor would resemble myosin, in which the actin-binding site and ATPase activity reside in the globular portion of heavy meromyosin (Mueller and Perry, 1962).

*Electrophoresis of Receptors Fractionated by Ion Exchange and Gel Filtration.* The labeled complexes in some receptor preparations (the protamine and low salt DEAE filter extracts) were not "stacked" or concentrated into a sharp zone in the upper gel of the multiphasic system described by Miller et al. (1975). Therefore, to permit analyses of all receptor forms under the same conditions, no stacking gels were used in this study. Electrophoresis was performed in highly cross-linked (15% $C$) separation gels of several total acrylamide concentrations (5, 8, and 11% $T$ or 5, 7, 9, and 11% $T$) with human hemoglobin and bovine serum albumin–bromophenol blue complex as visible standards in every gel. The radioactivity detected in the gels after electrophoresis of labeled cytosol under these conditions accounted for about 88% of the bound [$^3$H]progesterone, as measured by the charcoal-dextran assay (Miller et al., 1975).

The results for the various receptor preparations in 5% $T$ gels are compared in Figure 6. The distribution of labeled steroid in cytosol was characterized by a sharp peak of relatively high mobility (the cytosol fast form, $R_f$ 0.45 ± 0.01) and a heterodisperse slow peak ($R_f$ 0.2 to 0.35), that varied in shape among different preparations and with protein load (Figure 6a). Electrophoresis of pool II from gel filtration of cytosol in high salt buffer (Figure 3a) revealed the cytosol fast form, nearly free of the slow components (Figure 6b). Pool III from the same column contained a component of lower mobility ($R_f$ 0.30 ± 0.01). On the basis of these results, the electrophoretic components characterized by $R_f$'s of about 0.45 and 0.30 under these conditions were tentatively identified as receptor forms II and III, respectively. The lower $R_f$ of form III, in combination with its smaller size as measured by gel filtration, indicated a lower net (negative) charge/surface area at the operative pH in these gels (see Rodbard and Chrambach, 1971).

The electrophoretic patterns of the complexes eluted from DEAE filters by different salt solutions are compared

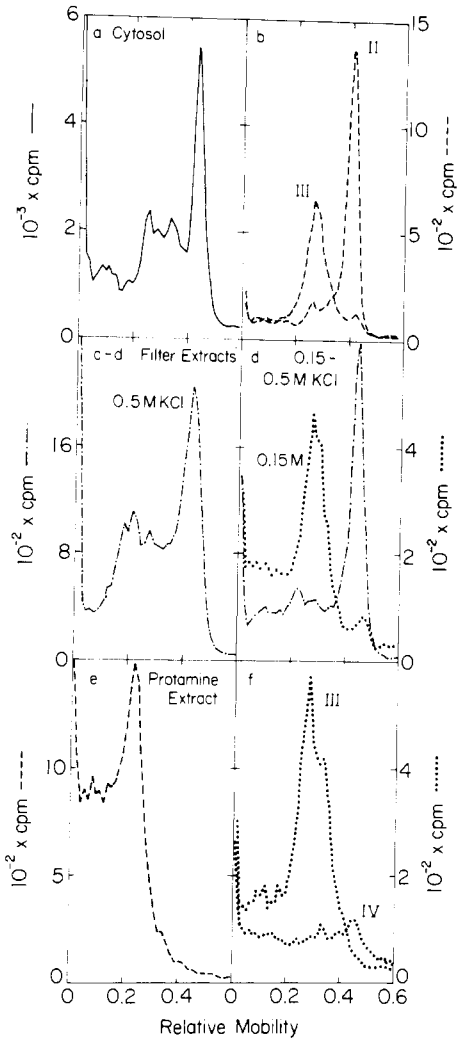


FIGURE 6: Electrophoretic identification of receptor forms in whole and fractionated cytosol. Each sample was analyzed in triplicate 110-mm gels containing 5, 7, 8, 9, or 11% total acrylamide ($T$); data are shown for 5% $T$ gels. (a) [$^3$H]Progesterone-labeled cytosol, 40 $\mu$l; (b) separate analyses of pools II and III from gel filtration of cytosol on agarose in high salt buffer (cf. Figure 3a), 150 $\mu$l; (c) 0.5 M KCl extract of cytosol adsorbed to DE-81 filters, 150 $\mu$l; (d) separate analyses of 0.15 M KCl filter extract and 0.5 M KCl filter extract following 0.15 M KCl extraction (cf. Figure 3b), 200 $\mu$l; (e) 0.5 M KCl extract of protamine-precipitated cytosol, 50 $\mu$l; (f) separate analyses of pools III and IV from agarose filtration of 0.5 M KCl protamine extract in high salt buffer (cf. Figure 3c), 200 $\mu$l.

in Figures 6c and 6d. The 0.5 M KCl extract, without prior low salt extraction, appears to contain a mixture of complexes similar to those in untreated cytosol (cf. Figure 6a). This result is consistent with the gel filtration data (Figures 3a and 3c). The electrophoretic patterns for the 0.15 M KCl DEAE filter extract and the subsequent 0.5 M KCl extract, by contrast, each contain a single major peak of radioactivity (Figure 6d). The 0.15 to 0.5 M KCl extract of the DEAE filters appears to contain the same high mobility complex ($R_f$ 0.44 ± 0.01) as pool II from gel filtration (cf. Figure 6b). The mobility of the complex in the low salt DEAE filter extract ($R_f$ 0.24 ± 0.02) was slightly lower than that of pool III from gel filtration ($p < 0.1$).

These electrophoretic analyses and the gel filtration patterns in Figure 3b demonstrate that stepwise elution from DEAE filters efficiently fractionates the two major receptor forms in crude cytosol. The larger complex (form II) is extracted from DEAE filters only by high concentrations of KCl (>0.3 M). Therefore, it is identified as the B component of Schrader and O'Malley (1972). The interactions of form II with DEAE and protamine (see below) in neutral buffers, and its high electrophoretic mobility in alkaline buffer, imply a high net negative charge. Since the smaller complex, III, is extracted from DEAE filters by 0.15 M KCl, it is identified as the A component of Schrader and O'Malley (1972). Its low $R_f$ at pH 10.2 and ease of extraction from DEAE and protamine (see below) are consistent with a low net negative charge.

*Electrophoresis of Receptors Fractionated by Protamine Precipitation.* Electrophoretic analysis was also used to characterize the KCl and $CaCl_2$ extracts of protamine-precipitated cytosol, fractions of these extracts obtained by gel filtration in high salt buffer, and the complexes remaining in the supernatant after protamine precipitation. As shown in Figure 6e, the 0.5 M KCl protamine extract contained a complex of low mobility ($R_f$ 0.24 ± 0.01) and a trail of radioactivity between the complex and the top of the gel. The absence of high mobility complex was consistent with the gel filtration patterns for the protamine extract, which revealed a single major complex (III) in place of the double peak in whole cytosol (Figure 3c). When gel filtration fractions from the protamine extract were electrophoresed (Figure 6f), more than one low mobility complex was apparent in pool III, while a barely detectable complex in pool IV migrated with the mobility of the cytosol fast component ($R_f$ ~0.45). The majority of the complexes in pool IV, that apparently failed to enter the gel, are presumably less acidic than those in pool III.

The 0.1 M $CaCl_2$ extract of protamine-precipitated cytosol and pools IV and V from gel filtration of this extract (cf. Figure 3a) were analyzed similarly (results not shown). In the whole extract, a small peak of radioactivity at $R_f$ ~0.22 was unresolved from a series of more slowly migrating complexes, presumably aggregates. No labeled complexes were detected by electrophoresis of the gel filtration fractions of this extract. The failure to detect the mero-receptor (form V) in this electrophoretic system was interpreted in conjunction with other experimental data: (1) The mero-receptor was stable during prolonged centrifugation in the separation phase buffer (Table I). (2) It was also stable during filtration on agarose in the separation phase buffer, but was eluted later than expected from its elution volume in high salt buffer and sedimentation coefficient. Such retardation on agarose in an alkaline buffer was previously noted for cytochrome $c$, a basic monomeric protein (Miller et al., 1975; Figure 8). (3) In isoelectric focusing experiments under the conditions used by Sherman et al. (1974), the mero-receptor had an apparent isoelectric point of greater than 8. (4) The mero-receptor was released from protamine precipitates by solutions of relatively low ionic strength (e.g. 20 mM $CaCl_2$, Figure 3a).

This ensemble of data is consistent with the hypothesis that the fragment of the receptor that contains the steroid-binding site is a basic polypeptide. As such, it should be studied in an electrophoretic system with the reverse polarity from that used here. Since receptor forms II, III, and V apparently contain the same steroid-binding moiety (Sherman et al., 1974), the more acidic portions of the larger units must be lost in the formation of V.

The high mobility receptor form, II, was essentially absent from KCl and $CaCl_2$ protamine extracts as analyzed by either agarose gel filtration or electrophoresis (Figures

Table III: Relative Mobilities ($R_f$'s) of Receptor Forms in 15% *C*, 5% *T* Gels.[a]

| Preparation | Form II (B) | Form III (A) |
|---|---|---|
| Cytosol | 0.45 ± 0.01 (3) | |
| DEAE filter extracts | | |
| 0.5 M KCl | 0.43 ± 0.01 (3) | |
| 0.15–0.5 M KCl | 0.44 ± 0.01 (2) | |
| 0.15 M KCl[b] | | 0.24 ± 0.02 (4) |
| Protamine precipitation | | |
| Supernatant[b] | | 0.21 ± 0.01 (3) |
| 0.5 M KCl extract[b] | | 0.24 ± 0.01 (2) |
| Gel filtration | | |
| Cytosol | 0.44 ± 0.01 (6) | 0.30 ± 0.01 (6) |
| Protamine extract | | 0.29 ± 0.01 (2) |

[a] Samples were electrophoresed in a Tris–glycine–HCl buffer system with a Tris–thioglycolate underlayer; mobilities of [$^3$H]progesterone complexes were measured relative to the chloride–thioglycolate boundary (see Materials and Methods). Mean values of $R_f$ ± SEM are shown for the number of gels in parentheses. Alternate nomenclature for receptor forms is shown in brackets. [b] Complexes with $R_f < 0.27$ under these conditions are designated as form III* (cf. Figure 8).

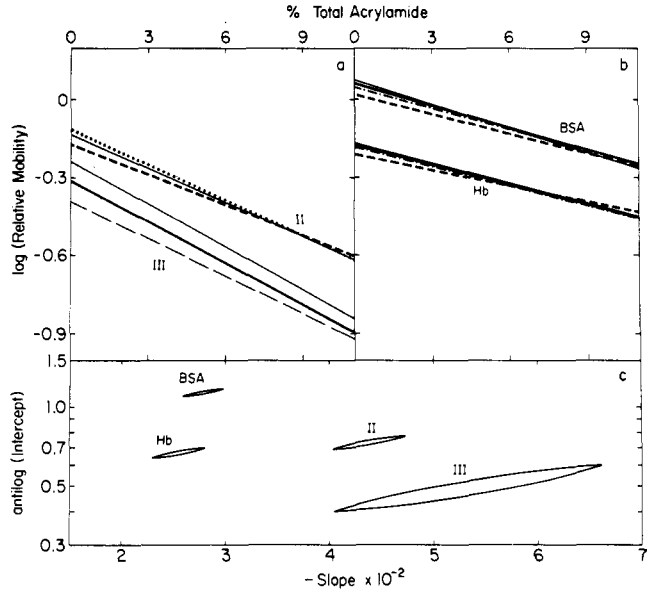


FIGURE 7: Relative mobilities ($R_f$'s) of various receptor forms and standard proteins as a function of total acrylamide concentration (*T*). Weighted linear regressions of log $R_f$ on *T* (Ferguson plots) and the joint 95% confidence limits of the slopes and intercepts of these plots were computed from data in 5–11% *T* gels with the programs of D. Rodbard. (a) Ferguson plots for receptor form II were calculated from combined data for the high mobility complex in untreated cytosol (Figure 6a) and in KCl-treated cytosol (- - -), from data for pool II from filtration of cytosol on agarose in high salt buffer (—; Figure 6b) and from combined data for 0.5 M KCl extracts of cytosol adsorbed to DEAE-coated filters, with or without prior low salt extraction (• • •, Figures 6c and 6d). Ferguson plots for the lower mobility receptor forms (labeled III) were calculated from data for the 0.15 M KCl filter extract (— —; Figure 6d), for the 0.5 M KCl extract of protamine precipitated cytosol (▬; Figure 6e) and for pool III from agarose filtration of cytosol in high salt buffer (—; Figure 6b). (b) Ferguson plots are shown for bovine serum albumin (BSA) and human hemoglobin (Hb) which were used as internal standards in the receptor experiments shown in (a). Data for each standard run with low and high salt filter extracts of cytosol were combined (–•–•–). (c) The joint confidence limits of the slopes (–retardation coefficient, $K_R$) and intercepts (log $Y_0$) of the Ferguson plots were computed from the combined data from all studies of receptor forms II and III and the standard proteins.

3a, 3c, and 6e). These findings suggested that either form II was not precipitated by the polyamine, or it was precipitated but not resolubilized from the pellet by the salt solutions tested. The gel pattern of the protamine supernatant, which resembled that of the KCl protamine extract (Figure 6e), favored the second interpretation.

*Mobilities of the Major Receptor Forms as a Function of Total Gel Concentration (T).* As shown in Table III, there were no consistent differences in the $R_f$'s in 5% *T* gels of the high mobility complex (form II) in various preparations: untreated cytosol, KCl-treated cytosol, 0.15 to 0.5 M KCl DEAE filter extract, 0.5 M KCl DEAE filter extract, pool II from agarose filtration in high salt buffer, or the included peak from agarose filtration in the separation phase buffer. Moreover, the $R_f$ values in gels of systematically varied concentration (5 to 11% *T*) were as consistent for receptor form II in these various preparations as for bovine serum albumin and hemoglobin analyzed in the same gels. These results are summarized as graphs of log $R_f$ vs. *T* (Ferguson plots) in Figures 7a and 7b (Ferguson, 1964; Rodbard and Chrambach, 1971). The joint 95% confidence limits for the slopes and intercepts of the Ferguson plots for the combined data for each component (in all preparations) are shown in Figure 7c (Rodbard and Chrambach, 1974; Miller et al., 1975). The small size of the ellipse of the joint confidence limits for the charge and size parameters of the high mobility complex implies that the same receptor form (II) was obtained in all of the preparations listed above.

In contrast, both the $R_f$ and the shape of the low mobility peak (form III) depended on the method of preparation. For example, the complex in pool III from agarose filtration of whole cytosol (5.9 pmol of [$^3$H]progesterone/mg of protein) had an $R_f$ of 0.30 ± 0.01, compared with 0.24 ± 0.02 for the low salt DEAE filter extract[3] (0.6 pmol of [$^3$H]progesterone/mg of protein), $p < 0.1$. When data from the 5% *T* gels were pooled according to the specific activity of the receptor, the difference between the $R_f$'s in the more highly purified preparations (i.e., fractions from agarose columns ± protamine precipitation, $R_f$ 0.30 ± 0.01) and those of higher protein content (i.e., whole protamine extracts and 0.15 M KCl extracts of DEAE filters, $R_f$ 0.24 ± 0.01) was significant at the level of $p < 0.05$. The impression that the mobility of the slow component varied among preparations was confirmed by analyses of $R_f$ as a function of gel concentration. Thus, the Ferguson plots for complex III obtained in the low salt DEAE filter extract, the protamine extract, and by agarose gel filtration were not superimposable (Figure 7a), although the joint 95% confidence limits for the slopes and intercepts overlapped to some extent. As shown in Figure 7c, the confidence ellipse for the combined data was much larger than for the other proteins.

There are several possible interpretations of these findings: (1) The same receptor form is present in each of the preparations but its low net charge prevents reproducible $R_f$ determinations. (2) Form III may exist in several low mobility variants. (3) A single form is present, but its apparent mobility in some preparations is altered by association with adventitious proteins, protamine, or DEAE from the filter. (4) The receptor is cleaved by proteolytic enzymes in some preparations.

The first interpretation was investigated by experiments with mixed preparations, one of which is shown in Figure 8.

[3] To permit the evaluation of the concentration of endogenous proteins in the DEAE filter extracts, ovalbumin was omitted from the extracting solutions in this experiment.

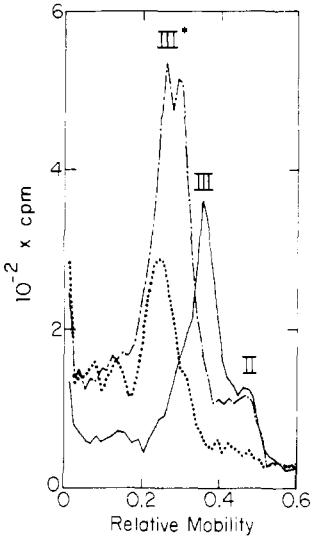


FIGURE 8. Discrimination between the low mobility receptor forms prepared by gel filtration (III) and by ion exchange filtration (III*). Samples containing 100 μl of pool III from agarose filtration of whole cytosol in high salt buffer (—; cf. Figures 3a, 6b) or 100 μl of 0.15 M KCl extract of cytosol from DEAE-coated filters (• • •; cf. Figures 3b, 6d) or a mixture of 100 μl each of pool III and filter extract (– – –) were electrophoresed on quadruplicate 5% *T*, 15% *C* gels. A small amount of the fast component characteristic of pool II from gel filtration was detectable in pool III. In the mixed samples, III was apparently converted to III*.

In this experiment, 100- and 200-μl aliquots of pool III from gel filtration (Figure 3a) and 0.15 M KCl extracts of cytosol from DEAE filters (0.5 ml of extract/0.1 ml of cytosol on filter) were analyzed separately and in 1:1 mixtures. Of the total radioactivity placed on the gels shown in Figure 8, the recoveries were greater than 80% in each case. The mobilities of the complexes in triplicate analyses of the two preparations were noticeably different: the major complex in pool III had a mean $R_f$ of 0.37, compared with 0.25 for the DEAE filter extract. Most striking in these results was the apparent conversion of the complex in pool III to the lower mobility form (labeled III*) when mixed with the filter extract ($R_f$ 0.27).

This experiment clearly showed that at least two low mobility forms of the progesterone receptor (III and III*) can be distinguished. It also provided insight into the variable and heterodisperse patterns observed for the low mobility components of whole cytosol. Thus, the slow peak in Figure 6a may encompass forms III and III*, as well as form I (cf. Miller et al., 1975).

*Relationship of the Electrophoretic Components to the 6S and 8S Receptors.* Schrader et al. (1975a) reported the preparation of a 6S form of the progesterone receptor. This complex was described as a dimer of the A and B components, although the elution pattern from DEAE-cellulose with KCl was indistinguishable from that of the B component alone. Of the receptor forms characterized in our laboratory, forms I and III* were the most likely candidates for such a complex. If either form corresponded to the 6S dimer, gel filtration in 0.4 M KCl should have revealed an equal mixture of forms II (B) and III (A). As shown in Figures 3b and 4b, however, neither the low salt filter extract (form III*) nor the complex excluded from agarose in Tris–glycine buffer (form I) yielded a significant amount of form II. The sedimentation coefficients of the remaining forms described in this report (Table I) excluded their identification with the 6S dimer. On the other hand, the 8S receptor detected in our first analyses of this system (Sherman et al., 1970) may be among the low mobility components in unfractionated cytosol (Figure 6a). The macromolecular composition of the 8S complex has not yet been determined, but it may include nonreceptor components.

## Conclusions

By a variety of physical-chemical techniques, the progesterone receptor of chick oviduct cytosol has been detected in at least five forms. Two of these, forms I and III*, may be produced by the aggregation of form III to other macromolecules in hypotonic media. Studies are in progress concerning the molecular mechanism of the conversion of III to III* and its possible relationship to the activation of cytoplasmic receptors. Forms IV and V (the mero-receptor) may be the products of proteolytic enzymes to which the native receptor is not exposed in the intact cell. Studies of all available forms of the receptor are relevant to understanding its regulatory function. For example, we have separated the globular mero-receptor from a more acidic, asymmetric portion of the molecule and established the role of the mero-receptor in steroid binding (Sherman et al., 1974). The asymmetric moiety must be isolated next and its possible role in the interaction of the receptor with chromatin investigated.

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## References

Baxter, J. D., Santi, D. V., and Rousseau, G. G. (1975), *Methods Enzymol. 36,* 234.

Castellino, F. J., and Barker, R. (1968), *Biochemistry 7,* 2207.

Champagne, M. (1950), *J. Chim. Phys. Phys.-Chim. Biol. 47,* 693.

Davis, B. J. (1964), *Ann. N.Y. Acad. Sci. 121,* 404.

Ferguson, K. A. (1964), *Metabolism 13,* 985.

Gelotte, B. J. (1960), *J. Chromatogr. 3,* 330.

Heimberger, N., Heide, K., Haupt, H., and Schultze, H. E. (1964), *Clin. Chim. Acta 10,* 293.

Jovin, T. M. (1973), *Ann. N.Y. Acad. Sci. 209,* 477.

Kendall, M. G., and Stuart, A. (1969), The Advanced Theory of Statistics, Vol. 1, 3rd ed, New York, N.Y., Hafner Publishing Co., p 245.

Kuhn, R. W., Schrader, W. T., Smith, R. G., and O'Malley, B. W. (1975), *J. Biol. Chem. 250,* 4220.

Loeb, G. I., and Scheraga, H. A. (1956), *J. Phys. Chem. 60,* 1633.

Martin, R. G., and Ames, B. N. (1961), *J. Biol. Chem. 236,* 1372.

Miller, L. K., Diaz, S. C., and Sherman, M. R. (1975), *Biochemistry 14,* 4433.

Mueller, H., and Perry, S. V. (1962), *Biochem. J. 85,* 431.

Oncley, J. L., Scatchard, G., and Brown, A. (1947), *J. Phys. Chem. 51,* 184.

Riveros-Moreno, V., and Wittenberg, J. B. (1972), *J. Biol. Chem. 247,* 895.

Rodbard, D., and Chrambach, A. (1971), *Anal. Biochem. 40,* 95.

Rodbard, D., and Chrambach, A. (1974), in Electrophoresis and Isoelectric Focusing on Polyacrylamide Gel, Allen, R. C., and Maurer, H. R., Ed., Berlin, Walter de

Gruyter & Co., p 28.
Rosner, W. (1975), *Anal. Biochem. 67,* 422.
Santi, D. V., Sibley, C. H., Perriard, E. R., Tomkins, G. M., and Baxter, J. D. (1973), *Biochemistry 12,* 2412.
Schachman, H. K. (1959), Ultracentrifugation in Biochemistry, New York, N.Y., Academic Press, p 239.
Schrader, W. T., Buller, R. E., Kuhn, R. W., and O'Malley, B. W. (1974), *J. Steroid Biochem. 5,* 989.
Schrader, W. T., Heuer, S. S., and O'Malley, B. W. (1975a), *Biol. Reprod. 12,* 134.
Schrader, W. T., Kuhn, R. W., Heuer, S. S., and O'Malley, B. W. (1975b), Abstracts of the Endocrine Society 57th Annual Meeting, New York, N.Y., p 332.
Schrader, W. T., and O'Malley, B. W. (1972), *J. Biol. Chem. 247,* 51.
Schumaker, V. N., and Schachman, H. K. (1957), *Biochim. Biophys. Acta 23,* 628.
Sherman, M. R. (1975), *Methods Enzymol. 36,* 211.
Sherman, M. R., Atienza, S. B. P., Shansky, J. R., and Hoffman, L. M. (1974), *J. Biol. Chem. 249,* 5351.
Sherman, M. R., Corvol, P. L., and O'Malley, B. W. (1970), *J. Biol. Chem. 245,* 6085.
Sherman, M. R., and Miller, L. K. (1975), *Physiologist 18,* 387.
Squire, P. G., Moser, P., and O'Konski, C. T. (1968), *Biochemistry 7,* 4261.
Stellwagen, E. (1968), *Biochemistry 7,* 2893.
Waddell, W. J. (1956), *J. Lab. Clin. Med. 48,* 311.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244,* 4406.
Wolf, B., Michelin Lausarot, P., Lesnaw, J. A., and Reichmann, M. E. (1970), *Biochim. Biophys. Acta 200,* 180.

# Lipoteichoic Acid from *Bacillus licheniformis* 6346 MH-1. Comparative Studies on the Lipid Portion of the Lipoteichoic Acid and the Membrane Glycolipid†

David Button* and Norman L. Hemmings

ABSTRACT: A lipoteichoic acid and a membrane glycolipid were isolated from *Bacillus licheniformis* 6346 MH-1. The fatty acid composition of the two preparations were similar. Most of the fatty acids were of the branched chain type. The glycolipid was shown to be a diacyl derivative of *O*-β-D-glucopyranosyl-(1→6)-*O*-β-D-glucopyranosyl-(1→3)-glycerol. The lipoteichoic acid contained lipid, polyglycerol phosphate, and glucosamine. The lipid was released by treatment with hydrofluoric acid and by hydrolysis in dilute acid and was shown to have a structure identical with that of the membrane glycolipid.

1,2-diglycerides, substituted with either monosaccharide or oligosaccharide residues, are components of the membranes of gram-positive bacteria (Shaw, 1970). Diglycosyl diglycerides comprise a major class of glycolipids and have taxonomic significance, in that the disaccharide residues, which are contained in the glycolipids of member organisms of a given genus, are identical (Shaw and Baddiley, 1968). The sugar components of glycolipids frequently occur as structural units of polymers, that are located in the cell envelope. For example, in *Micrococcus lysodeikticus,* the same dimannosyl residue occurs in both glycolipid and membrane polysaccharide (Lennarz and Talamo, 1966) and the glycolipid and capsular polysaccharide of *Pneumococcus* type XIV contain D-galactopyranosyl-(1→2)-*O*-α-D-glucopyranosyl residues (Kaufman et al., 1965; Barker et al., 1961).

Lipoteichoic acids from *Lactobacillus fermenti* and *Streptococcus faecalis* NCIB 8191 consist of membrane teichoic acid, covalently linked to lipid. In *L. fermenti,* the lipid is identical with the membrane glycolipid (Wicken and Knox, 1970). In *S. faecalis,* the lipid moiety in the lipoteichoic acid is phosphatidylkojiobiosyl diglyceride (Ganfield and Pieringer, 1975). This lipid is a minor component of the membrane lipids and is a derivative of the principal glycolipid of *S. faecalis,* kojiobiosyl diglyceride (Fischer et al., 1973; Brundish et al., 1966).

Structural information on lipoteichoic acids from other organisms is required before the extent of the relationship between membrane lipid and lipoteichoic acid lipid can be assessed. However, chromatographic evidence has been presented, which indicates that, in both *Bacillus subtilis Marburg* strain 168 and *Micrococcus sp.* 24, the lipid portion of the lipoteichoic acid differs from either of the respective membrane glycolipid or a phosphatidyl derivative of the glycolipid (Coley et al., 1972).

In the following communication, a partial structure for the lipoteichoic acid from *B. licheniformis* 6346 MH-1 is reported, and comparative studies of the membrane glycolipid and the lipid portion of lipoteichoic acid are described.

## Materials and Methods

*Materials.* Alkaline phosphatase (EC 3.1.3.1), hexokinase (EC 2.7.1.1), glucose-6-phosphate dehydrogenase (EC 1.1.1.49), glycerokinase (EC 2.7.1.30), and glycerolphosphate dehydrogenase (EC 1.1.1.8) were obtained from the Boehringer Corporation (London) Ltd. α-Glucosidase (EC 3.2.1.20) and β-glucosidase (EC 3.2.1.21) were obtained

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