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High-Affinity Binding of Progesterone, Estradiol-17β and Testosterone by Plasma Proteins of the Reptile *Lacerta vivipara* J.

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Steroid–protein interactions were studied in the lizard, *Lacerta vivipara* J. The detection of specific steroid-binding plasma proteins was determined by equilibrium dialysis, Sephadex filtration, sucrose gradient centrifugation, and polyacrylamide gel electrophoresis. Two high-affinity binding systems are described: a transcortin-type protein and an SBP-type protein. A high concentration of progesterone-binding sites was found ($N = 1.1 \times 10^{-5}$ M), whereas binding activity for corticosterone was weak. The estradiol-17β-binding affinity to the lizard SBP ($K_A = 2 \times 10^9\ M^{-1}$) is similar to that found in man and amphibians. Testosterone binds to both the above specific binding systems.

INTRODUCTION

Plasma binding of steroids has been documented for a variety of vertebrate species (Seal and Doe, 1965; Corvol and Bardin, 1973; Wenn *et al.*, 1977). A corticosteroid-binding globulin (CBG or transcortin) and a sex hormone-binding protein (SBP) were identified in man and found widely distributed in a great number of mammalian vertebrates (Westphal, 1971; King and Mainwaring, 1974); CBG and SBP are the main plasma proteins which bind circulating steroids with high affinity, limited capacity, and great ligand specificity. Steroid-binding proteins with many properties similar to those of human CBG and SBP were demonstrated in the plasma of various classes of nonmammalian vertebrates; thus, a transcortin-like molecule was identified and characterized in birds (Cochet and Chambaz, 1976; Monet *et al.*, 1976; Martin *et al.*, 1977; Gould and Siegel, 1978), amphibians (Martin and Ozon, 1975), and teleost fishes (Fostier and Breton, 1975); and an SBP-like molecule was identified in amphibians (Martin and Ozon, 1975; Martin and Collenot, 1975) and teleost fishes (Fostier and Breton, 1975).

The physiological significance of these proteins is still controversial (Funder, 1977), although it seems that the steroid which is not bound to the protein (the free fraction of the plasma hormone) is active in target organs; in that case, the steroid bound to the protein would be biologically inactive.

Our interest in the reptile, *Lacerta vivipara,* as a model for studying ovoviviparous reproduction and our desire for an improved endocrinological approach prompted us, in the initial phase of the present work, to characterize the proteins which bind to steroids in the lizard's plasma.

Evidence is given for the presence in this plasma of at least two distinct steroid-binding proteins: an SBP-like molecule and a CBG-like molecule, two kinds of proteins previously found in another reptile, *Lacerta viridis* L. (Martin, 1974).

MATERIAL AND METHODS

Animals and plasma proteins. Adult female lizards, *L. vivipara,* were freshly collected from Monts d'Auvergne (1000 m), France. Blood samples were taken from the infraorbital sinus with the help of a heparinized Pasteur capillary pipet and collected into heparinized tubes. Plasma was obtained by centrifugation at 4° (1000g for 15 min), removed, and stored at −30° until assay. In some experiments, plasma was treated with Norit A charcoal according to Heyns *et*

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al. (1967), in order to remove most of the endogenous steroids.

Steroids. The following labeled steroids were obtained commercially from New England Nuclear, Boston, Massachusetts: [1, 2, 6, 7,-^{3}H]progesterone (sp act, : 85 Ci/mmol), [2, 4, 6, 7 -^{3}H]estradiol-17β (sp act, 95 Ci/mmol), and [1, 2, 6, 7 -^{3}H]corticosterone (sp act, 82 Ci/mmol). These steroids were shown to be homogeneous by thin-layer chromatography. Radioinert steroids were supplied by Roussel-Uclaf.

Buffer. A 0.01 *M* Tris–HCl buffer (pH 7.4) was used (Tris buffer).

Radioactivity measurement. Radioactivity was determined in a Tricarb Packard liquid scintillation spectrometer, Model 3320, with external standardization. Each sample was dissolved in toluene-based scintillator. Small volumes ($\leq$0.5 ml) of aqueous solutions were counted in 10 ml of a mixture of PPO (5.5 g), POPOP (0.1 g), Triton X-100 (333 ml), and toluene (667 ml) solution.

Equilibrium dialysis. Dialysis tubing was washed with distilled water and Tris buffer. For all experiments, 1 ml of diluted whole plasma (1/1000, 1/800, or 1/160) was placed inside the bag and the radioactive steroid was placed outside the bag in 4 ml Tris buffer. In competition experiments, the unlabeled and labeled steroid were both placed outside the bag. To each dialyzing system were added various amounts of nonradioactive steroid ranging from 10^{-10} to 10^{-6} *M* and a fixed amount of labeled steroid (1×10^{-10} *M*). All binding experiments were performed at 4° with stirring for a set time of 48 hr.

Sephadex filtration. Microcolumns (0.5 × 8 cm) of fine Sephadex G-25 were equilibrated at 4° in Tris buffer and tested with Dextran Blue 2000 (Pharmacia). The plasma diluted 1:50 with Tris buffer was incubated with steroids for 3 hr at 4°; 0.2 ml of the incubated solutions was placed on top of the column and eluted with the Tris buffer at 4°. The bound steroid was collected into counting vials.

Sucrose gradient. Plasma diluted with Tris buffer was incubated with steroids for 2 hr at 4°; 0.2 ml of the incubated solutions was layered on a 5 to 20% sucrose gradient in Tris buffer (4.9 ml). The cellulose nitrate tubes were centrifuged at 38000 rpm for 16.30 hr at 4° in a SW-50.1 rotor (Spinco $L_2$50). The radioactivity of two-drop fractions collected from the bottom of the tube was counted. Bovine serum albumin solution (2 mg) was run systematically as a reference for approximate determination of the sedimentation coefficient. Protein content was measured by optical absorption at 280 nm.

Polyacrylamide gel electrophoresis. Analytical polyacrylamide gel electrophoresis (Davis, 1962; Ritzen *et al.*, 1974) was performed with 7.5% acrylamide gel in Tris–glycine buffer at pH 8.3. Stacking gel was made with 3.125% acrylamide–bisacrylamide. Radioactive and nonradioactive steroids were added to the polyacrylamide gel solutions before polymerization. The gels (7 × 60 mm) were polymerized in round glass tubes; 100-μl plasma samples, previously incubated with steroids at 4° for 12 hr, were layered. The electrophoresis was run at 0° for 120 min at 2 mA/tube until the bromophenol blue marker had come to the end of the gel. Gels were then sliced into 1-mm segments and placed in counting vials. After standing overnight at room temperature, all the radioactive steroid in the gel was extracted into the toluene counting fluid. Relative protein mobility (R_f) was calculated using the dye as a reference. Bromophenol blue R_f was referred to as 1.0.

Calculation of steroid binding. The percentage of steroid binding was determined by the following equation: percentage bound = $100\,[1 - (D \cdot V_r) / (R \cdot V_d)]$, where R and D are the total amounts of radioactivity present inside and outside the dialysis bag, respectively, and V_r and V_d are the corresponding volumes (Sandberg *et al.*, 1966). The relative affinity for the protein of each steroid tested compared to the affinity of progesterone and/or estradiol was determined at the 50% competition level by the method of Korenman (1968). Apparent steroid binding constants, binding capacity (N), and dissociation constant (K_D) were determined from the results of equilibrium dialysis binding experiments and Sephadex gel filtration. Estimation and statistical evaluation of binding parameters were made on the assumption that each binding system was independent and follows the law of mass action. Binding constants were computed using a C II 10070 computer (Raynaud, 1973) and by graphic analysis according to Scatchard (1949) as modified by Rosenthal (1967). Similar results were obtained with both methods.

RESULTS

Progesterone and Estradiol Apparent Binding Constants

Equilibrium dialysis was used to investigate progesterone and estradiol-17β binding in the plasma of female *L. vivipara*. Figure 1 represents the Scatchard plot corresponding to the binding of progesterone (Fig. 1A) and estradiol (Fig. 1B) by lizard plasma. The pattern obtained with progesterone indicates the presence of one type of binding site (saturable binding sites), whereas that obtained with estradiol indicates the coexistence of at least two types of binding sites (one saturable and the second unsaturable under our experimental conditions). The respective binding capacities and dissociation constants for proges-

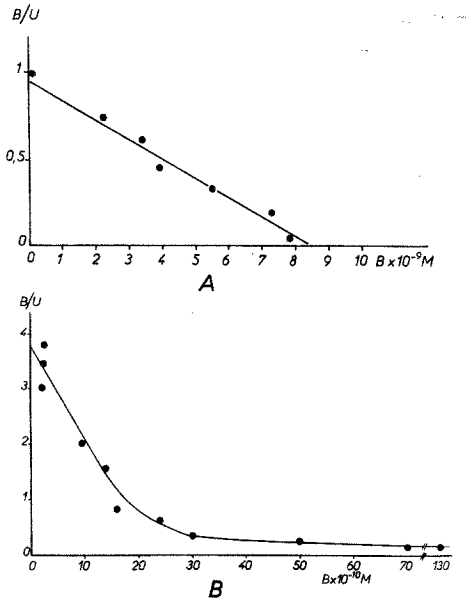

FIG. 1. Binding of progesterone (A) and estradiol-17β (B) to lizard plasma as determined by equilibrium dialysis experiments with plasma diluted 1:1000 for (A) and 1:160 for (B). Scatchard-type plot. B/U: concentration of bound steroid/concentration of unbound steroid.

terone and estradiol differed considerably. Thus, the mean ± SD binding capacities (N) and dissociation constants (K_D) obtained at 4° after four determinations (one animal per determination) were: progesterone, $N = 11133 \pm 2914 \times 10^{-9}\ M$ and $K_D = 10.7 \pm 1.2 \times 10^{-9}\ M$; and estradiol-17$\beta$, $N = 295 \pm 48 \times 10^{-9}\ M$ and $K_D = 0.5 \pm 0.1 \times 10^{-9}\ M$. Lower values for binding affinities and binding capacities were obtained by Sephadex gel filtration (for progesterone: $N = 5000 \times 10^{-9}\ M$ and $K_D = 1 \times 10^{-7}\ M$).

Electrophoresis and Sucrose-Gradient Centrifugation

In an attempt to identify the protein(s) responsible for the binding processes demonstrated by equilibrium dialysis and Sephadex gel filtration, we analyzed the electrophoretic behavior and the sucrose-gradient ultracentrifugation patterns of [^{3}H]progesterone and [^{3}H]estradiol binding in lizard plasma.

Figure 2 shows the radioactive patterns obtained after polyacrylamide gel electrophoresis of plasma incubated either with [^{3}H]progesterone or [^{3}H]estradiol. Progesterone and estradiol were found to bind to different protein systems. The relative mobility of the progesterone-binding system is 0.56. The [^{3}H]estradiol diagram exhibits one major peak of bound radioactivity (Rf = 0.33) preceded by a trail of radioactivity. This trail may be due either to partial dissociation of the steroid–protein complexes during electrophoresis or to the weak "nonsaturable" interactions visible on the Scatchard plot. No binding was observed for corticosterone (Fig. 2).

On the sucrose gradient, the position of the molecule binding [^{3}H]progesterone was not different from the position of the molecule binding [^{3}H]estradiol (Fig. 3). Comparison of the sedimentation pattern to that of bovine serum albumin ($S = 4.6$) gave approximately the same S coefficient.

Specificity Studies

The specificities of the two major proteins involved in the binding of [^{3}H]-progesterone and [^{3}H]estradiol, respectively, were measured by competition experiments. The extent of competition was determined by equilibrium assays, by observing the sedimentation pattern on sucrose gradients, and by the electrophoretic behavior. Nonradioactive progesterone, testosterone, cortisone, corticosterone, dexamethasone, and estradiol-17β were tested for their ability to compete with [^{3}H]progesterone for the binding sites of the plasma protein–progesterone complex. Nonradioactive estradiol, testosterone, dihydrotestosterone, and progesterone were tested for their ability to compete with [^{3}H]estradiol for the binding sites of the plasma protein–estradiol-17β complex.

The sucrose-gradient sedimentation and the electrophoresis revealed the following (Figs. 3, 4, and 5). When [^{3}H]progesterone was incubated with an excess of proges-

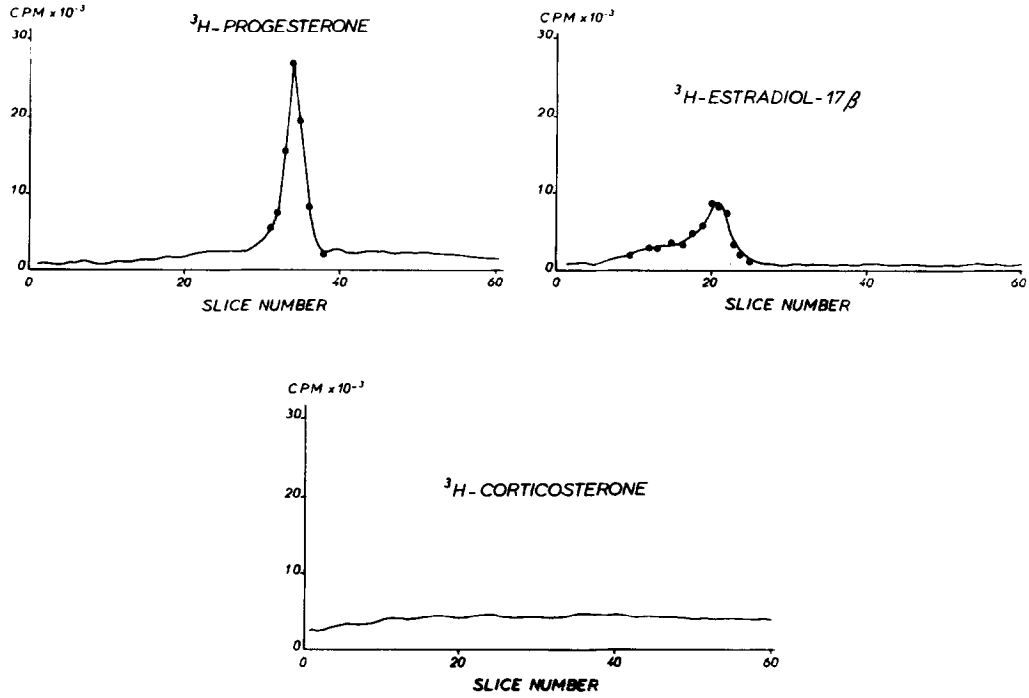

FIG. 2. Polyacrylamide gel electrophoresis of plasma proteins labeled with ^{3}H-steroids. Female plasmas diluted 1:20 (v/v) were incubated for 12 hr at 4° with [^{3}H]progesterone (2 n*M*), [^{3}H]estradiol (2 n*M*), and [^{3}H]corticosterone (2 n*M*), respectively, and analyzed by steady-state polyacrylamide gel electrophoresis. One hundred microliters was applied on polyacrylamide gel containing, respectively, 1 n*M* [^{3}H]progesterone, 0.7 n*M* [^{3}H]estradiol, and 2 n*M* [^{3}H]corticosterone in the stacking and in the separating gels.

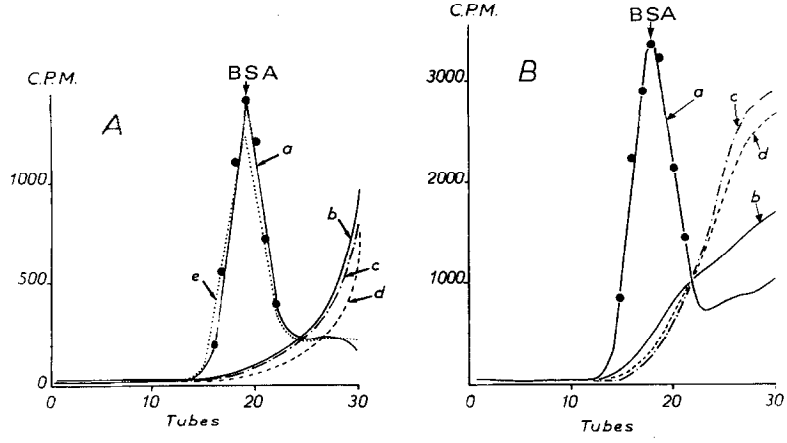

FIG. 3. Sucrose-gradient ultracentrifugation of [^{3}H]progesterone (A) and [^{3}H]estradiol-17β (B) with diluted female plasma. Plasma proteins diluted 1:100 (v/v) and 1:30 (v/v) previously incubated with 1 n*M* [^{3}H]progesterone and 1 n*M* [^{3}H]estradiol, respectively, were applied to a 5–20% sucrose gradient and centrifuged.

(A) Plasma containing: (a) only [^{3}H]progesterone; (b) [^{3}H]progesterone with 10,000 n*M* of progesterone; (c) testosterone; (d) cortisone; (e) estradiol. (B) Plasma containing: (a) only [^{3}H]estradiol-17β; (b) [^{3}H]estradiol with 10,000 n*M* of estradiol; (c) dihydrotestosterone; (d) testosterone.

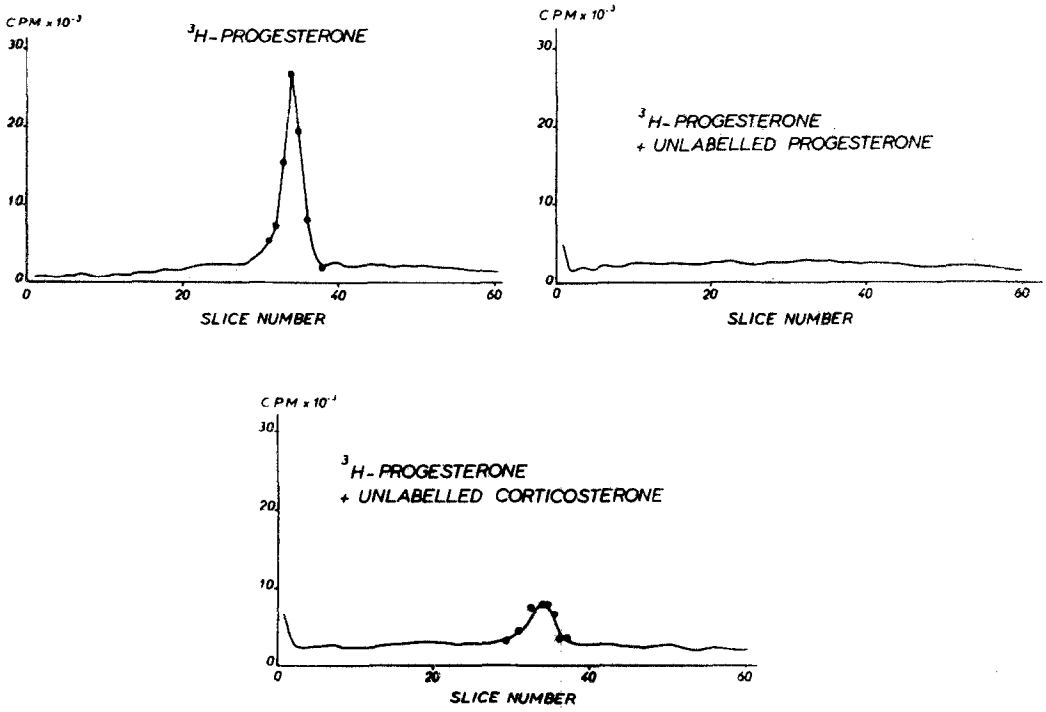

FIG. 4. Specific binding of [^{3}H]progesterone by plasma protein analyzed by steady-state polyacrylamide gel electrophoresis. Female plasma diluted 1:20 (v/v) was run into gels containing 1 n*M* [^{3}H]progesterone in the stacking gel and 1 n*M* [^{3}H]progesterone in the separating gel. Nonradioactive progesterone (10^{-5} *M*) and corticosterone (10^{-5} *M*) were added in the stacking and separating gels for competitive experiments. Above left: [^{3}H]progesterone alone (2 n*M*). Above right: [^{3}H]progesterone and 10,000 times excess of unlabeled progesterone. Under: [^{3}H]progesterone and 10,000 times excess of unlabeled corticosterone.

terone, testosterone, or cortisone (10^{-5} *M*), the radioactive peak ([^{3}H]progesterone bound to the transcortin-like molecule) disappeared. Estradiol-17β did not compete for the progesterone-binding components whereas corticosterone competed poorly. When [^{3}H]estradiol-17β was incubated with an excess of estradiol, dihydrotestosterone, or testosterone (10^{-5} *M*), the radioactive peak ([^{3}H]-estradiol bound to the SBP-like molecule) disappeared.

Equilibrium dialysis at 4° using diluted plasma was performed with a fixed amount of labeled steroids and increasing amounts

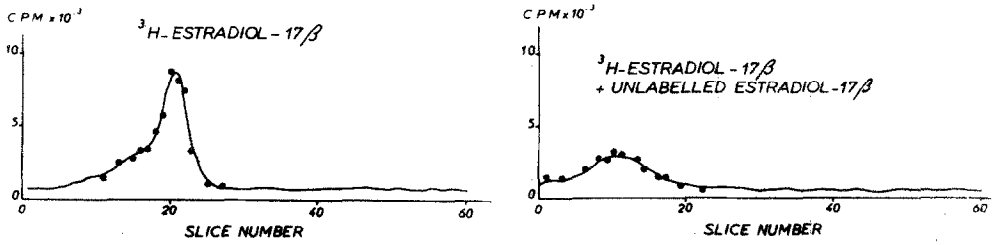

FIG. 5. Specific binding of [^{3}H]estradiol by plasma protein analyzed by steady-state polyacrylamide gel electrophoresis. Female plasma diluted 1:20 (v/v) was run into gels containing 0.7 n*M* [^{3}H]estradiol in the stacking and in the separating gels. Nonradioactive estradiol (10^{-5} *M*) was added in the stacking and in the separating gels for competitive experiments. Left: [^{3}H]estradiol alone (2 n*M*). Right: [^{3}H]estradiol and 10,000 times excess of unlabeled estradiol.

of several unlabeled steroids. Figure 6 shows the effect with a 1:800 diluted plasma of adding increasing amounts of progesterone, testosterone, cortisone, corticosterone, dexamethasone, or estradiol-17β upon the binding of a fixed amount of [^{3}H]-progesterone. Using 1:160 diluted plasma, we show, in Fig. 7, the effects of adding increasing amounts of estradiol, testosterone, dihydrotestosterone, or progesterone to the binding of a fixed amount of [^{3}H]estradiol. It was found that progesterone, testosterone, cortisone, and to a lesser degree corticosterone and dexamethasone, competed with [^{3}H]progesterone for binding sites (in contrast, estradiol-17β had no effect), and that estradiol-17β, testosterone, dihydrotestosterone, and to a lesser degree progesterone, competed with [^{3}H]estradiol-17β for binding sites.

The parallel nature of the competition curves shown in Figs. 6 and 7 is characteristic of competitive inhibition and made it possible to determine relative potency by estimating the amount of steroid necessary to reduce ^{3}H-steroid binding by 50%. When [^{3}H]progesterone was the standard for binding activity (Relative Potency Estimate (RPE) = 100), comparative relative potencies were calculated for various steroids: testosterone (100), cortisone (100), dexamethasone (≈3), and corticosterone (≈3). When [^{3}H]estradiol was the standard for binding affinity (RPE = 100), the comparative relative potency for testosterone and dihydrotestosterone was approximately 35.

DISCUSSION

This report demonstrates that two high-affinity steroid-binding components exist in *L. vivipara* plasma. One of them has a high affinity for progesterone ($K_{AA^\circ} = 1 \times 10^8$ M^{-1}) and saturable binding sites ($N = 1.1 \times 10^{-5}$ M), and roughly resembles a transcortin-type protein. The second component has a high affinity ($K_{AA^\circ} = 2 \times 10^9$ M^{-1}) and a limited capacity ($N = 3 \times 10^{-7}$ M) for binding estradiol-17β and might be a protein of the sex hormone-binding protein type. The poor agreement between the binding constants obtained by dialysis and gel filtration, respectively, can be explained by the continuous dissociation occurring as the steroid–protein complex moves down the Sephadex column. The binding capacity obtained by gel filtration should be considered as minimal.

As regards specificity, plasma concentration, and binding affinity, the two steroid-binding systems described are comparable to those previously studied in the green lizard, *L. viridis* (Martin, 1974). For

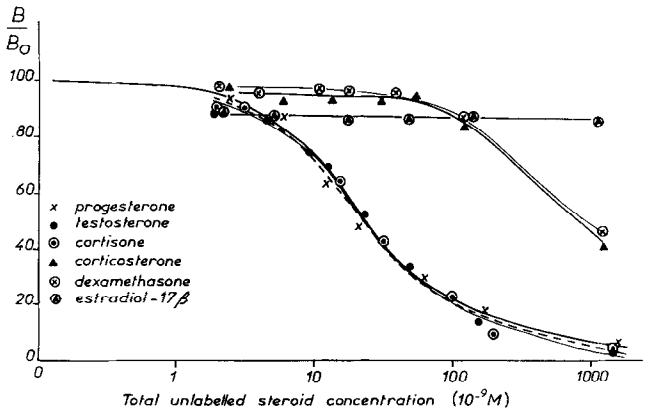

FIG. 6. Competition for the bound [^{3}H]progesterone in diluted plasma (1:800) as a function of unlabeled steroid concentration. B/B_0 : ratio of labeled steroid bound in presence of unlabeled steroid, to the labeled steroid bound in absence of unlabeled steroid; equilibrium dialysis experiments.

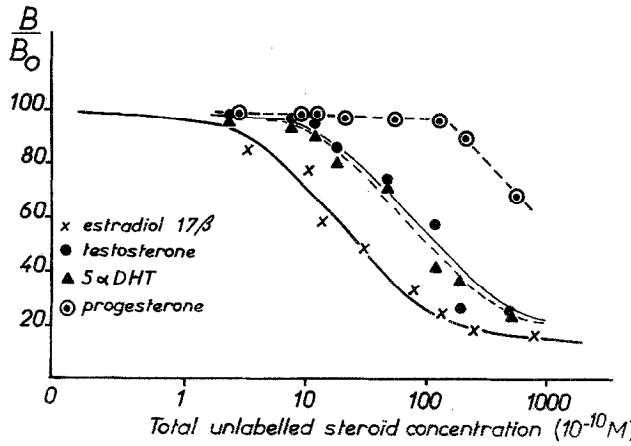

FIG. 7. Competition for the bound [^{3}H]estradiol-17β in diluted plasma (1:160) as a function of unlabeled steroid concentration. B/B_0 : ratio of labeled steroid bound in the presence of unlabeled steroid, to the labeled steroid bound in absence of unlabeled steroid; equilibrium dialysis experiments.

example, progesterone shows a high affinity for *L. viridis* transcortin ($K_{A4^\circ} = 2 \times 10^8$ M^{-1}) and a high binding capacity ($N = 1.5 \times 10^{-5}$ M).

In addition to the properties common to these lizard steroid-binding proteins and to those characterized in amphibians, birds, and mammals, there are also some differences between them. As regards transcortin, these differences concern the plasma concentration and ligand specificity. The transcortin concentration in lizard plasma is about 20 times higher than that in human plasma (Westphal, 1971), 60 times higher than that in hens and roosters (Monet *et al.*, 1976), and 30 times higher than that in amphibians (Martin and Ozon, 1975).

Competition experiments with lizard transcortin revealed that steroid hormone specificity was higher for progesterone and cortisone (RPE = 100) than with either corticosterone or dexamethasone (RPE = 3). This differs from human, amphibian, or hen transcortin which have high affinities for corticosterone (Lebeau *et al.*, 1969; Martin and Ozon, 1975; Monet *et al.*, 1976). Such differences in the relative affinity for corticosterone were reported previously in various reptiles (Seal and Doe, 1965). Particularly striking was the iguana, which showed no affinity for corticosterone.

It should be pointed out that in our results, testosterone shows a marked relative affinity for both transcortin and SBP in lizard plasma. A similar observation was reported in various species of amphibians (Martin and Ozon, 1975), whereas in mammals, testosterone is preferentially bound to SBP (Westphal, 1971). High levels of circulating testosterone—five times higher than the estradiol concentration—were observed in the preovulatory females of the freshwater turtle, *Chrysemys picta* (Callard *et al.*, 1978). Consequently, the high concentration of testosterone binders may be of great physiological importance in female reptiles.

High plasma progesterone levels are associated with the maturation of ovarian follicles in oviparous reptiles (Callard and Lance, 1977; Arslan *et al.*, 1978; Callard *et al.*, 1978; Lance and Callard, 1978; Lewis *et al.*, 1979). In the ovoviviparous and viviparous reptiles, plasma progesterone begins to increase during the preovulatory phase, but only reaches maximum levels during pregnancy in connection with luteal function (Callard *et al.*, 1972; Chan *et al.*, 1973; Veith, 1974; Highfill and Meade, 1975; Callard and Lance, 1977). A similar secretory pattern is observed in the ovoviviparous lizard, *L. vivipara,* in which plas-

ma progesterone levels are highest during pregnancy (unpublished data). The large amount of transcortin-type protein in *L. vivipara* plasma (10^{-5} *M*) might be commensurate with the elevated levels of circulating progesterone and might explain why this reptile is able to tolerate large quantities of steroids.

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