Molecular Weights of Estrogen and Androgen Binding Proteins in the Liver of *Xenopus laevis*†

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ABSTRACT: [^{3}H]Estradiol-17β and [^{3}H]dihydrotestosterone binding proteins in the cytosol fraction of liver from both male and female *Xenopus laevis* were characterized by electrophoresis on polyacrylamide gels. These binding proteins, which were indistinguishable based upon their mobilities on gels of different acrylamide concentrations, migrated as single components with a molecular weight of 2.0×10^4. Separation of native or sodium dodecyl sulfate denatured specific estrogen-binding components on dodecyl sulfate free acrylamide gels gave similar results, i.e., a single species of molecular weight $2.0\text{–}2.5 \times 10^4$. The same molecular weight also was obtained when cytosol was prepared in the presence of either diisopropyl fluorophosphate or phenylmethanesulfonyl fluoride, protease inhibitors. Evidence that the liver components binding either [^{3}H]estradiol-17β or [^{3}H]dihydrotestosterone were not plasma contaminants was provided by the observation that the plasma sex-steroid binding globulin of *Xenopus* had a different mobility when separated by polyacrylamide gel electrophoresis.

Administration of estrogens to male or female *Xenopus laevis* led to the appearance of the yolk protein, vitellogenin, in blood (Wallace and Bergink, 1974). Vitellogenin, the precursor of the yolk proteins, lipovitellin and phosvitin (Bergink and Wallace, 1974), was synthesized in the liver (Wallace and Jared, 1969; Wittliff and Kenney, 1972). Induction of yolk protein synthesis was preceded by an increase in the rate of synthesis of "pulse-labeled" RNA in liver which was uridylate rich (Wittliff et al., 1972). Further evidence of a requisite for RNA synthesis was shown since administration of actinomycin D within 6 hr after estrogen treatment inhibited vitellogenin induction (Wittliff et al., 1972). The influence of estradiol-17β[1] on vitellogenin synthesis in vitro using liver explants from estrogen-primed frogs has been shown recently, further supporting the idea that estradiol-17β interacts directly with the liver (Wallace and Bergink, 1974).

Currently it is accepted that the primary event following the interaction of a steroid hormone with a target cell is the formation of a steroid-binding protein complex (Jensen and DeSombre, 1972). Specific estrogen-binding sites have been observed in the liver of several yolk-producing vertebrates. Arias and Warren (1971) reported the presence of an estrogen-binding macromolecule in the cytosol fraction of chicken liver. Using the same system, the chicken liver, Mêstér and Baulieu (1972) were unable to detect a receptor for estradiol in cytosol, but demonstrated in vitro specific estrogen binding of high affinity by nuclear components. Direct binding in vitro of [^{3}H]estradiol-17β to sites in nuclei of chicken liver has been reported also by Lebeau et al. (1973) and by Gschwendt and Kittstein (1973). Additionally there is disagreement in the literature concerning the presence of high affinity binding sites for estradiol-17β in the cytosol of frog liver. Ozon and Bellé (1973) found estrophilic components only in the nuclear fraction of the liver of the amphibian, *Discoglossus pictus*. However, we reported recently the specific binding of [^{3}H]estradiol-17β to a macromolecular fraction from the cytosol of liver from *Xenopus laevis* which was excluded by gel filtration on Sephadex G-200 using low ionic strength buffers and which sedimented at 11–12 S on linear sucrose gradients (Wittliff and Zelson, 1974). Additionally, we demonstrated the presence of specific [^{3}H]dihydrotestosterone[2] binding components in the cytosol of *Xenopus* liver which have similar properties to those of the estrogen-binding proteins (Zelson and Wittliff, 1974a,b).

In this paper we describe a polyacrylamide gel electrophoresis procedure for the separation of the cytoplasmic components of *Xenopus* liver which bind either [^{3}H]estradiol-17β or [^{3}H]dihydrotestosterone specifically. Using this method we determined that the molecular weights of these entities were $\sim 2.0 \times 10^4$.

Materials and Methods

Chemicals and Reagents. Unlabeled steroids were obtained from Calbiochem while bovine serum albumin, Tris (Trizma base), chymotrypsinogen, pepsin, ovalbumin, diisopropyl fluorophosphate, diethyl pyrocarbonate, and phenylmethanesulfonyl fluoride were purchased from Sigma Chemical Co. Sheep prolactin was supplied without charge by the National Institute of Arthritis, Metabolism and Digestive Diseases of the National Institutes of Health. [2,4,6,7-^{3}H]Estradiol-17β (110 Ci/mmol), [1,2-^{3}H]-5α-dihydrotestosterone (44 Ci/mmol), and Omnifluor were purchased from New England Nuclear Corp. Triton X-100 for scintillation counting was bought from Research Products International Corp. Acrylamide (electrophoresis grade) and *N,N*^{1}-bisacrylamide were obtained from Eastman while *N,N,N*^{1},*N*^{1}-tetramethylethylenediamine and ammonium persulfate were purchased from Canalco. Purified sucrose, urea, and sodium dodecyl sulfate were from Schwarz/Mann.

†From the University of Rochester Cancer Center and Department of Biochemistry, The University of Rochester Medical Center, Rochester, New York 14642. *Received January 13, 1975.* A preliminary report of these studies was presented at the 59th annual meeting of the Federation of American Societies for Experimental Biology (Bergink and Wittliff, 1975). Supported in part by U.S. Public Health Service Grant CA-11198, E.W.B. is a Senior Research Fellow.

[1] Estra-1,3,5(10)-triene-3,17β-diol.

[2] 5α-Androstan-17β-ol-3-one.

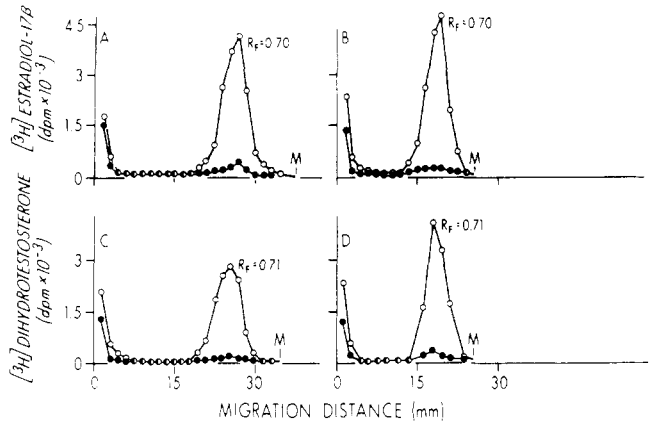

FIGURE 1: Separation of estrogen- and androgen-binding components from the liver by acrylamide gel electrophoresis. Cytosol was incubated 16 hr, 0–3°, with either [^{3}H]estradiol-17β (40 nM) or [^{3}H]dihydrotestosterone (50 nM) alone (○). A second set of reactions for each ^{3}H-labeled ligand was performed in the presence of 2 μM unlabeled steroid (●) and then these mixtures were layered onto gels of different acrylamide concentrations (A,C-5%; B,D-10%). Electrophoresis and fractionation were performed as described in the text. No dodecyl sulfate was used in either samples or gels in these experiments.

Animals. Xenopus laevis which were purchased from South African Snake Farm, Fish Hoek, South Africa, were kept in tanks provided with running charcoal-filtered tapwater and fed once a week with beef liver. Animals were put on ice 30 min prior to decapitation. Bleeding was performed by heart incision; 3–5 ml of blood was collected into ice-cooled polyethylene tubes containing 0.1 ml of heparin (1000 units/ml) solution. The plasma was separated by centrifugation and used immediately.

Preparation of Liver Cytosol. Livers from adult male or female *Xenopus* were excised and placed in ice-cold 0.12 M NaCl. Liver slices (1 mm) were made using a McIlwain tissue chopper (Brinkmann Instruments, Inc.) and stirred in cold Krebs-Ringer-Henseleit buffer (pH 7.3) for 30 min (Arias and Warren, 1971). The slices were blotted dry, weighed, and homogenized in four volumes of 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA and 0.25 M sucrose with a Duall glass homogenizer (Kontes Glass Co.) at 0–3°. The homogenates were centrifuged at 105,000g for 30 min to obtain a supernatant fraction (cytosol). The protein concentration of the cytosol was determined by the method of Lowry et al. (1951).

Labeling of Steroid-Binding Components. The cytosol (0.1 ml) was added immediately to glass vials (0.8 × 3 cm) containing steroid hormone previously dried down and the mixture incubated for 16 hr at 0°. Maximal binding for [^{3}H]estradiol-17β and [^{3}H]dihydrotestosterone was observed under these conditions (Wittliff and Zelson, 1974; Zelson, 1974). In some experiments the excess unbound steroid was removed following incubation by addition of 0.2 ml of dextran-coated charcoal suspension (0.5% Norit A, 0.05% dextran C in 10 mM Tris-HCl (pH 8.0) containing 1 mM EDTA and 0.25 M sucrose). With intermittent shaking, the vials were incubated at 0° for 10 min and then centrifuged at 700g, 10 min; the supernatant was applied immediately to acrylamide gels.

Polyacrylamide Gel Electrophoresis. Steroid-binding components were separated on gels (5.0 × 0.5 cm) of various concentrations ranging from 5 to 15% acrylamide using stock solutions of 24% acrylamide and 0.65% N,N^1-bismethyleneacrylamide. The concentration of the Tris-HCl buffer (pH 8.6) in the gel and electrode compartments was 0.29 M. Stacking gel was not used. Polymerization was accomplished using ammonium persulfate (0.06% final concentration) and tetramethylethylenediamine (0.08% final concentration). Prior to application of the labeled cytosol or plasma, 100 μl of sample was mixed with 40 μl of a solution containing 0.015% Bromophenol Blue in 50% aqueous glycerol; 40 μl of this mixture (200–400 μg of protein) was layered onto the gel and electrophoresis was carried out for 1.5 hr at 4° using a current of 4 mA/gel with an Ortec pulsed-power supply. Unless described otherwise, electrophoresis of samples containing 1% dodecyl sulfate was performed at room temperature in the absence of dodecyl sulfate in the gel and electrode compartment. The gel and buffer compositions were identical with those of the undenatured samples. Where described, marker proteins were dissolved in the same Tris-HCl buffer as the cytosols and separated on separate acrylamide gels of identical composition as those used for steroid-binding components. R_f values were expressed as the relative mobility with respect to the tracking dye.

Measurements of Radioactivity. Following electrophoresis, each gel was sliced into 1-mm discs and placed in glass scintillation vials. The radioactivity in the gel was extracted by incubation with 1 ml of 1% dodecyl sulfate solution for 4 hr at 70° (Senior, 1975); 9 ml of a toluene-base scintillation fluor (30% Triton X-100, 0.4% Omnifluor) was added to each vial and counted in liquid scintillation counter (Nuclear-Chicago Corp.).

Results

Separation of Steroid-Binding Components. When cytosol from *Xenopus* liver was incubated with either 40 nM [^{3}H]estradiol-17β or 50 nM [^{3}H]dihydrotestosterone and subjected to electrophoresis on polyacrylamide gels, in each case a single peak of bound radioactivity was observed (Figure 1). Under the conditions described both binding components separated with identical relative mobilities (R_f 0.7) on either 5 or 10% acrylamide gels. Apparently the binding of [^{3}H]estradiol-17β or [^{3}H]dihydrotestosterone was specific since incubation in the presence of excess unlabeled steroid resulted in a decrease in the amount of radioactivity found in the peak area. The amount of [^{3}H-labeled]ligand recovered in each peak on the gel (Figure 1A) represented >95% of the total amount of specifically bound steroid layered onto the gel initially. Therefore, no significant dissociation of either the estrogen-binding protein complex or dihydrotestosterone-binding protein complex occurred during electrophoresis. Furthermore, virtually all of the specifically bound steroid migrated into the gel as a single species. Earlier we reported that the estrogen-binding components were excluded by gel filtration on Sephadex G-100 or G-200 columns using 10 mM Tris-HCl buffer (pH 7.4) containing 1.5 mM EDTA (Wittliff and Zelson, 1974). Polyacrylamide gel electrophoresis (using 5% gel) of constituents in the excluded fractions separated with an R_f value of 0.7 also indicating a molecular weight of 2×10^4 (data not shown).

Concentrations of [^{3}H]estradiol-17β and [^{3}H]dihydrotestosterone which were unsaturating were chosen to diminish the amount of labeled steroid bound nonspecifically to sites of lower affinity. It was observed that the specific estrogen binding capacities of cytosol preparations used in this study varied considerably (0.3–2.5 pmol/mg of protein). Likewise, specific androgen binding capacity ranged from 1.5 to 3.5 pmol/mg of protein similar to that reported

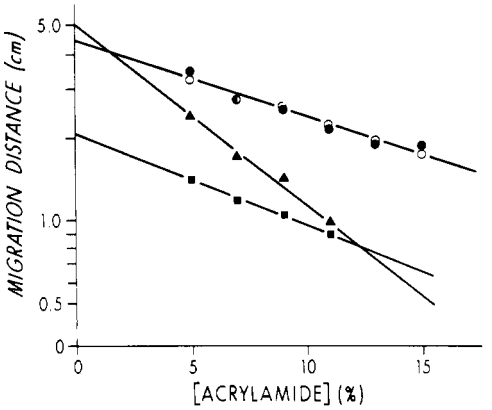

FIGURE 2: Ferguson plot of electrophoretic data showing the relationship between the logarithm of distance migrated by a protein and the acrylamide gel concentration. Liver cytosol was incubated with [^{3}H]estradiol-17β either at 0°, 16 hr (●) or at 25°, 1 hr (○) prior to electrophoresis. Also shown are the results of experiments using two marker proteins, prolactin (■) and bovine serum albumin monomer (▲), of known molecular weight.

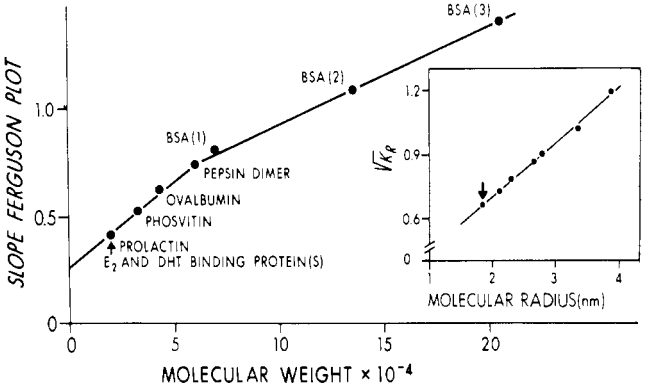

FIGURE 3: Estimation of the molecular weights of [^{3}H]estradiol-17β and [^{3}H]dihydrotestosterone binding proteins using results from Ferguson plots. The values of the retardation coefficients (K_R), estimated from the slopes of Ferguson plots, were determined for each of the marker proteins indicated on this graph using the analyses outlined in Figure 2. Note that the molecular weights of the ^{3}H-labeled steroid binding components were identical (arrow). A plot of the K_R vs. molecular radius as suggested by Rodbard and Chrambach (1971) yielded a straight line as shown in the insert.

by Zelson (1974).

Effective Molecular Radii. The mobility of the [^{3}H]estradiol-binding component was measured in gels of different acrylamide concentrations, each cross-linked to the same extent. According to Ferguson (1964) the logarithm of the electrophoretic mobility (M) is a linear function of the gel concentration (T) which may be expressed using the equation

$$\log M = \log M_0 - K_R T$$

The free electrophoretic mobility (M_0) is dependent upon the net charge and molecular radius of the molecule as well as the pH and ionic strength of the environment. The retardation coefficient (K_R) is a measure of the effective molecular surface area and, therefore, directly related to the radius of the unsolvated equivalent sphere. Both parameters are physical constants characteristic of each molecule in any one system (Rodbard and Chrambach, 1971). The results presented in Figure 2 demonstrate the experimental validity of this relationship. Although all of the marker proteins used in this study were examined by the method of Ferguson (1964) and gave linear relationships, only the results of analyses with prolactin and bovine serum albumin monomer are presented in Figure 2. The mobility of estrogen-binding protein complexes formed at 0° (16 hr) was compared with that of complexes made at 25° (1 hr). The latter incubation conditions are reported to bring about a temperature-dependent transformation of estrogen receptors in rat uterine cytosol resulting in an increase in molecular weight (Jensen et al., 1971: Notides and Nielsen, 1974). Plots of mobility vs. T for the tracking dye, Bromophenol Blue, gave identical slopes in each experiment, thus serving as an internal control. Extrapolation of the Ferguson plot yielded the same free electrophoretic mobility value for either binding component as for the bovine serum albumin monomer, dimer, or trimer, indicating the same charge per surface area for these proteins at pH 8.5. The retardation coefficients of estrogen-binding protein incubated either 1 hr at 25° or 16 hr at 0° were indistinguishable from that of prolactin indicating a similar molecular weight of 2.0×10^4. The relationship of the retardation coefficient vs. the molecular weight of marker proteins using the method of Hedrick and Smith (1968) was not linear below molecular weights of 60,000 (Figure 3). However, a linear relationship was obtained by plotting the square root of the retardation coefficient vs. the effective molecular radius (Figure 3, insert) as was suggested by Rodbard and Chrambach (1971). The observed molecular radius for the estrogen-binding proteins is 1.8 nm. This value corresponds to a molecular weight of 2.0×10^4 assuming a partial specific volume ($\bar{v}$) of 0.74.

Effects of Sodium Dodecyl Sulfate Treatment. Cytosol incubated either with [^{3}H]estradiol-17β (40 nM) or with [^{3}H]dihydrotestosterone (40 nM) was denatured in 1% dodecyl sulfate containing 10 mM mercaptoethanol for 5 min at 100° and analyzed for the presence of undissociated complex by electrophoresis on polyacrylamide gels (Figure 4). As described earlier each type of ligand-binding protein complexes migrated as a single peak with R_f 0.7.

The interaction between estradiol-17β and its binding site or between dihydrotestosterone and its binding site was sufficiently strong to withstand the denaturing effect of the dodecyl sulfate since there was no loss in steroid-binding capacity (Table I). However, if binding protein preparations were treated with 1% dodecyl sulfate prior to the loading of binding sites with either [^{3}H]estradiol-17β or [^{3}H]dihydrotestosterone, no formation of ligand-binding protein complexes was observed (Table I). It was observed that dodecyl sulfate treatment of ligand-binding protein complexes formed under different concentrations of [^{3}H]estradiol-17β did not alter the number of binding sites (data not shown).

Evidence that the binding of dihydrotestosterone influences the association of [^{3}H]estradiol-17β with its binding sites and vice versa is presented in Figure 4. Assurance of the specificity of binding was provided by the observation that following incubation of [^{3}H]estradiol-17β (40 nM) with a binding protein preparation in the presence of 4 μM diethylstilbesterol, little [^{3}H]estradiol-binding was observed (Figure 4A). A similar observation was made using [^{3}H]dihydrotestosterone (40 nM) as the ligand and unlabeled dihydrotestosterone (1 μM) as a competitor (Figure 4B). From our previous observation that dihydrotestosterone inhibited [^{3}H]estradiol-17β binding in *Xenopus* liver (Zelson and Wittliff, 1974b), separate reactions as described above were incubated either with 1 μM dihydrotestosterone (reactions containing 40 nM [^{3}H]estradiol-17β)

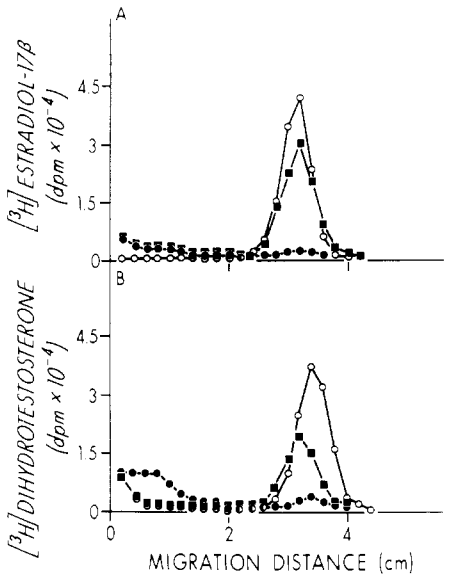

FIGURE 4: Electrophoretic mobility of steroid-binding proteins previously treated with 1% dodecyl sulfate. Cytosols were incubated with either [^{3}H]estradiol-17β (A) or [^{3}H]dihydrotestosterone (B) alone (○) and separated on 10% acrylamide gels. Shown in A, separate portions of cytosol were incubated with [^{3}H]estradiol-17β in the presence of either diethylstilbesterol (●) or dihydrotestosterone (■) and subjected to electrophoresis. In another set of reactions, cytosol was incubated with [^{3}H]dihydrotestosterone in the presence of either dihydrotestosterone (●) or estradiol-17β (■) and separated by electrophoresis (panel B). In all cases, following loading of the binding sites with ^{3}H-labeled steroid or competitor, the complexes were incubated with dodecyl sulfate prior to electrophoresis in dodecyl sulfate free gels as described in the text.

or with 1 μM estradiol-17β (reactions containing 40 nM [^{3}H]dihydrotestosterone) and subjected to electrophoresis. As seen in Figure 4A a 25-fold excess of dihydrotestosterone only partially inhibited the binding of [^{3}H]estradiol-17β to the component migrating at R_f 0.7. A 25-fold excess of estradiol-17β brought about >50% inhibition of [^{3}H]dihydrotestosterone binding (Figure 4B).

Electrophoresis of Unbound Steroid. It was observed that electrophoresis of dodecyl sulfate-treated cytosol containing steroid-loaded binding sites did not separate bound from unbound ^{3}H-labeled steroid when dodecyl sulfate was present in the gel system. This was due to the observation that, on dodecyl sulfate containing acrylamide gels, estradiol-17β migrated as a single peak with R_f 0.81 (Figure 5B). However, prior incubation of [^{3}H]estradiol-17β with dodecyl sulfate and electrophoresis on dodecyl sulfate free gels gave the radioactivity profile seen in Figure 5A. The radioactivity was equally distributed in the first 2 cm of the gel, suggesting that the estradiol–dodecyl sulfate micelles were unstable when subjected to electrophoresis on dodecyl sulfate free gels. Thus, it was necessary to first denature ligand-binding protein complexes with dodecyl sulfate and then separate on dodecyl sulfate free acrylamide gels. Protein–dodecyl sulfate complexes are stable under these conditions as has been reported by Stocklosa and Latz (1974).

Estimation of Molecular Weights of Denatured Steroid-Binding Proteins. The molecular weights of the dodecyl sulfate denatured steroid-binding proteins were determined by comparing their mobilities on 10% polyacrylamide gels (dodecyl sulfate free) with that of certain marker proteins. There was a linear relationship between the logarithm of the molecular weight of marker proteins (17,000–68,000) and their electrophoretic mobilities. The estimated molecular weight for either the [^{3}H]estradiol-binding protein or the [^{3}H]dihydrotestosterone binding protein of liver cytosol was ~25,000.

Table I: Stability of the Estrogen-Binding Protein in the Presence and Absence of Bound [^{3}H]Estradiol-17β.

Treatment	[^{3}H]Estradiol-17β Binding Capacity (% of Control)[a]	
	Binding Protein with Unoccupied Sites[b]	Binding Protein with Occupied Sites[c]
100°, 5 min	<1	100
100°, 5 min in 1% dodecyl sulfate	<1	96
20°, 5 min in 1 mM diethyl pyrocarbonate	5	94
20°, 5 min in 1 mM *p*-hydroxymercuribenzoate	5	103

[a] Liver cytosol (5 mg of protein/ml) either treated or untreated as described was incubated with 50 nM [^{3}H]estradiol-17β overnight. The [^{3}H]estradiol-binding capacity was estimated by polyacrylamide gel electrophoresis (see Materials and Methods). The binding capacities of the reactions in each treatment group were expressed as percent of the untreated cytosol (control). The specific binding capacity of this preparation was 2.5 pmol/mg of cytosol protein. [b] Treatments of preparations of binding protein were performed prior to incubation with [^{3}H]estradiol-17β overnight (3°) to measure residual binding capacity. [c] Binding sites were loaded with [^{3}H]estradiol-17β overnight at 3° before treatment was applied.

Effects of Modifying Agents on the Size and the Amount of Estradiol-Binding Components. A remarkable difference existed between the stability and reactivity of the binding protein from cytosol of *Xenopus* liver with steroid-loaded sites compared to the component with unoccupied binding sites. As shown earlier, treatment of cytosol containing complexes of binding protein and [^{3}H]estradiol-17β did not result in their dissociation when treated with dodecyl sulfate as was shown by electrophoresis on polyacrylamide gels (Table I). Furthermore, heating of the estrogen binding complexes at 100° for 5 min in the absence of dodecyl sulfate was ineffectual in dissocitating the complexes. However, all of the [^{3}H]estradiol-binding capacity was destroyed when the binding component with unoccupied sites was incubated at 100°, 5 min in the presence or absence of dodecyl sulfate (Table I).

Treatment of the estrogen-binding proteins from *Xenopus* liver with diethyl pyrocarbonate, a carbethoxylating agent (Wolf et al., 1970), also revealed that these complexes were more stable than the component with unoccupied sites (Table I). Similar results were obtained using *p*-hydroxymercuribenzoate, a substance known to react with sulfhydryl groups.

Notides et al. (1972) have shown that uterus contains a potent protease which may act upon the estrogen-binding protein degrading it to a lower molecular weight component. Diisopropyl fluorophosphate, an inhibitor of this protease activity (Notides et al., 1973), was used to test whether the estrogen-binding protein of 25,000 observed in *Xenopus* liver cytosol resulted from degradation of a larger molecular weight component. Liver cytosol was prepared by homogenization in 10 mM Tris-HCl buffer (pH 8.4) containing 1 mM EDTA and either 5 mM diisopropyl fluorophosphate or 10 mM phenylmethanesulfonyl fluoride, another inhibitor of "serine-type" proteolytic enzymes (Fahrney and Gold, 1963). Following preparation of the cy-

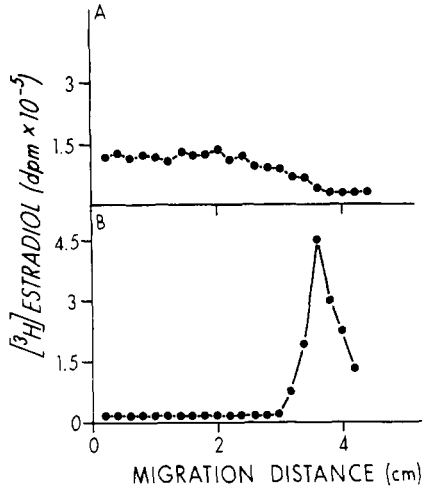

FIGURE 5: Electrophoretic mobility of unbound [^{3}H]estradiol-17β. Free [^{3}H]estradiol-17β was treated with 1% dodecyl sulfate as described in the text and separated on 10% acrylamide gels which were dodecyl sulfate free (A) or contained 0.1% dodecyl sulfate (B).

tosol, each was incubated with 40 nM [^{3}H]estradiol-17β and separated by electrophoresis on 10% gels. No change was observed in the apparent molecular weight of the estrogen binding protein prepared in a protease-inhibited environment (data not shown).

As another test of the molecular integrity of the [^{3}H]estradiol-binding complexes, the possibility of a time-dependent proteolysis of a higher molecular component to the 25,000 species was examined. As shown earlier, incubation of cytosol with [^{3}H]estradiol-17β for 16 hr, 0°, resulted in a binding component with R_f 0.7 (Figure 4A). Although binding ^{3}H-labeled ligand to a lesser extent, separation of complexes incubated only 30 min, 0°, gave a peak with the same R_f value indicating that the species binding [^{3}H]estradiol-17β throughout the time course was the 25,000 unit. However, simultaneous incubation of cytosol and [^{3}H]estradiol-17β with 8 mM $CaCl_2$ resulted in a 50% reduction of steroid-binding protein complexes when separated by gel electrophoresis. Notably there was no change in the molecular weight of the complexes formed in the presence of calcium ions.

Sex-Steroid Binding Capacity of Plasma. An important consideration of this study is that the binding of ^{3}H-labeled sex steroids observed may be due in part to association with sex-steroid binding globulin from plasma contamination of liver cytosol. It is known that certain nonmammalian vertebrates such as lamprey (Boffa et al., 1972), skate (Freeman and Idler, 1969), and newt (Ozon et al., 1971) contain these sex-steroid binding globulins.

Using the gel electrophoresis system, we demonstated that components capable of binding both [^{3}H]estradiol-17β and [^{3}H]dihydrotestosterone were present in *Xenopus laevis* plasma (Figure 6). Plasma from a female was diluted tenfold with 10 mM Tris-HCl buffer (pH 8.0) containing 5 mM $CaCl_2$ and 0.3 M glycerol and incubated for 16 hr at 0–3° with either 10 nM [^{3}H]estradiol-17β or [^{3}H]dihydrotestosterone. Calcium ions and glycerol were added to the buffer to retard inactivation of testosterone–estradiol binding globulin (Rosner et al., 1974). After electrophoresis on 10% acrylamide gels, a peak of bound radioactivity was observed with an R_f value of 0.18 using either ^{3}H-labeled steroid as the ligand (Figure 6A and B). Simultaneous incubation of [^{3}H]dihydrotestosterone with 1 μM unlabeled dihydrotestosterone completely displaced the bound radioactivity (Figure 6B). Likewise [^{3}H]dihydrotestosterone binding was displaced after incubation with 1 μM unlabeled estradiol-17β but not with 1 μM diethylstilbesterol (Figure 6C). This suggests that the ^{3}H-labeled steroid binding component in *Xenopus* plasma is testosterone–estradiol binding globulin based upon its ability to bind both dihydrotestosterone and estradiol-17β and its inability to bind diethylstilbesterol (Murphy, 1968; Soloff et al., 1971).

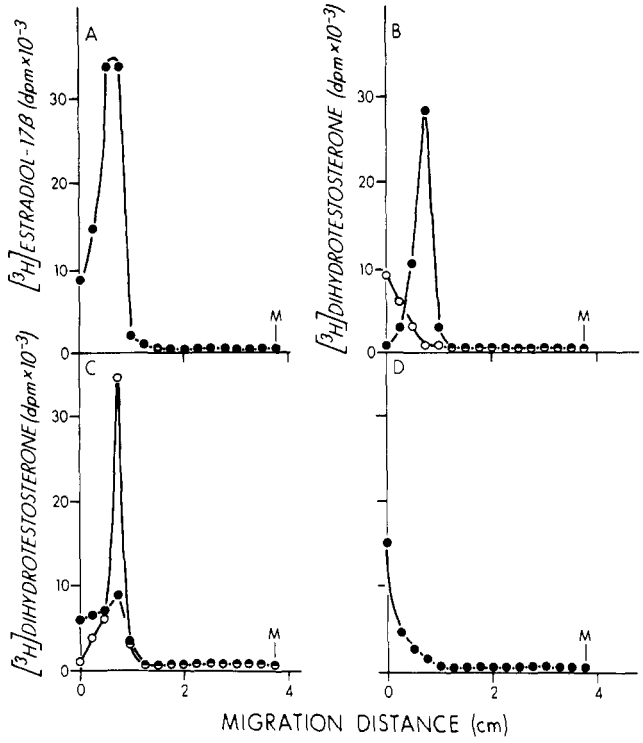

FIGURE 6: Electrophoretic mobility of plasma constituents binding ^{3}H-labeled steroids. Plasma was incubated with either [^{3}H]estradiol-17β (A) or [^{3}H]dihydrotestosterone (B) alone (●) and separated by gel electrophoresis as described in the text. Separate reactions containing [^{3}H]dihydrotestosterone were incubated 16 hr, 0° in the presence of either unlabeled dihydrotestosterone (B, ○), diethylstilbesterol (C, ○) or estradiol-17β (C, ●) and layered onto acrylamide gels. A final reaction was treated with 1% dodecyl sulfate after formation of [^{3}H]dihydrotestosterone-binding protein complexes and separated by gel electrophoresis (D).

To determine further the nature of the steroid binding complexes in plasma, these components were treated with 1% dodecyl sulfate after binding sites were occupied by [^{3}H]dihydrotestosterone. As seen in Figure 6D, no peak of bound radioactivity was observed after electrophoresis indicating dissociation of the complexes.

Discussion

Results from our earlier studes on the molecular properties of the estrogen-binding components in cytosol of *Xenopus* liver were perplexing. These components were excluded by Sephadex G-200 gel filtration using low ionic strength buffer (Wittliff and Zelson, 1974). However, separation on linear gradients of sucrose containing low salt concentrations gave two estrogen-binding species, one sedimenting at 11–12 S and the second sedimenting at <4 S (Wittliff and Zelson, 1974). To further discern the molecular nature of these binding components we studied their electrophoretic mobility on acrylamide gels.

Using acrylamide gel electrophoresis, we demonstrated a single type of estrogen-binding component in liver cytosol

with a molecular weight of 2×10^4. This entity bound [^{3}H]estradiol-17β specifically as indicated by inhibition of binding by unlabelled estradiol-17β and diethylstilbesterol which was reported earlier (Zelson and Wittliff, 1974a). Dihydrotestosterone partially inhibited [^{3}H]estradiol-17β binding by the 2×10^4 component similar to our previous observation (Zelson and Wittliff, 1974b). Treatment of the estrogen-binding component with dodecyl sulfate prior to loading of binding sites with [^{3}H]estradiol-17β completely inactivated it. However, if the binding sites were first loaded and then the complexes were treated with dodecyl sulfate and separated on dodecyl sulfate free acrylamide gels, no dissociation occurred indicating that the steroid ligand conferred stability upon the binding component. Separation of native or dodecyl sulfate denatured estrogen-binding components of dodecyl sulfate free acrylamide gels gave similar results, i.e., a single species of molecular weight $2.0–2.5 \times 10^4$. Evidence that the 2×10^4 component is not the result of proteolytic degradation was provided by the observation that the same molecular weight was obtained when cytosol was prepared in the presence of either diisopropyl fluorophosphate or phenylmethanesulfonyl fluoride, protease inhibitors. Thus it appears that the molecular weight of the protomer is $\sim 2 \times 10^4$ and that the larger molecular weight species reported earlier (Wittliff and Zelson, 1974) represents aggregates of these entities.

While investigating the characteristics of [^{3}H]estradiol-17β binding components in liver it was observed that there were also specific binding sites with high affinity for [^{3}H]dihydrotestosterone (Zelson and Wittliff, 1974b). We found that these binding components migrated as a single species on acrylamide gels with a mobility corresponding to a molecular weight of 2×10^4. The molecular weight was identical when dihydrotestosterone-binding complexes were treated with dodecyl sulfate and separated on dodecyl sulfate free gels. [^{3}H]Dihydrotestosterone binding by the 2×10^4 component was partially inhibited by estradiol-17β.

Since it is known that plasma of certain amphibia contain sex-steroid binding globulins (e.g., Ozon et al., 1971) similar to those of mammals (Anderson, 1974), it was possible that a portion of the binding of [^{3}H]estradiol-17β and [^{3}H]dihydrotestosterone was attributed to plasma contamination. However, this was not the case for the following reasons. Firstly, the mobility of sex-steroid binding globulin of *Xenopus* plasma was different from that of the liver components. Secondly, diethylstilbesterol was an excellent competitor for [^{3}H]estradiol-binding sites but had no effect on ^{3}H-labeled ligand binding by the plasma component. In mammals, Murphy (1968) has shown that diethylstilbesterol also did not inhibit ligand binding to sex-steroid binding globulin. Finally the liver estrogen-binding component remained intact after exposure to denaturing agents such as dodecyl sulfate while the plasma component dissociated readily.

The cytosolic components of liver binding either [^{3}H]estradiol-17β or [^{3}H]dihydrotestosterone gave indistinguishable Ferguson plots when analyzed on acrylamide gels in 0.3 *M* Tris-HCl buffer (pH 8.5). Even though these binding components have similar molecular weights, i.e., 2×10^4, there are several arguments that the estrogen and androgen-binding sites reside on separate molecules. Firstly, we have observed that the ratio of [^{3}H]estradiol-17β and [^{3}H]dihydrotestosterone binding sites varied considerably with the individual liver preparation. Zelson (1974) has reported similar results. Secondly, from detailed ligand specificity studies, it has been shown that only a portion of the specific [^{3}H]estradiol-17β binding sites in liver cytosol from male or female *Xenopus* were inhibited by dihydrotestosterone, regardless of the concentration (Zelson, 1974). Testosterone was ineffective as an inhibitor of [^{3}H]estradiol-17β binding but was a good inhibitor of [^{3}H]dihydrotestosterone binding. Zelson also showed that the [^{3}H]dihydrotestosterone binding sites could be inhibited by either estradiol-17β or diethylstilbesterol at 100-fold excess. These data suggest that there are cytosolic components containing only estrogen-binding sites and others, perhaps, containing sites with affinities for both [^{3}H]estradiol-17β and [^{3}H]dihydrotestosterone. Support of this conclusion is provided by the recent report of Roy et al. (1974). These workers demonstrated an androgen receptor of high affinity in cytosol of rat liver which sedimented at 3.5 S and bound estradiol-17β with a lower affinity.

If there are separate protein entities which bind either estrogens or androgens, one may be able to demonstrate a variable sensitivity to temperature inactivation. In preliminary experiments of the temperature stability of unloaded binding sites, we observed that the [^{3}H]estradiol-17β binding sites had a temperature inactivation coefficient (T_i) of $\sim 40°$ while that of the [^{3}H]dihydrotestosterone binding sites was $\sim 20°$ (Bergink and Wittliff, unpublished). These data support the idea that there are separate components in *Xenopus* liver which bind either estradiol-17β alone or both estradiol-17β and dihydrotestosterone.

This study represents one of the few in which acrylamide gel electrophoresis has been used successfully to separate steroid-binding proteins. Sherman et al. (1970) calculated, from the variation of electrophoretic mobility with the acrylamide concentration, molecular weights of 1×10^5 and 3.6×10^5, respectively, for the 5S and 8S progesterone receptors in cytosol of chick oviduct. Recently Sherman et al. (1974) demonstrated that the progesterone-binding component from oviduct was dissociated in the presence of calcium ions, into a "subunit" of 2×10^4. Using purified preparations of the calcium-stabilized estrogen-binding unit of calf uterus which sedimented at 4 S, DeSombre et al. (1971) demonstrated a single protein band on acrylamide gel electrophoresis. Sex-steroid binding components of plasma have also been separated using this technique (Corvol et al., 1971).

The presence of estrogen-binding proteins in *Xenopus* liver suggests that these entities may be involved in the estrogen-induced synthesis of yolk proteins. The role of the 2×10^4 species in this process is under investigation.

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