Estradiol and Progesterone Binding to a Fraction of Ovine Endometrial Cytoplasm*

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ABSTRACT: Endometrial scrapings from mature sheep uteri have been used as a source of the estrogen-receptor protein to elucidate some of its binding properties for 17β-estradiol and progesterone. As measured by equilibrium dialysis, endogenous 17β-estradiol in the mature sheep uterus does not mask tight binding sites for this hormone. Chromatography on Sephadex gel columns separated the 8S receptor from the 4S receptor on a preparative scale. Binding of the 17β compound to the 8S receptor in equilibrium dialysis at 5° showed two binding modes. The first is observed at relatively low concentrations of free 17β-estradiol and is consistent with homogeneous independent binding sites. Above a critical concentration of free estradiol, a second binding mode occurs. This mode is characterized by dependent cooperative binding due to interaction either of the binding sites themselves, or of ligands, one of which was already bound. A mathematical expression of this complex system has been derived.

Although on analysis by centrifugation through sucrose gradients progesterone did not appear to bind to the 8S receptor, equilibrium dialysis revealed binding in a manner which is consistent with the formation of a complex consisting of two molecules of 8S receptor combined with a single molecule of progesterone.

Pursuit of the estrogen binder in target tissue, especially in the uterus, has by now fairly well established that a receptor in the cytoplasm serves to concentrate the hormone from peripheral blood (Glascock and Hoekstra, 1959; Jensen and Jacobson, 1962; Noteboom and Gorski, 1965; Talwar *et al.*, 1968). In any target tissue, entrapment of the hormone is of special interest since it is possibly the first step in the mechanism that leads to the physiological effects of the hormone. Analyses of the cytoplasmic fraction from immature rat or calf uteri by sucrose gradient centrifugation, has shown the existence of two macromolecules that bind estradiol. One of these moves in the centrifugal field with a sedimentation coefficient of 8 S and appears to bind estradiol specifically (Toft and Gorski, 1966; Toft *et al.*, 1967; Jensen *et al.*, 1968; Erdos, 1968). Another fraction sedimenting at 4 S also binds estradiol but binds other steroids such as progesterone and testosterone as well (Toft *et al.*, 1967). Whether the 4S binder is a subunit of the 8S molecule (Korenman and Rao, 1968; Erdos, 1968), is not known definitely at this time.

We have isolated a fraction (designated the a peak) from the cytoplasm of mature sheep endometrium, which on centrifugation through sucrose gradients appeared to contain only the 8S binder and none of the 4S material. Binding of estradiol to the a peak has been studied using the technique of equilibrium dialysis. This analysis has revealed two different binding entities for estradiol in this fraction, even though analysis by sedimentation patterns in sucrose gradients indicated a single binder. The binding at low concentrations of the free steroid appears to be characterized by independent homogeneous binding sites, whereas at higher free steroid concentrations, the binding is heterogeneous with stabilizing interaction at the sites that result in augmentation of the binding.

When centrifuged through sucrose gradients, the a peak did not bind progesterone. However, using equilibrium dialysis, the a peak was found to bind progesterone in a way that suggests the possibility of the formation of a complex consisting of two protein molecules and a single steroid molecule.

The results suggest that the a peak contains two modes of binding for estradiol and one for progesterone, but the relationship, if any, that exists between these binding molecules is, at present, unknown.

Materials and Methods

Preparation of Endometrial Cytosol. Uteri from mature sheep were excised at the abattoir and immediately put on ice. They were delivered to the laboratory usually 2 hr, and no later than 3 hr, from the time of slaughter and were promptly dissected clean of fat and surrounding tissue, including blood vessels. The uterine horns were slit, and the endometrium was scraped onto glass plates and put at once into a measured amount of cold homogenizing medium. In experiments with uteri from immature sheep, the inner layer was peeled from the outer layer. The homogenizing medium for early experiments was 0.25 M sucrose, which was 0.003 M with respect to $CaCl_2$. For the binding studies reported here, the medium was 0.01 M Tris buffer (pH 8.0) which was 0.0015 M with respect to EDTA (Tris-EDTA). Pools of endometrial scrapings from 12 mature sheep uteri were used for each preparation.

The scrapings were homogenized in an all-glass motor-driven Potter-Elvehjem homogenizer with continual cooling

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in ice using three series of ten strokes with 30-sec cooling periods between each series of strokes. The volume to weight ratio of the homogenate was approximately 1.5:1.

The homogenate was immediately centrifuged at 27,000*g* for 20 min in a refrigerated Sorval centrifuge Model RC 2b. The supernatant was further centrifuged at 258,000*g* for 60 min, using a Spinco ultracentrifuge Model L2-65B, to produce the cytosol.

Materials. 17β-Estradiol-6,7-*t*, with a specific activity of 42.4 Ci/mmole, and progesterone-1,2-*t*, with a specific activity of 33.5 Ci/mmole, were obtained from the New England Nuclear Corp. The 17β-*t* compound was purified by paper chromatography (Kushinsky and Demetriou, 1963) every 3 months or sooner and stored in ethanol–benzene (9:1) at 5°. Progesterone-1,2-*t* was also purified on paper using a modified Bush A system (Bush, 1952). Sheep endometria were obtained as described above. Dextran gels (Sephadex G-25 and G-200) were obtained from the Pharmacia Co. Dioxane counting solution was prepared from freshly distilled dioxane which had been refluxed overnight over NaOH pellets. The counting solution contained 100 g of naphthalene, 4 g of 2,5-diphenyloxazole, and 0.3 g of 1,4-bis[2-(5-phenyloxozolyl)]benzene in 1 l. of dioxane. The scintillators were obtained from Packard Instrument Co., Inc. The counting mixture contained 5 ml of counting solution and 0.2 ml of sample solution in buffer. Yeast alcohol dehydrogenase was obtained from the Worthington Biochemical Corp., Freehold, N. J.

In Vitro Incubations. Hormone–receptor complexes were obtained by *in vitro* incubations. Aliquots of the tritiated hormone, usually containing 1×10^6 to 2×10^6 cpm per optical density unit of protein in the solution to be incubated were removed from stock solutions as needed, blown to dryness under nitrogen, and redissolved in the incubation medium. Incubations were usually carried out at 5° for 1 hr, after which the mixture was filtered through a Sephadex G-25 column of appropriate size at 5° to remove the free 17β-estradiol-6,7-*t* or progesterone-1,2-*t*. The columns were monitored in the ultraviolet range and for radioactivity. Early eluates containing the macromolecules complexed with the tritiated hormones were pooled.

Sephadex Gel Filtration. Both analytical and preparative chromatograms were run in water-jacketed columns at 5°, using Sephadex gels and buffers which had been deaerated under reduced pressure overnight. Buffers used were 0.01 M phosphate buffer (pH 7.4) which was 0.15 N with respect to NaCl (standard buffer), Tris-EDTA, and Tris-EDTA, which was 0.3 M with respect to KCl (Tris-EDTA-KCl). Analytical columns of Sephadex G-200, using samples of 17β-estradiol-6,7-*t*–receptor complexes after *in vitro* incubations were 160-ml total volume (2×60 cm) and were run under gravity flow at 5–8 ml/hr. Preparative columns (Pharmacia Co.) were 1500-ml total volume (5×90 cm) and were run by inverse flow at 30 ml/hr, using a peristaltic pump. All fractions were collected at 5°. Fractions were monitored for protein concentration by ultraviolet absorption at 278 mμ or at 215 mμ, and aliquots of tubes of the analytical columns were monitored for radioactivity.

Centrifugation through Sucrose Gradients. Gradients of between 5 and 20% sucrose were prepared in an automatic gradient maker. The protein solution to be analyzed was incubated for 1 hr at 5° with appropriate amounts of tritiated hormone, after which a 0.2-ml sample of the mixture was layered over 4.8 ml of sucrose gradient. The tubes were centrifuged at 43,000 rpm for 16 hr in a Spinco Model L2-65B ultracentrifuge using the SW65 rotor. After centrifugation, the tubes were punctured and samples were collected directly into counting vials using gravity flow. About 26 fractions of equal numbers of drops (usually 15) were collected from a total volume of 5 ml and counted in dioxane counting solution. Sedimentation patterns of yeast alcohol dehydrogenase were analyzed by determining the dehydrogenase activity in each fraction by a modification of the method of Racker (1955). Sedimentation patterns of albumin and γ-globulin were monitored by reading the ultraviolet absorbance at 278 mμ.

Concentration of Protein Solutions. In order to reestablish adequate protein concentrations for equilibrium dialysis or sedimentation in sucrose gradients after the gel filtration chromatography, concentrators using reduced pressure as described by Hofsten and Falkbring (1960) or those provided by Schleicher & Schuell, Inc., in their collodion bag apparatus were used. The concentrator tubes were water-jacketed at 5°. The collodion bag apparatus was kept in ice.

Equilibrium Dialysis. Equilibrium dialysis was carried out in specially constructed cells in which a volume of 0.3 ml was used on each side of the membrane. The cell design previously used (Zimmering *et al.*, 1967) was further modified to minimize the nonspecific adsorption of estradiol to the walls of the system. The parafilm caps used previously were not suitable with this hormone and new cells were designed using glass covers which were clamped to precision ground surfaces of the cells by metal bars. Since low concentrations of estradiol are adsorbed onto Pyrex glass, a series of glasses received from Bellco Glass, Inc., was tested. The recovery of low concentrations of estradiol when test tubes made of flint, borosilicate, and Kimax glasses were used, was about 95%. The equilibrium dialysis cells were therefore fabricated from Kimax glass. The Schleicher & Schuell membranes used in previous studies for dialysis of testosterone derivatives were not suitable for dialysis of 17β-estradiol since 85% of the free 17β compound was adsorbed by these membranes. Dialyzer tubing 4465-A2 from A. H. Thomas Co., however, adsorbed only about 5% of the hormone. To fit the cells, the tubing was cut into disks which were then washed with stirring for 2 days in 20% ethanol and for 5 days in distilled water with daily changes of the wash. Disks were stored in water at 5°. The overall recovery using these various precautions was 80–85% of the estradiol of the original solutions. At 5°, equilibrium occurred after 20 hr. The dialysis was routinely carried out for 24 hr in standard buffer. During this time, the cells were rotated in a water bath at 5° in a specially constructed apparatus which tumbled the cells in each rotation at the rate of 5 rotations/min.

After equilibrium had been reached, 0.2-ml aliquots were removed from each side of the cell and counted in dioxane counting solution. The aqueous phase represented 4% of the total counting volume. For this ratio, the counting efficiency for estradiol was 85% that found using toluene solution without polar solvent. The small amount of protein in the samples did not affect the counting efficiency.

To study 17β-estradiol binding, equilibrium dialysis was carried out using solutions of nonradioactive 17β-estradiol to which small amounts of 17β-estradiol-6,7-*t* were added.

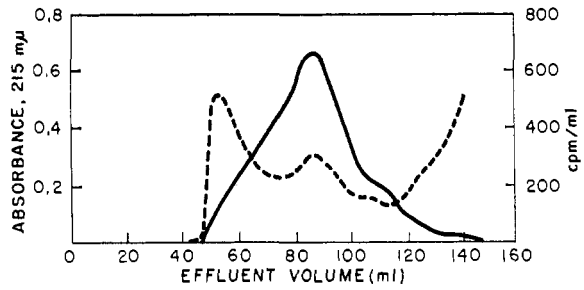

FIGURE 1: Chromatographic pattern of cytosol prepared from whole fresh 24-day-old Sprague–Dawley rat uteri. Cytosol was incubated with 17β-estradiol-6,7-*t* at 5° for 1 hr and filtered through Sephadex G-25. The protein–17β-estradiol complex was pooled and chromatographed through a 2 × 60 cm column of deaerated Sephadex G-200 at a flow rate of 8.2 ml/hr. (——) Absorbance at 215 mμ. (-----) Radioactivity in counts per minute per milliliter.

The bound 17β-estradiol was obtained by subtracting the radioactivity on one side of the membrane from that on the side containing the binding protein, and the data were converted into picomoles per milliliter of 17β-estradiol, bound or free. Protein concentrations were estimated by ultraviolet adsorption at 278 mμ, and the data for bound 17β-estradiol were corrected to the same protein absorbance.

For binding studies, dialysis was performed using a series of concentrations of free 17β-estradiol. Dissociation experiments were performed as follows; cells were set up at relatively high concentrations of free 17β-estradiol and allowed to come to equilibrium. After 24 hr, the solutions on the side containing free 17β-estradiol only were removed, the cell compartment was blotted dry, and 0.3 ml of bufferwas put in to replace the free 17β-estradiol solutions. The cells were then returned to the rotator in the bath and brought to a new equilibrium during another 24 hr, after which the solutions on the two sides of the membrane were analyzed in the usual way.

Progesterone binding was studied in the same manner, but the variation in protein concentration was corrected as described in Results.

Results

Comparison of Binding of 17β-Estradiol to Cytosol from Mature and Immature Sheep. As determined by sedimentation through sucrose gradients, the *in vivo* binding of the 17β compound to uterine 8S receptors uses only a small fraction of the total capacity of these macromolecules (Jensen *et al.*, 1968). On the assumption that receptor molecules are comparable in mature and immature sheep uteri, it would be expected that binding sites which may be obscured in cytosol taken from mature animals by the presence of endogenous 17β compound would be available for binding to cytosol which was extracted from immature animals. This difference would possibly become apparent at low concentrations of free hormone where tight binding sites may be masked by endogenous 17β compound in cytosol from mature animals and yet be available for binding when the cytosol is prepared from immature uteri. In a comparative study of the binding of the 17β compound to whole cytosol prepared from the pooled inner layers peeled from uteri of mature ewes with the binding to cytosol prepared in the same way from uteri of

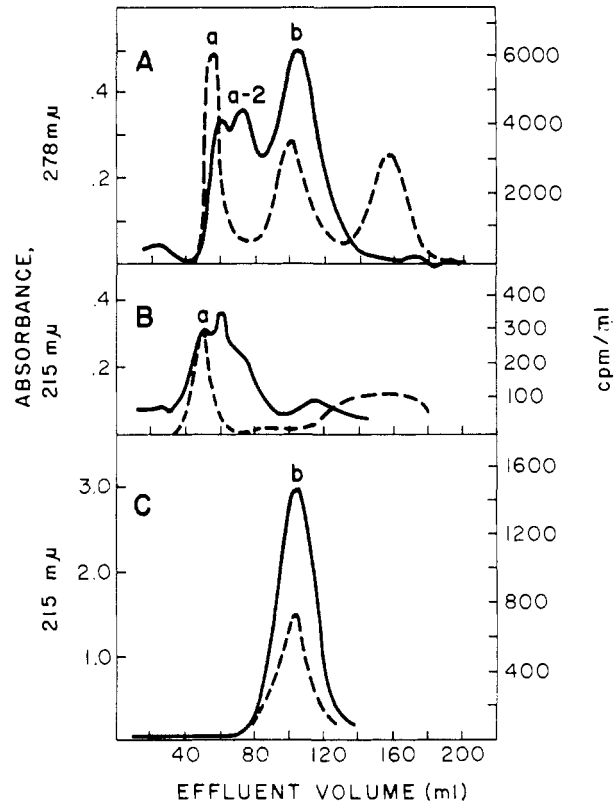

FIGURE 2: Chromatographic pattern of cytosol prepared from peeled inner layers of frozen uteri from mature sheep. Cytosol was incubated with 17β-estradiol-6,7-*t* and filtered through Sephadex as in Figure 1. (——) Absorbance at 215 or 278 mμ. (-----) Radioactivity in counts per minute per milliliter. (A) Whole cytosol as above. (B) Rechromatogram of 7.3 ml of a peak on Sephadex G-200 at a flow rate of 4.8 ml/hr. (C) Rechromatogram of 10 ml of b peak on Sephadex G-200 at a flow rate of 6.7 ml/hr.

immature sheep, no difference in the binding was observed and therefore mature sheep endometrial cytosol was used for the present study.

Sephadex G-200 Chromatography. When cytosol from immature rat uteri which had been incubated with 17β-estradiol-6,7-*t* was chromatographed on Sephadex G-100 (Talwar *et al.*, 1964), the bound hormone was eluted with the bulk of the protein material in the forerun fraction. Essentially the same results were obtained using Bio-Gel P-200 columns (Vonderhaar and Mueller, 1968). In this laboratory, cytosol from whole immature rat uteri was first incubated with 17β-estradiol-6,7-*t*. A sample of this cytosol, now containing the receptor–estradiol complex, was put on Sephadex G-200 columns in standard buffer. The chromatographic analysis was carried out using gel which had been deaerated and with deaerated standard buffers. Under these conditions, the bulk of the protein was retained on the column and the protein-bound 17β-estradiol-6,7-*t* was eluted in two distinct fractions (Figure 1). Cytosol from frozen rabbit uteri and from frozen human endometrial scrapings, when treated in the same way, gave similar chromatographic patterns.

When endometrial scrapings from frozen sheep uteri were treated in this manner, three distinct protein peaks were eluted from the columns (Figure 2A). The first (a) and third (b) peaks both retained 17β-estradiol-6,7-*t*, while the second

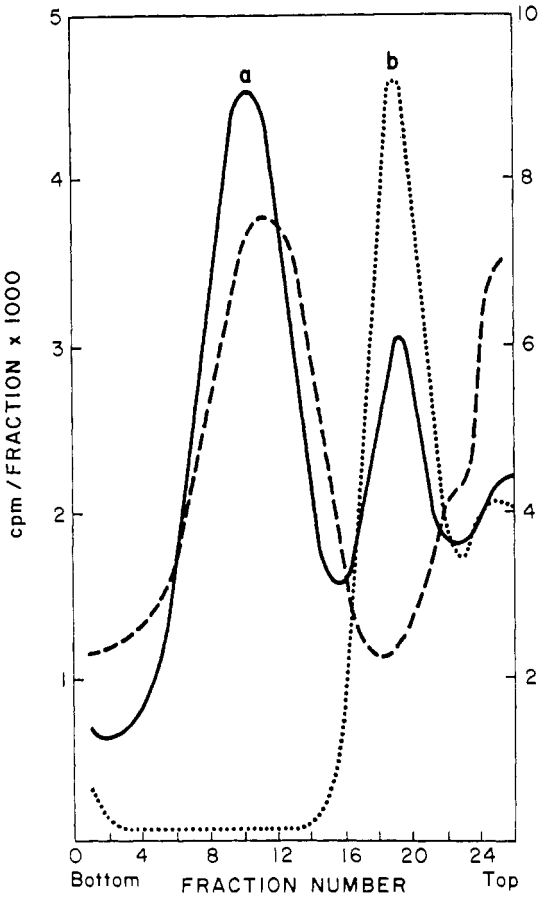

FIGURE 3: Sucrose gradient centrifugation patterns of cytosol and of the a and b peaks taken from mature sheep endometrium. Sample (0.2 ml) incubated with 60,000 cpm of 17β-estradiol-6,7-*t* (2.2 pmoles) was layered over 4.8-ml sucrose gradient (5–20%) and centrifuged at 43,000 rpm for 16 hr in a Spinco SW-65 head. (——) Whole cytosol. (-----) a peak, concentrated to $^1/_{50}$ volume. (· · · · ·) b peak, concentrated to $^1/_{13.5}$ volume. Scale at left is for cytosol and a peak. Scale at right is for b peak.

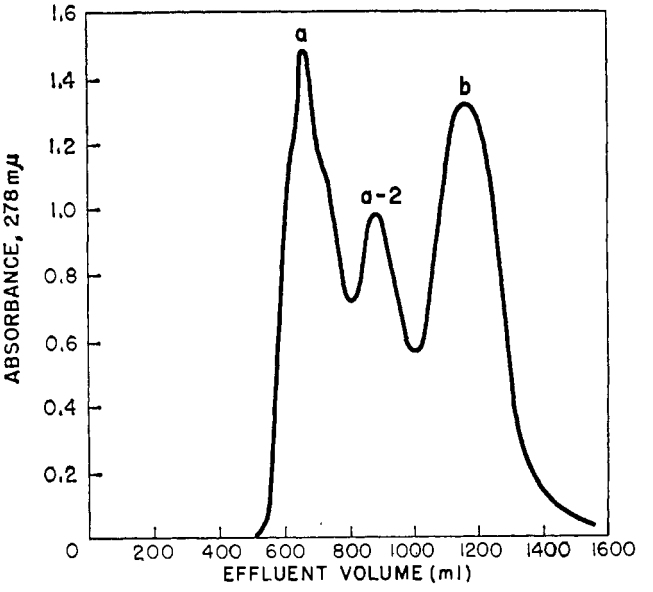

FIGURE 4: Ultraviolet absorbance pattern of a preparative chromatogram of fresh cytosol from a pool of fresh mature sheep uteri on Sephadex G-200 column (volume 1500 ml) using inverse flow at a rate of 31.2 ml/hr. Buffer is Tris-EDTA.

(a-2) peak carried no counts. The effluent pattern in this medium was essentially the same when incubation of the cytosol was carried out at 5° for 1 hr or at 37° for 15 min. When the first peak was rechromatographed in the same medium, a considerable amount of protein that did not bind 17β-estradiol-6,7-*t* was separated from the fraction containing the binding macromolecule, but the elution pattern still showed a single peak of bound estradiol (Figure 2B). On centrifugation through a sucrose gradient, the a peak from fresh sheep endometrium sedimented as 8 S with no evidence of the lighter binding material in the sedimentation pattern (Figure 3).

The a-2 peak of the Sephadex G-200 chromatogram, which did not retain 17β-estradiol on the chromatogram, was incubated with 17β-estradiol-6,7-*t* and filtered through Sephadex G-25. Again, no evidence of binding was observed with this protein fraction.

When the b peak was rechromatographed on Sephadex G-200, the protein–estradiol complex was eluted in the same effluent volume as in the first chromatogram (Figure 2C). On centrifugation through sucrose gradients, the b peak from fresh sheep endometrium had no 8S component, but sedimented as 4 S only (Figure 3).

The chromatographic patterns on Sephadex G-200 varied with the ionic strength of the medium. In Tris-EDTA, all of the bound 17β-estradiol-6,7-*t* was in the a peak, and the a-2 peak was not well separated from the b peak. In Tris-EDTA–KCl buffer, the binding macromolecules were retained on the column and were not eluted exclusively with any of the main protein fractions. Chromatograms from sheep heart cytosol showed no large molecules which bound 17β-estradiol. For binding studies using equilibrium dialysis, cytosol from fresh sheep endometrium was chromatographed on 1500-ml preparative columns of Sephadex G-200 with inverse flow in Tris-EDTA. Under these conditions, separation of the three peaks was considerably improved (Figure 4).

Sucrose Gradient Centrifugation. Sucrose gradient centrifugation of mature sheep uterine cytosol showed two binding moieties similar to those found by Toft *et al.* (1967) and by Jensen *et al.* (1968) for immature rat uteri. In our system, human γ-globulin, with a sedimentation constant of 6.6 (Fahey, 1962) or 7.0 S (Porter, 1960) and human albumin, with 4.3–4.6 S (Phelps and Putnam, 1960), had peaks which appeared in tubes 13 and 18, respectively, when monitored in the ultraviolet at 278 mμ. Alcohol dehydrogenase with 7.6 S (Martin and Ames, 1961) was monitored by enzyme assay (Racker, 1955) and the peak found in tube 12. Sheep uterine cytosol, incubated with 17β-estradiol-6,7-*t* and sedimented through sucrose gradients under the same conditions, showed two peaks, in tubes 11 and 19, respectively, corresponding to average sedimentation coefficients of 7.9[1] and 4 S as calculated by the method of Noll (1967). After concentration to $^1/_{50}$th of the original volume, a peak from G-200 columns, sedimented as the 8S peak and gave

[1] This peak is referred to as having a sedimentation coefficient of 8 S.

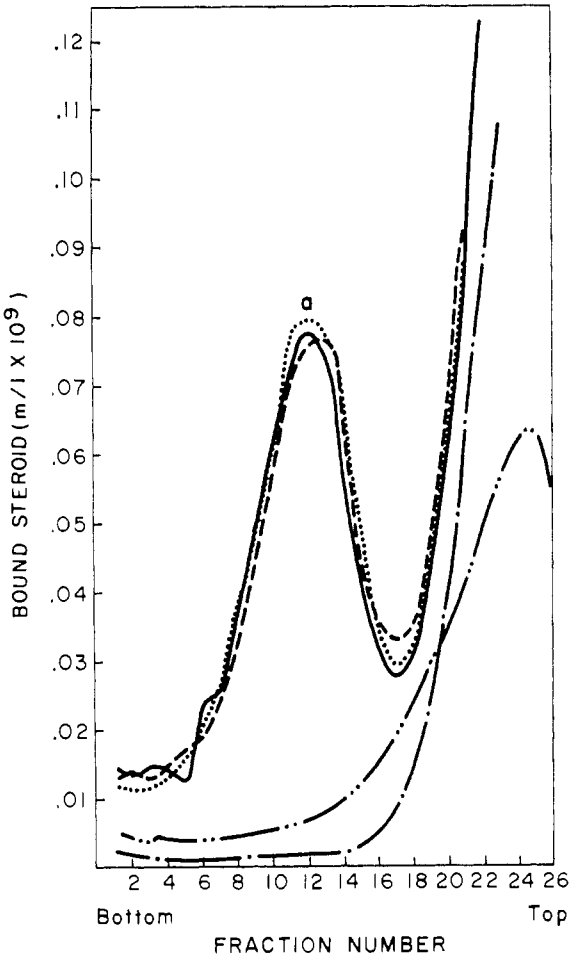

FIGURE 5: Sucrose gradient centrifugation patterns of the a peak from Sephadex G-200 columns, concentrated to $^1/_{18.3}$ volume and incubated as for Figure 3 with (——) 2.2 pmoles of 17β-estradiol-6,7-*t* alone. (-----) 2.2 pmoles of 17β-estradiol-6,7-*t* in the presence of 220 pmoles of nonradioactive progesterone. (·····) 2.2 pmoles of 17β-estradiol-6,7-*t* in the presence of 220 pmoles of nonradioactive testosterone. (-·-·-·-) 2.2 pmoles of progesterone-1,2-*t* (-···-) 2.2 pmoles of tritiated testosterone.

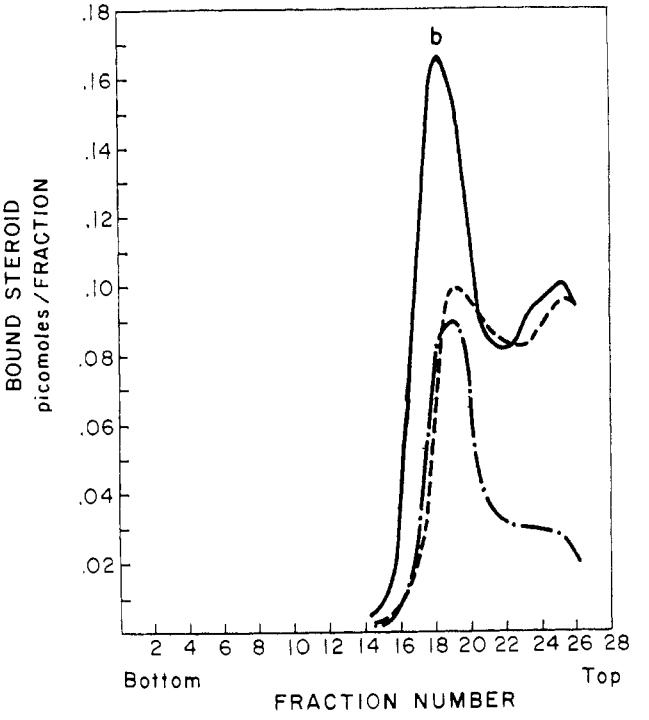

FIGURE 6: Sucrose gradient patterns of the b peak concentrated to one-ninth volume. (——) Incubated with 2.2 pmoles of 17β-estradiol-6,7-*t* alone. The identical patterns resulted when the incubation with 17β-estradiol-6,7-*t* was done in the presence of 220 pmoles of nonradioactive progesterone or testosterone. (-···-) Incubated with 2.2 pmoles of progesterone-1,2-*t*. (-----) Incubated with 2.2 pmoles of tritiated testosterone. There was no binding below fraction 15.

no evidence of a 4S peak (Figure 3). Although it was not seen in the sedimentation pattern of Figure 3, this a peak on other occasions showed evidence of aggregation in the same manner as observed by Erdos (1968) for calf endometrial cytosol. Neither tritiated progesterone nor tritiated testosterone bound to the a peak, when sedimented through sucrose gradients and when progesterone and testosterone were added to the sample in 100-fold excess over 17β-estradiol-6,7-*t*, they did not interfere with the specific binding of the 17β-estradiol-6,7-*t* (Figure 5).

The b peak from the G-200 columns sedimented as the 4S peak and gave no evidence of an 8S peak. Neither testosterone nor progesterone interfered with 17β-estradiol-6,7-*t* binding to this peak, although these hormones alone did bind to this fraction (Figure 6).

Equilibrium Dialysis. For binding experiments with estradiol, four different a peaks were prepared from pools of endometrial cytosol, each pool being derived from 12 fresh sheep uteri. After chromatography, the a peak was concentrated to one-eighth to one-tenth its volume by reduced pressure dialysis, and the solutions were clarified by centrifugation at 257,000*g* for 1 hr. The resulting solutions had optical density readings of 1.44–1.85 at 278 mμ, and were used for equilibrium dialysis with a series of estradiol solutions. For one of the four preparations shown in Figure 7, the tissue was washed three times in isotonic sucrose solution containing calcium ion before it was homogenized in Tris-EDTA. In the binding studies, the free hormone in equilibrium with the protein–estradiol complex ranged from 1×10^{-9} to 9×10^{-7} M. When the total protein concentration was corrected to the same ultraviolet absorbance at 278 mμ, these four separate experiments resulted in curves which were superimposable. Since both washed and unwashed tissues produced the same curve, it would appear that any plasma proteins which remained in the homogenates of unwashed tissue were removed during the subsequent purification procedures.

When the amount of hormone bound at a given protein concentration was plotted against the concentration of free estradiol, the resulting curve gave evidence of biphasic binding, with the binding directly dependent upon the protein concentration (Figure 7). Equilibrium was approached from both sides, either by dialyzing the protein solutions against estradiol solutions or different concentrations containing some 17β-estradiol-6,7-*t*, as shown in Figure 7, or by dialyzing the protein–estradiol complex in a solution of free estradiol against standard buffer as shown in Figure

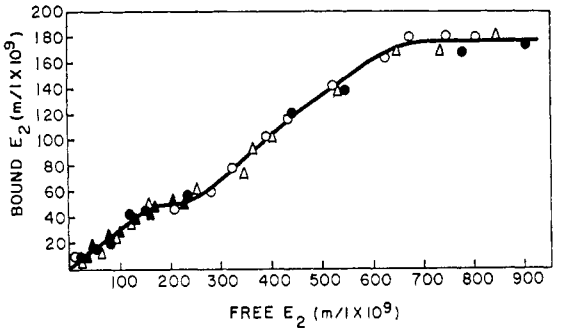

FIGURE 7: Saturation curve of the binding of 17β-estradiol by the a peak from Sephadex G-200 chromatograms. The curve represents four binding experiments using four different protein preparations, each with a series of concentrations of free 17β-estradiol. The a peaks were concentrated by reduced pressure dialysis and the solutions were clarified by centrifugation at 257,000*g* for 1 hr. Ultraviolet absorbances at 278 mμ of the concentrated solutions of the a peaks were 1.445 (○○○○○), 1.620 (●●●●●), 1.440 (△△△△△), and 1.850 (▲▲▲▲▲). The data labeled △△△△△ were derived from tissue which had been washed in 0.25 M sucrose before homogenization in Tris-EDTA. Curves are normalized to the same protein concentration (absorbance of 1.620 at 278 mμ). Binding was measured by equilibrium dialysis (see Methods section).

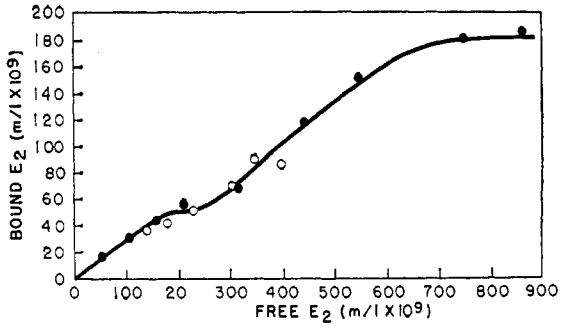

FIGURE 8: Saturation curve of 17β-estradiol and a peak prepared by dissociation of the 17β-estradiol complex as described in Methods section. The curve is a tracing from Figure 7; the points are experimental points using two protein preparations, different from those used for the direct binding shown in Figure 7, concentrated and clarified as for Figure 7. Optical densities of the concentrated solutions of the a peaks were 1.620 (○○○○○) 1.260 (●●●●●). Data for the binding to the latter preparation were corrected to correspond to a protein absorbance of 1.620.

8. The pattern of the binding curves was the same when equilibrium was approached from either side, showing that binding at this temperature is a true equilibrium process. This is illustrated in Figure 8 where the curve is taken from Figure 7 and the points are those determined by dialysis of the protein–estradiol complex as outlined in the Methods section.

We have attempted to find a mathematical expression which will describe the two binding modes observed in the saturation curve. When applied to our data, the Scatchard (1949) plot frequently used to analyze binding phenomena, did not give a descending curve from which the equilibrium constant could be estimated. The Sips equation (1948), which has been used previously to describe the binding of steroids to anti-steroid antibodies (Zimmering *et al.*, 1967), fitted the second binding mode but not the first. This analysis was originally derived from a consideration of the binding of oxygen to hemoglobin by Hill (1913), for the binding to catalytic surfaces by Sips (1948) and was applied to equilibria in solution by Klotz (1953) and by Weber (1965). For equilibria in solution, the Sips equation takes the form

$$\frac{r}{n} = \frac{(KC)^\alpha}{1 + (KC)^\alpha} \tag{1}$$

where r is the number of moles of ligand bound per mole of binding protein, C is the concentration of free ligand, n is the number of binding sites per molecule of protein, and K is a binding parameter which is described by the above equation. When $\alpha = 1$, the equation reduces to one descriptive of a homogeneous system, with independent binding sites of the same binding affinities. When $\alpha < 1$, it is an index of the degree of heterogeneity of binding affinities, and when $\alpha > 1$, it serves as an indication of a more complex system of binding. When there are stabilizing interactions between binding sites, so that the reaction at one site leads to an increase in the binding affinity for an adjacent or nearby site, α is always greater than unity (Wyman, 1948) and conversely if $\alpha > 1$, augmented or cooperative binding always occurs.

Another form of the equation is[2]

$$\log \frac{B}{U} = \alpha \log K + \alpha \log C \tag{2}$$

where B is the number of bound sites, U the number of unbound sites, and the remaining symbols are as defined above. U was evaluated by subtracting the value of B at a given C from the saturation value of $B(B_\infty)$. The results of the experiments illustrated in Figures 7 and 8 had been adjusted to correspond to those for a protein concentration such that the ultraviolet absorbance at 278 mμ would be 1.62. Under these conditions, the saturation value, B_∞, is 1.79×10^{-7} M. Using this value, a plot of log (B/U) as a function of log C gave a curve which could be resolved into two straight lines, providing further evidence for two binding modes for estradiol.

The mathematical form which best fits the results found in the saturation curve is a simple expression of the two types of binding found. Below a critical value of $C(C_c)$, binding occurs in the first mode only and the saturation curve for this portion of the binding appears to be a straight line. Binding in the second mode follows the form of eq 1 and begins to take place after C_c has been reached. In this analysis

$$\frac{r}{n} = \begin{cases} K_1 C & C < C_c \\ K_1 C_c + \dfrac{[K_2(C - C_c)]^\alpha}{1 + [K_2(C - C_c)]^\alpha} & C \geq C_c \end{cases} \tag{3}$$

where K_1 and K_2 are the binding association constants for the two different modes of binding. Note that for $C < C_c$,

[2] Equation 2 can readily be derived by letting r, the number of moles of ligand bound per molecule of protein, = B/P; and n, the number of binding sites per protein molecule = (B + U)/P. Substitution into eq 1 leads to eq 2.

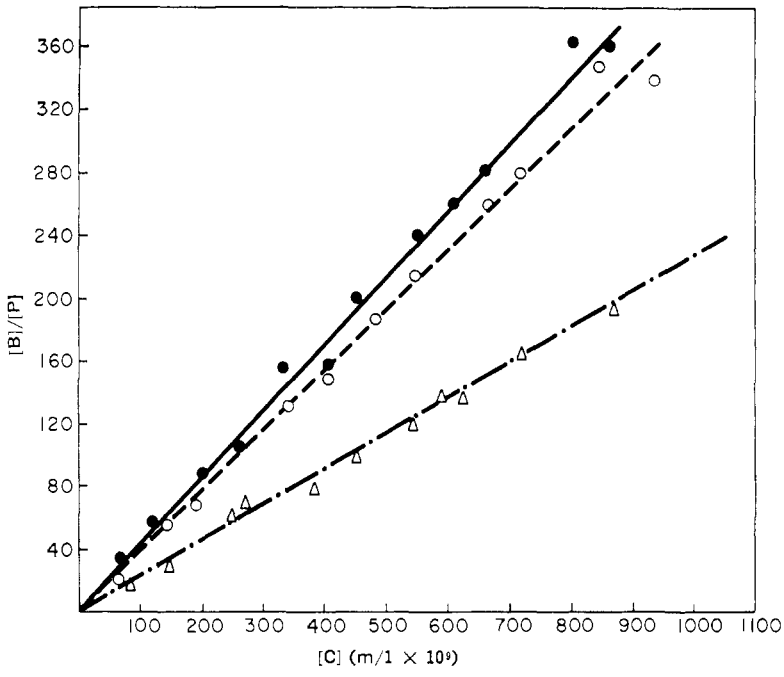

FIGURE 9: Binding curves for progesterone and the a peak from Sephadex G-200 columns as measured by equilibrium dialysis. Ultraviolet absorbance at 278 mμ of the concentrated a peaks were 1.680 (-●-●-●), 1.430 (-○--○--○), and 0.860 (-···△···△). The data have been corrected to the same protein concentration. Each experiment represents a different preparation of the a peak. [B] is the concentration of bound progesterone in moles/l. × 10^{-9}, [P] is the concentration of protein normalized to a single ultraviolet absorbance of 1.620 at 278 mμ. [C] is the concentration of free progesterone in moles/l. × 10^{-9}.

r/n plotted against C yields a straight line. K_1 was evaluated by plotting B/B_∞ (as a measure of r/n) against C for all values of B below 52 × 10^{-9} M, using the total saturation value of 179 × 10^{-9} M for B_∞. A straight line was determined by the method of least squares and the slope, which is K_1 was found to be 1.64 × 10^6 l./mole. C_c was approximated from the first part of eq 3 at B = 52 × 10^{-9} M and was found to be 177 × 10^{-9} M. K_2 and α were evaluated from the second part of eq 3 in the following way. Let $r/n - K_1C_c = f$. Then

$$\log \frac{f}{1-f} = \log K_2 + \alpha \log (C - C_c) \qquad (4)$$

Equation 4 is derived in the same way as eq 2 by substituting f into the second part of eq 3. By the method of least squares applied to a plot of log $f/(1-f)$ against log $(C - C_c)$ using the data for values of C above the critical value of 177 × 10^{-9} M, α was found to be 2.50 and K_2 to be 2.74 × 10^6 l./mole.

The binding behavior of the a peak with progesterone is quite different. Although binding studied by centrifugation through sucrose gradients showed no binding to the a peak (Figure 5) in agreement with the results found for rat uterine cytosol (Toft *et al.*, 1967), this hormone was bound by the a peak in equilibrium dialysis. Over the range of concentration of free progesterone of 1 × 10^{-9} to 1 × 10^{-6} M, the amount of binding remained proportional to the free ligand concentration with no evidence of saturation. Also, there was no evidence of more than one type of binding for progesterone in the a peak in this range of free ligand. When the binding data taken at different protein concentrations were corrected to a single protein concentration, as was done for estradiol binding, the resulting straight lines still showed different apparent binding for different original protein concentrations (Figure 9). When the binding data were corrected as the square of the protein concentration, the resulting values were directly proportional to the free ligand concentration (Figure 10). The equation for this straight line as determined by least squares was found to be: $[B]/[P]^2 = 0.4655[C]$, where [P] is the concentration of total protein in the solution, expressed in optical density units, [B] is the concentration of bound progesterone in moles per liter, and [C] is the concentration of free progesterone in the same units. This equation can be derived from the reaction of one molecule of progesterone with two molecules of protein: C + 2P $\rightleftarrows$ B and $K = [B]/[C][P]^2$. K cannot be evaluated, since the curve does not reach saturation and the data therefore give no measure for $[P]^2$.

Discussion

The uterine receptor for estradiol has hitherto been studied either *in vivo* through the uptake of tritiated estradiol by various subcellular fractions (Noteboom and Gorski, 1965; Talwar *et al.*, 1964), or *in vitro* by examining the sedimentation behavior of cytosol through sugar gradients (Toft *et al.*, 1967; Jensen *et al.*, 1968; Shyamala and Gorski, 1969; Erdos, 1968). From these observations, complex theories of hormone action which involve these binding entities in the uterus have been developed (Jensen *et al.*, 1968: Gorski *et al.*, 1968). Quantitative measurements that lead to a better understanding of the nature of the binding macromolecule will bear on these theories. In the currect study, some investigations into the binding properties of the 8S uterine receptor of ewes have been carried out.

It has already been shown by sugar gradient analysis that there are two binding macromolecules in uterine cytosol of a number of animals; one binder has a sedimentation coefficient of 8 S and the other of 4 S. In this investigation, cytosol from mature sheep endometrium has been found to exhibit the same sedimentation behavior on centrifugation through sugar gradients. Careful gel filtration of the cytoplasmic fraction from mature sheep endometrium has successfully separated these two cytoplasmic binding molecules on a

preparative scale. In spite of the fact that rechromatography of the fraction sedimenting as 8 S has shown that this fraction was still impure, some of the binding properties of the 8S receptor for estradiol could be deduced using this material (a peak), since the saturation binding level was reached. The binding of both estradiol and progesterone to this fraction has been studied at 5°. Centrifugation through sucrose gradients at this temperature produced a pattern showing only one binding moiety for estradiol, and no binding for progesterone. On the other hand, more detailed analyses using equilibrium dialysis has provided evidence for two distinct binding modes for estradiol and a complex binding behavior with respect to progesterone.

In general, the saturation value for binding is a measure of the total binding capacity of a sample and with this information data at lower binding levels can be used to evaluate some aspects of the nature of the binding. The method cannot give information about individual molecules and therefore the number of sites per molecule cannot be estimated. Nevertheless, the saturation curve for the binding of estradiol to the 8S fraction from uterine endometrial cytosol (Figures 7 and 8) showed two distinct binding phenomena to this fraction of cytosol even though it sediments as a single 8S component on sugar gradients. In the first type of binding, occurring at low concentrations of free ligand, the amount bound remains directly proportional to the concentration of free hormone until saturation is reached. This is the kind of behavior characteristic of the binding of small molecules to albumin. The binder, however, is found in the early eluates of the Sephadex G-200 columns and therefore, is probably not albumin which is eluted at a higher effluent volume. After a critical concentration of free estradiol, a second phase of the binding begins to occur. This second mode is potentiated binding which follows an s curve described by the Sips equation, with $\alpha = 2.50$. This means that in the second set of binding sites there is interaction such that once a hormone molecule is bound, the binding of an additional molecule is facilitated. A number of examples of this increase in binding affinity with increased binding have been observed. Most notable is the uptake of oxygen by hemoglobin (Wyman, 1948). The behavior has also been observed in the combination of reduced diphosphopyridine nucleotide with beef muscle lactate dehydrogenase (Anderson and Weber, 1965), in the binding of 1-anilino-8-naphthalene sulfate to albumin at low pH (Daniel and Weber, 1966), in the linking of testosterone to anti-testosterone antibodies (Zimmering *et al.*, 1967) and in a number of allosteric systems (Monod *et al.*, 1965).

To return to the 17β-estradiol binder, the macromolecules responsible for both of the binding modes appear to be derived from endometrial tissue and not from contaminating plasma proteins. If plasma proteins were present, the a peak obtained from tissue which had been washed in isotonic sucrose before homogenization in Tris-EDTA, would not be expected to exhibit binding properties identical with those of the a peak derived from unwashed tissue. However, the data illustrated in Figure 7 show that binding to solutions derived from washed tissue is identical with that from unwashed tissue.

The mathematical equation which seems to fit the data requires that binding in the second mode occur only after saturation of the first mode. This sequence may be fortuitous,

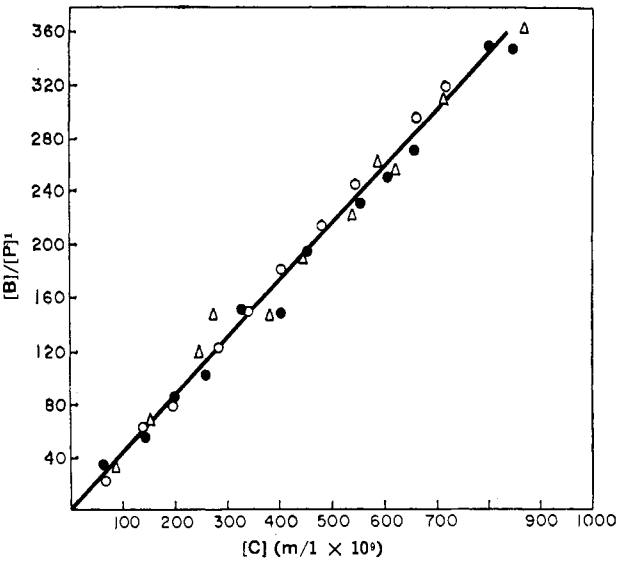

FIGURE 10: Binding curve for progesterone and the a peak. The data in Figure 9 have been corrected as the square of the protein concentration as measured by absorbance at 278 mμ. The symbols of the experimental points are the same as those of Figure 9. The line is derived from the corrected data by the method of least squares.

but, on the other hand, it suggests that the second binding mode is dependent upon, and possibly occurs, only as the result of the first binding.

Values popularly accepted as being indicative of specific binding to target receptors are usually within the range of 10^8–10^9 l./mole. Thus, Toft *et al.* (1967), using sucrose gradient patterns done at 5° on rat cytosol, report a dissociation constant of approximately 7×10^{-10} (binding constant of 1.4×10^9) and Baulieu (1968), measuring direct binding of 17β-estradiol to cytosol from immature sows at room temperature reported a dissociation constant of 2×10^{-9} (binding constant of 5×10^8). These constants, however, are derived assuming simple interactions between the ligand and a macromolecule characterized by independent, homogeneous binding sites. The first binding mode found here is also characteristic of simple binding. The possibility exists that the estradiol receptor present in untreated ovine uterine cytosol binds with an affinity constant of the order of 10^8 l./mole. That the values determined for the a peak in this study were lower (10^6 l./mole) may be due to the purification procedures used here, even though the protein was at no time warmed above 5°. The purification procedure, however, did not alter the behavior of the a peak on centrifugation through sucrose gradients, where it moved at the same rate as the 8S component of whole cytosol. It is possible that the concentration step which is done by dialysis under reduced pressure partially removed some factor which exists in whole cytosol and is necessary for binding. This could account for the lower affinities found here for K_1 even though cytosol which has been treated in the same way did form an estradiol–protein complex which sedimented as 8 S.

The second affinity constant, K_2, can be compared with other constants only in the context of the equation from which it is derived. It is an apparent binding parameter which averages many values, starting with low affinities at low

concentrations of free estradiol, then increasing as potentiation of the binding occurs.

Cooperative binding can be due to a number of different effects. It may be due to the interaction of the binding sites themselves as reaction with the ligand proceeds, or to the interaction of ligands or other factors, one of which is already bound. The equilibrium dialysis studies reported here provide no experimental evidence that would demonstrate that the cooperative character of the binding is the result of either tautomeric or allosteric changes in the macromolecule. Since the lability of the 8S fraction prevents further purification at the present time, no attempt could be made to determine whether each of the two binding modes for estradiol was associated with an individual macromolecule.

Progesterone binding to the a peak could not be analyzed by the Sips equation since saturation of the binding did not occur within the limitations of the experimental method. The binding curves of Figure 9 which represent experiments done at different protein concentrations could be superimposed only when they were constructed by plotting $[B]/[P]^2$ *vs.* [C] (Figure 10). This relationship can be rationalized by assuming that one molecule of progesterone is bound by two macromolecules. The fact that progesterone does not bind with any component sedimenting in the 8S region through sugar gradients may be due to the formation of aggregates of sufficient size to sediment the complex against the bottom of the centrifuge tube. It is also possible that sugar in the gradient solution masks the binding. The estimated concentration of sucrose in the fraction containing the 8S peak is 13%. On equilibrium dialysis in this amount of sucrose, the binding of progesterone was greatly reduced, although not completely prevented.

The relationship found between the binding of progesterone and the protein concentration could also be explained by again applying eq 1, with $\alpha = 0.5$. This would mean that there is heterogeneity of the binding affinities ranging from very tight binding sites which are saturated at relatively low concentrations of free ligand to more loosely binding sites which bind ligand only at higher concentrations of free ligand, and as the tighter sites are saturated. Since the saturation curves could not be outlined completely, the applicability of the Sips equation could not be tested in this system. However, the linear relationship between bound and free steroid over a 1000-fold span of concentrations of free steroid makes this type of heterogeneity in binding affinities unlikely.

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