*A RECEPTOR MOLECULE FOR ESTROGENS: STUDIES USING A CELL-FREE SYSTEM**

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When immature rats are injected with tritium-labeled estradiol-17β, the hormone is preferentially bound in the uterus (Jensen and Jacobson, 1962). One interpretation of these observations is that the uterus contains specific "receptors" that combine with estrogens to form a complex which is necessary for the sequence of events that constitute the uterine response to estrogens.

We have described a soluble factor from the rat uterus which, because of its binding of estrogens *in vivo*, is believed to be a specific hormone receptor (Toft and Gorski, 1966). This component binds estrogenic compounds, but not nonestrogenic steroids. Because of its binding specificity, its size (mol wt $\sim$ 200,000), and its sensitivity to proteolytic enzymes, this substance is probably a protein.

Since the hormone is bound so precisely *in vivo*, an *in vitro* cell-free system was sought which would duplicate the *in vivo* results. In the experiments presented here, estrogen binding was studied after simply adding the hormone directly to an aqueous extract from the uterus. The binding of estrogens in this cell-free extract appears to be identical to that which was observed in the *in vivo* experiments.

Immature Holtzman rats (20–24 days old) were decapitated and the uteri were removed and homogenized in 0.04 *M* tris-Cl, 0.0015 *M* EDTA, pH 7.4 (10 uteri per ml). A soluble fraction was obtained by centrifuging the homogenate at 105,000 $\times$ *g* for 1.5 hours or 270,000 $\times$ *g* for 30 minutes. This high-speed supernatant fraction contained 6–9 mg of protein per ml. Steroids dissolved in ethanol were added to the soluble fraction, which was then incubated for 15 minutes at 0–4°C. The final ethanol concentration did not exceed 0.5 per cent by volume. Samples were then layered on 5–20 per cent sucrose gradients. Sucrose gradient and counting techniques were as reported previously (Toft and Gorski, 1966) except for the centrifugation which was performed in an International model B-60 using an SB-405 rotor. Centrifugation was for 12–13 hours at 220,000 $\times$ *g* at 3°C. Isotopes were measured in a Packard model 3003 scintillation counter with 20 per cent efficiency.

The tritium-labeled compounds (New England Nuclear) were checked for purity by paper chromatography and were purified when necessary. The following specific activities were used: estradiol, 167 μc/μg; progesterone, 32.3 μc/μg, and testosterone, 167 μc/μg.

The sedimentation patterns of the soluble fraction containing various amounts of H^3-estradiol are shown in Figure 1. There are two regions in the gradient where estradiol is bound. The faster-sedimenting component, which has properties of a hormone receptor, has a sedimentation constant of approximately 9.5*S*. When higher concentrations of estrogen are used, a component with a sedimentation constant between 3 and 6 *S* also binds the estrogen. This 3–6*S* binding material quite likely consists of serum proteins, since when rat serum containing H^3-estradiol is analyzed on sucrose gradients, a single peak of bound estradiol appears in the

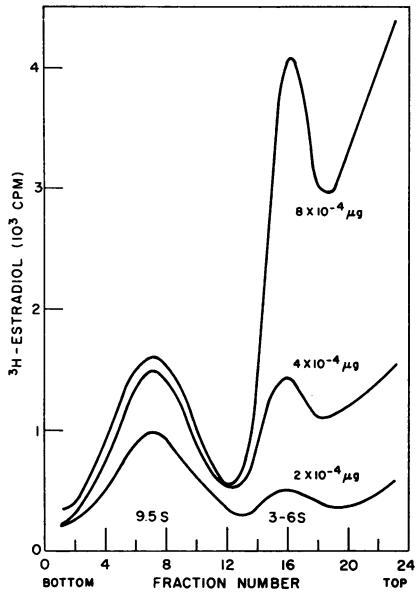

FIG. 1.—Density gradient patterns of uterine-soluble fraction with increasing concentrations of H^3-estradiol-17β. One-tenth ml of soluble fraction containing the amount of estradiol designated in figure was layered on 5–20% sucrose gradients and centrifuged for 13 hr at 200,000 × g, 3°C.

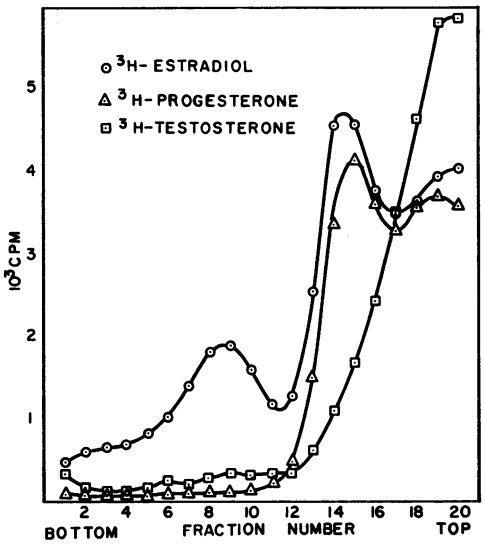

FIG. 2.—Density gradient patterns showing binding of estradiol, progesterone, and testosterone. Two-tenths ml of soluble fraction containing isotope was layered on 5–20% sucrose and centrifuged for 12 hr at 200,000 × g, 3°C.

3–6S region. When uteri are incubated for one hour in Eagle's medium, the 3–6S binding material appears to be washed out whereas the 9.5S component remains, suggesting that the former might be of extracellular origin. In addition, the 3–6S material binds other nonestrogenic steroids (see Fig. 2) and therefore does not possess the basic criteria for identification as a specific estrogen "receptor."

The 9.5S component was shown to be specific for estrogenic compounds in the *in vivo* studies previously reported. This property is also exhibited in the cell-free system. Tritium-labeled testosterone and progesterone are not bound in the 9.5S region; however, progesterone is bound in the 3–6S region (Fig. 2). In additional studies, estriol, hexestrol, and diethylstilbestrol have been found to compete *in vitro* with estradiol for binding to the 9.5S component. Both estriol and hexestrol have a high affinity for the uterus *in vivo* (Jensen, 1966). Determinations of the dissociation constants of these compounds and other properties of their interaction with the receptor are in progress.

In Figure 3 the amount of estradiol bound in the 9.5S region, the 3–6S region, and the free estradiol are plotted versus the total estradiol added to the sample. This plot clearly illustrates the high estrogen affinity of the 9.5S component, which is saturated at very low concentrations of the hormone. On the other hand, only a small amount of estrogen is bound in the 3–6S region at low hormone concentrations, and the amount bound continues to increase with the highest concentration that is used.

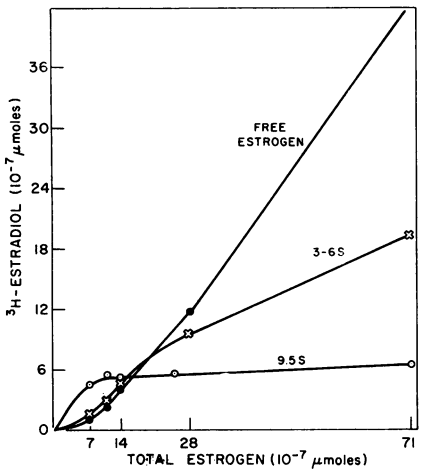

FIG. 3.—Titration plot from density gradient analysis of estradiol binding in the uterine-soluble fraction. The gradients contained 0.1 ml of soluble fraction. The amount of H^3-estradiol in the 9.5*S* region, 3–6*S* region, and free estradiol were determined and plotted versus total estradiol concentration.

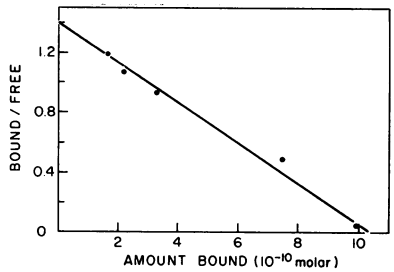

FIG. 4.—Determination of the dissociation constant and the number of binding sites. Using sucrose gradient analysis, measurements were made of the amount of estradiol bound to the 9.5*S* component and the amount of "free" estradiol. These values are expressed as molar concentrations in the soluble fraction and are adjusted to correspond to a protein concentration of 1 mg/ml. The actual protein concentration was 8.6 mg/ml. The relationship used is:

$$[\text{Bound}]/[\text{Free}] = 1/K([\text{Binding sites}] - [\text{Bound}]).$$

From the slope, K is $\sim 7 \times 10^{-10}$ M. From the X intercept, the number of binding sites is 10×10^{-10} moles/liter or 10×10^{-13} moles/mg protein.

A Scatchard plot was used to analyze the data from this and similar experiments (Scatchard, 1949). An example of such a plot is shown in Figure 4. The apparent dissociation constant for the 9.5*S* component is 7×10^{-10} M. For this approximation it was necessary to assume that estradiol not bound in the 9.5*S* region is essentially free, and that no significant dissociation occurs during centrifugation. Because of these assumptions, the determination tends to overestimate the dissociation constant, but it does provide a useful estimate of the strength of estradiol binding.

It has been reported (Daughaday and Mariz, 1960; Westphal, 1961) that circulating estrogens are bound to serum proteins with dissociation constants on the order of 10^{-5} to 10^{-6} M. The observed high affinity of the receptor molecule in the uterus would allow this molecule to compete successfully with serum proteins for the minute levels of hormone that exist in circulating blood.

Although the kinetics of estrogen binding have not been studied in detail, the data in Figure 4 fit a straight line. This can be interpreted as indicating that the binding sites in the 9.5*S* peak are homogeneous and, in turn, suggests that only one type of receptor site is involved. Such an interpretation is subject to error because the affinity for estrogen is so high that only large differences would be detected.

From the above data, the concentration of estrogen binding sites in the soluble fraction is estimated to be 10×10^{-13} moles per mg of protein, or about 8.9×10^{-13} moles per uterus. This is somewhat higher than we had estimated from the *in vivo* studies because in the cell-free system it is possible to produce more complete saturation of binding sites. If we assume that one molecule of estradiol is bound

per receptor molecule having a molecular weight of 200,000, a maximum value for the concentration of receptor is approximately 1.5 μg per ml of high-speed supernatant fraction, or about 1/5000 of the total protein. It must be noted that the above estimates do not represent the whole cell as *in vivo* experiments indicate that only 30 per cent of the bound estrogen is in the soluble fraction (Noteboom and Gorski, 1965).

Additional experiments have revealed some preliminary information on the properties of the 9.5*S* component. As shown in our previous report and confirmed in the cell-free system, the ability of this component to bind estrogen is destroyed by pronase and trypsin, but not by deoxyribonuclease or ribonuclease. The binding has a pH optimum of approximately pH 7 and is significantly reduced at pH 6 and 8. Heat treatment (60°, 4 min), detergents (0.5% sodium dodecyl sulfate or 1% taurocholate) and sulfhydryl-reacting agents (2 mM p-chloromercurobenzoate or 1 mM N-ethylmaleimide) remove all traces of binding. These properties are compatible with the supposition that the 9.5*S* component is at least in part composed of protein.

This cell-free system has all the properties which were described for estrogen binding at physiological levels *in vivo*. Of particular interest is the fact that binding occurs immediately in the cold and apparently requires no energy supply or processes which depend upon cellular organization. This system should be very useful in describing the direct interaction of estrogens with the uterine receptor, and we are presently attempting to purify this substance in quantities that will allow its further characterization.

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