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Specific Estrogen Receptors in the Lactating Mammary Gland of the Rat†

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ABSTRACT: Specific receptors of 17β-estradiol, which exhibited sedimentation coefficients of 8–9 S in sucrose gradients, were detected in the 105,000*g* supernatants of the lactating mammary gland of the Fischer rat. These receptors were specific for estrogens, as judged by competition studies, and demonstrated exceptionally high affinity for 17β-estradiol ($K_d \sim 10^{-9}$ M). They were protein in nature and dissociated into smaller binding components when separated on sucrose gradients containing 0.4 M KCl. While the lactating mammary gland demonstrated significant levels of these proteins, much lower levels were observed in glands obtained from pregnant and virgin animals. A receptor of lower molecular weight, sedimenting at ~4–5 S, was extracted from the nuclear pellets of mammary glands from pregnant and lactating rats treated *in vivo* with [2,4,6,7-^{3}H]-17β-estradiol.

Specific receptors for 17β-estradiol,[1] sedimenting at 8–9 S, have now been described in the cytoplasmic fraction from the uterus (Toft and Gorski, 1966; Jensen *et al.*, 1967a), anterior pituitary (Notides, 1970), and a variety of breast carcinomas, both of animal (Jungblut *et al.*, 1967; Kyser, 1970; McGuire *et al.*, 1971; Wittliff *et al.*, 1972a) and human origin (Jensen *et al.*, 1971; Wittliff *et al.*, 1972b). However, demonstration of a comparable estrogen receptor in normal mammary gland has been somewhat more elusive, in spite of the well known responsiveness of this tissue to estrogenic hormones. While Puca and Bresciani (1969) showed that quiescent mammary tissue of the mouse was capable of accumulating [^{3}H]-17β-estradiol *in vitro* in a specific fashion, information regarding the characteristics of a receptor mechanism at the molecular level was not presented. Recently, this laboratory (Wittliff *et al.*, 1972a) demonstrated the presence of a specific estrogen-binding component in the cytosol fraction of the lactating mammary gland of the rat. This receptor, which sedimented at ~8 S on sucrose gradients, was observed to possess high specificity and affinity ($K_d \sim 10^{-9}$ M) for 17β-estradiol. This paper presents a more extensive characterization of the estrogen-binding protein in the mammary gland and offers evidence for a probable physiological role for the receptor *in vivo*.

Materials and Methods

Chemicals and Reagents. All chemicals were reagent grade unless otherwise specified. [2,4,6,7-^{3}H]-17β-Estradiol (90–110 Ci/mmol) was obtained from New England Nulcear as were Omnifluor and PCS. Unlabeled 17β-estradiol, progesterone, hydrocortisone, estriol, testosterone, and aldosterone were obtained from Calbiochem. Unlabeled dihydrotestosterone was purchased from Steraloids, Inc. Deoxyribonuclease, ribonuclease, and catalase were obtained from Worthington Biochemical Corp. Tris (Trizma), Pronase, *p*-chloromercuribenzoate, and bovine serum albumin were purchased from Sigma Chemical Co. Norit A was obtained from Matheson, Coleman & Bell. CN-55,945-27[2] was a gift of Dr. Jerry Reel of Parke-Davis Co., while the cyanosteroid compound, CS-115,[3] was donated by Dr. Allen Goldman of The Children's Hospital in Philadelphia.

Preparation of Tissues. All animals were sacrificed by cervical dislocation. Tissues were removed as quickly as possible, placed in cold homogenizing medium (or alternatively, cold 0.15 M NaCl), and stirred to remove exogenous material. They were then debrided of necrotic and hemorrhagic segments, blotted free of fluid, weighed on a top-loading balance, and minced directly into a Duall glass homogenizing apparatus (Kontes Glass Co.).

Preparation of Tissue Cytosols. Tris buffer (10 mM Tris·HCl–1.5 mM EDTA, pH 7.4) or buffer A (0.01 M Tris·HCl–1 mM EDTA–0.25 M sucrose, pH 8.0) at a volume predetermined on the basis of tissue wet weight (usually 3:1, v/w for the lactating mammary glands) was added to the minced tissue.

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‡ Medical student Research Fellow supported by U. S. Public Health Service General Research Support grant.

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

[2] 1-[2-(*p*-[α-(*p*-Methoxyphenyl)-β-nitrostyryl]phenoxy)ethyl]pyrrolidine monocitrate.

[3] 3-Acetoxy-1,3,5(10)-16-estratetraene-17-carbonitrile.

Homogenization was effected in several 10–20-sec pulses separated by 20–30-sec cooling periods. A small ice bath was kept around the homogenizer at all times to prevent the accumulation of heat. The homogenate was then centrifuged at 105,000*g* for 30 min to prepare the postmicrosomal, or cytosol, fraction. The supernatant was drawn from beneath the lipid cap with a Pasteur pipet and placed on ice until used. Except where indicated, the nuclear pellet was discarded.

Sucrose Gradient Assay. The assay procedure closely paralleled that originally described by Toft and Gorski (1966) for the receptor from rat uterus and subsequently employed by Jensen *et al.* (1967a) and Wittliff *et al.* (1972a,b) with mammary tissue. In each of several small shell vials, an appropriate amount of [^{3}H]-17β-estradiol was evaporated to dryness. To each of these vials, 50 μl of Tris-EDTA buffer (10 mM Tris·HCl–1.5 mM EDTA, pH 7.4) alone, or in combination with a precalculated quantity of unlabeled steroid hormone or inhibitor, was added. A 200-μl volume of the 105,000*g* supernatant (prepared in Tris buffer) was combined with this mixture and incubated for an additional 60 min at 0° to ensure formation of the receptor–ligand complex. A 200-μl portion of the total reaction volume was then layered on a linear gradient of sucrose (5–20 or 5–40% sucrose, depending upon the experiment). These gradients were centrifuged in a Beckman L2-65B preparative ultracentrifuge for 15–16 hr (0°) at 308,000*g* using a Spinco SW-56 titanium rotor. Following centrifugation the gradient tubes were punctured and collected either from the bottom by gravity or from the top by an ISCO gradient fractionator. A total of 40 fractions/gradient were collected into scintillation vials containing 2 ml of 99% ethanol. Ten milliliters of toluene scintillation cocktail (4 g of Omnifluor/l. of toluene) was added to each fraction. The vials were then counted in a Mark II liquid scintillation counter (Nuclear-Chicago). Counting efficiency, calculated through external standardization of individual samples with a ^{135}Ba standard, ranged from 40 to 44%.

Dextran-Coated Charcoal Assay. Based on evidence to be discussed in the Results section of this paper, the following standard charcoal adsorption assay was devised. It is a modification of the earlier procedure reported by Korenman (1968). Individual variations in this assay, where employed, are described at length in the legends to the figures.

A known quantity of [^{3}H]-17β-estradiol, diluted in benzene, was evaporated to dryness in disposable glass test tubes. To each of these tubes, 50 μl of buffer A (10 mM Tris·HCl–1 mM EDTA–0.25 M sucrose, pH 8.0) alone or 50 μl of buffer A containing a standard concentration (usually 10 μM) of a competitive inhibitor, CN-55,945-27, were added. A 200 μl volume of the 105,000*g* supernatant, prepared in buffer A, was then added to each tube which contained the labeled steroid and buffer A with or without the inhibitor. These mixtures were incubated in a water bath (Bench-Scale Co.) at 25° for 20 min, conditions arrived at based on the data presented below. Following the incubation, 1 ml of buffer B (10 mM Tris·HCl–1 mM EDTA–0.25 M sucrose–0.50% Norit A–0.05% Dextran, pH 8.0) was added to each tube and shaken to promote mixing. This combination was then incubated an additional 15–20 min at 25° with intermittent shaking. At this point the tubes were removed from the bath, cooled to 0–4°, and centrifuged at 400*g* for 10 min. In most cases 0.5–1.0 ml of the supernatant was removed from the charcoal pellet and placed into a scintillation vial together with 10 ml of Bray's (1961) scintillation fluor or 10 ml of PCS for counting in a liquid scintillation counting system. Quenching was corrected for by external standardization of individual samples.

Studies in Vivo. A known quantity of [^{3}H]-17β-estradiol (0.26 μg, 100 μCi) was dissolved in two drops of absolute ethanol and brought to a predetermined volume (usually 1 ml) with 0.15 M NaCl. This solution was loaded into a syringe and injected into each animal in two to four individual subcutaneous deliveries. The animals were sacrificed 2 hr later and the mammary glands were removed. The tissues were washed in 0.15 M NaCl, blotted dry, weighed, and minced into a prechilled homogenizer. Following homogenization the tissue fractions were analyzed for estrogen-binding activity using the sucrose gradient assay.

Protein Determinations. Protein was estimated by the method of Lowry *et al.* (1951) using crystalline bovine serum albumin as a standard.

Calculation of Results. Data (as counts per minute) from either the sucrose gradient or the charcoal assays were printed out on a teletypewriter (Teletype Corp.) and simultaneously incorporated (in ASCII code) onto a paper punch tape. Using computer programs described elsewhere (Brooks and Wittliff, 1973), the data on the punch tape were converted to disintegrations per minute, and subsequently to steroid concentration, by an Olivetti Programma 101 interfaced to a solenoid deck (Nuclear-Chicago Corp.) and paper tape reader (Teletype Corp.). Steroid-binding substances were identified by the isotopic profiles on sucrose gradients. Estimates of specific binding were taken as the differences in the areas under the peaks sedimenting in the 8–9S region of the gradient, obtained in the presence or in the absence of the unlabeled steroid or a competitive inhibitor. Results were usually expressed as femtomoles per milligram of cytosol protein. Data obtained from the charcoal assay were expressed (on a per milligram of protein basis) as the total femtomoles of bound ligand less the femtomoles bound in the presence of the inhibitor.

Results

Use of the Dextran-Coated Charcoal Assay. To demonstrate that the charcoal adsorption assay gives valid approximations of estrogen-binding capacity in the cytosols from the lactating mammary gland, a series of several experiments was run.

A time course of association of [^{3}H]-17β-estradiol with its specific receptor protein is shown in Figure 1. The binding of the ligand to its receptor appeared to reach completion in 20 min at 25°. At 0°, the binding reaction was slower, reaching a comparable level 3 hr later. Incubation at 37° effected an initial rise in binding followed by a rapid loss in activity. This reflects a high degree of temperature sensitivity of the receptor–ligand complex or of the receptor alone.

The removal of unbound and nonspecifically bound [^{3}H]-17β-estradiol from reaction mixtures containing specific estrogen–receptor complexes was explored using dextran-coated charcoal. Following an initial incubation of supernatant from a homogenate of lactating mammary gland with [^{3}H]-17β-estradiol, dextran-coated charcoal (buffer B) was added and the mixtures were incubated for additional intervals at 25°. At this temperature, a 20-min incubation with charcoal appeared to remove most of the free and nonspecifically bound steroid (Figure 2). During this period, nonspecific binding, *i.e.*, that measured in the presence of CN-55,945-27, was reduced to basal levels (~10 fmol) while total binding remained at approximately 40 fmol. Specific binding may be calculated from the difference between these curves.

To check the stability of the steroid–receptor complex at 25°, supernatant prepared from lactating mammary gland

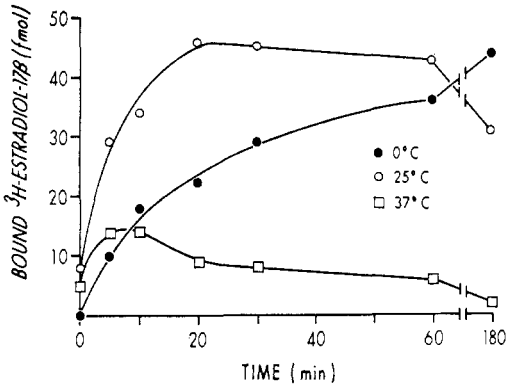

FIGURE 1: Time course of specific estrogen binding in the cytosol of lactating mammary gland as a function of temperature. Supernatant from the mammary gland of a rat 18-days postpartum was incubated at either 0, 25, or 37° with 2.2 nM [^{3}H]17β-estradiol, in the presence or in the absence of CN-55,945-27 (20 μM). Aliquots (200 μl) were taken at various times, placed in 1 ml of buffer B, and incubated at 25°. The mixture was centrifuged at 400*g* for 10 min (0–4°). A 0.5-ml sample was removed and digested in 0.5 ml of NCS, and 15 ml of Bray's scintillant was added and the contents were counted. Specific binding was determined as the difference between total binding and binding in the presence of CN-55, 945-27. Each point represents the mean of three determinations.

was preincubated in the presence and in the absence of [^{3}H]-17β-estradiol and then incubated for an additional 20 min at 25° in the presence of ligand. Figure 3 indicates that the presence of ligand afforded the receptor a significant stability against temperature inactivation. With the steroid present only a minimal loss of activity is seen after 20 min, the duration of the standard assay.

Characteristics of the Estrogen-Binding Protein in the

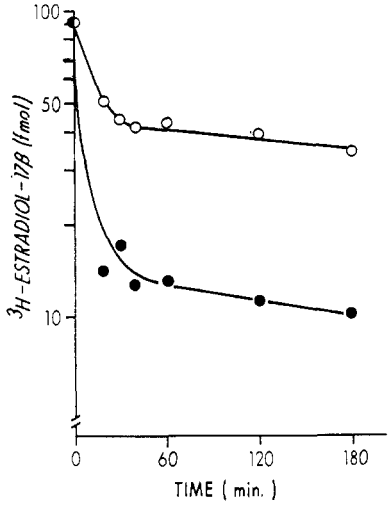

FIGURE 2: Dissociation of [^{3}H]-17β-estradiol from the estrogen-binding proteins during incubation with dextran-coated charcoal. Supernatant obtained from a homogenate of mammary gland from a 16 day lactating rat was incubated with 2.4 nM [^{3}H]-17β-estradiol at 25° for 20 min. Each incubation was carried out in the presence (closed circles) or in the absence (open circles) of the Parke-Davis compound, CN-55,945-27. The reaction was terminated by the addition of 1 ml of buffer B to each mixture. The dextran-coated charcoal in buffer B removed the unbound [^{3}H]-17β-estradiol. From the instant of addition of buffer B, taken as zero time on the graph, the mixtures were incubated for various intervals at 25°. After incubation, the suspension was centrifuged to sediment the charcoal and an aliquot was removed for counting. Each point is the mean of three determinations.

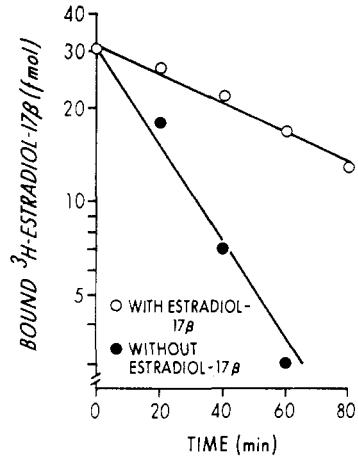

FIGURE 3: Stabilization of the estrogen-binding protein by 17β-estradiol following incubation at 25°. Supernatant from a homogenate of lactating mammary gland, 6-days postpartum, was preincubated either with or without 2.7 nM [^{3}H]-17β-estradiol for various time intervals at 25°. Following this reaction, the mixtures without 17β-estradiol were added to 2.7 nM [^{3}H]-17β-estradiol and incubated an additional 20 min at 25°. Those which contained [^{3}H]17β-estradiol during the preincubation were also incubated an additional 20 min at 25°. Each reaction was terminated by the addition of 1 ml of the buffer B followed by centrifugation at 400*g* for 10 min. An aliquot (0.5 ml) was added to 0.5 ml of NCS and 15 ml of Bray's fluor for counting. Each point represents the mean of three determinations.

Mammary Gland. Initial evidence for an estrogen-binding protein in the lactating mammary gland was provided by sucrose gradient analysis of the 105,000*g* supernatant. As shown in Figure 4, a macromolecular component binding [2,4,6,7-^{3}H]-17β-estradiol and sedimenting in the 8S region of the gradient was easily discernible. Data described previously (Wittliff *et al.*, 1972a) indicate that the components in this peak bind [^{3}H]-17β-estradiol specifically; a short incuba-

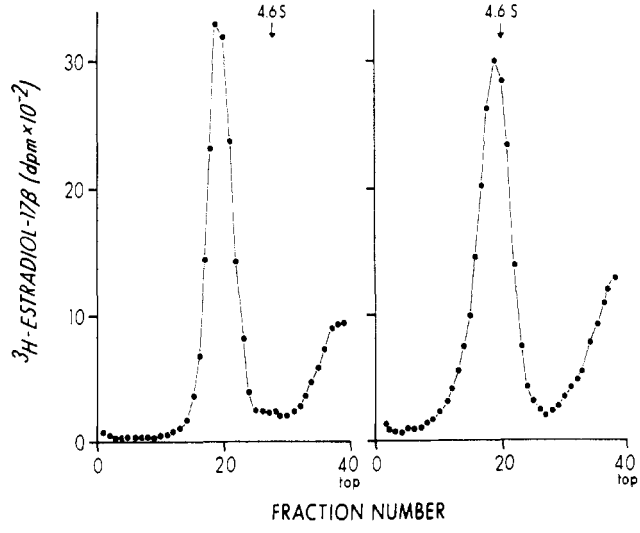

FIGURE 4: Separation of cytoplasmic estrogen-binding proteins from lactating mammary gland in the presence or absence of KCl. Freshly prepared supernatant from the mammary gland of a rat, lactating 12 days, was incubated with 0.7 nM [^{3}H]-17β-estradiol at 0° for 1 hr. A portion of the supernatant was then separated on a 5–40% sucrose gradient for 15 hr at 308,000*g* (profile on left). An equal volume of supernatant was separated on a 5–20% sucrose gradient containing 0.4 M KCl under identical centrifugation conditions (profile on right).

TABLE I: Specificity of [^{3}H]-17β-Estradiol Binding to the 8.6S Receptor from the Lactating Mammary Gland.[a]

Competitive Substance	Concn ($\times 10^{-6}$ M)	[^{3}H]-17β-Estradiol Bound (%)
None	0	100
CN-55,945-27	10	10
17β-Estradiol	1	9
Estriol	1	10
Estrone	1	12
CS-115	1	27
Hydrocortisone	1	91
Progesterone	1	100
Dihydrotestosterone	1	100
Testosterone	1	100
Aldosterone	1	100
Triamcinolone	20	100

[a] Each of the unlabeled competitive substances at the concentration designated was preincubated with the cytosol preparation from a lactating mammary gland for 10 min at 0–3°. [^{3}H]-17β-Estradiol at a concentration of 2–3 nM was added and the reaction was incubated at 0° for 1 hr. Following incubation, each mixture was layered on a 5–40% sucrose gradient and centrifuged at 308,000g for 15 hr. Total binding was determined as the radioactivity bound in the 8S region of the gradient. Competition is expressed as per cent of total binding in the untreated (control) gradient.

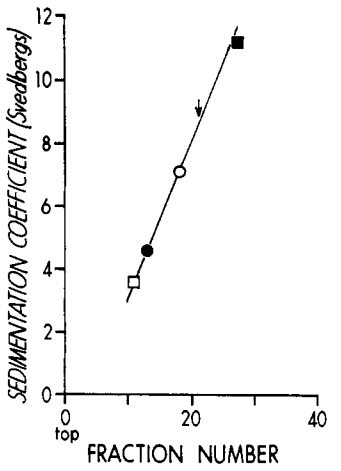

FIGURE 5: Estimation of the sedimentation coefficients of the estrogen-binding proteins in the cytoplasm of the lactating mammary gland. Supernatants were prepared from lactating mammary tissue and incubated with 2.4 nM [^{3}H]17β-estradiol, layered on 5–40% sucrose gradients and centrifuged for 15 hr at 308,000g. Likewise, purified protein standards (0.2 mg/gradient) were subjected to the same procedure. Estrogen-binding substances were identified by their isotopic profiles while the protein peaks were located using absorption at 254 nm. Position in the gradient was determined using the top of the gradient as a reference point. The estrogen-binding proteins from mammary gland sedimented to approximately tube 21 in the gradient under the conditions mentioned. (□) Ovalbumin (3.6 S), (●) bovine serum albumin (4.5 S), (○) human γ-globulin (7.1 S), and (■) catalase (11.2 S) were employed as standards.

tion with either unlabeled 17β-estradiol or the antiuterotropic agent, CN-55,945-27, prior to reaction with the labeled steroid reduced activity in this region to near basal levels.

In an effort to more closely determine the sedimentation coefficient of the estrogen-binding molecule in the mammary gland, several purified protein standards were centrifuged through linear gradients of 5–40% sucrose and their positions in the gradient were determined according to a modification of the method of Martin and Ames (1961). Figure 5 depicts the linear relationship between the sedimentation coefficient and the extent of migration into the gradient. Based on this plot, the soluble estrogen receptor of the lactating mammary gland has a sedimentation coefficient of approximately 8.6 S.

Centrifugation of the cytoplasmic estradiol–receptor complex through sucrose gradients containing 0.4 M KCl revealed an apparent subunit structure (Figure 4) comparable to that seen in the uterus. In the presence of high salt, a single component binding [^{3}H]-17β-estradiol sedimented in the 4–5S region of a 5–20% sucrose gradient (Figure 4, right panel). The 8.6S form of the receptor in the cytoplasm is shown on the left of Figure 4 for comparison. There was a conservation of bound [^{3}H]-17β-estradiol in the process of dissociation of the 8.6S form to the 4–5S form. Both incubations were conducted at estradiol concentrations which were below saturation to minimize the influence of nonspecific binding components. When the cytosol of uterine tissue from the same lactating animal was subjected to identical treatment, peaks of binding activity very similar to those seen in the mammary gland were obtained (data not shown). The steroid–receptor complex from either uterus or mammary gland sedimented as a single component (peak at fraction 8) when separated on KCl-free gradients of 5–20% sucrose. This finding precludes any effect of sucrose concentration on the sedimentation characteristics of the receptor and indicates a probable sedimentation coefficient of 4–5 S for the subunit.

Data which we reported earlier (Wittliff *et al.*, 1972a) indicate that the receptor saturates at a 17β-estradiol concentration of approximately 2 nM. The dissociation constant (K_d) derived from these data ranged from 10^{-9} to 10^{-10} M indicative of an exceptionally high affinity of the receptor for the steroid.

Binding specificity of the 8.6S component was analyzed by competition studies using sucrose gradient procedures. In all cases the unlabeled steroids were present in concentrations roughly 1000-fold excess over that of the [^{3}H]-17β-estradiol. The data in Table I are expressed as a percentage of the binding in the untreated (control) reaction. Of the steroids used, only those in the estrogen group (*i.e.*, estradiol, estriol, and estrone) brought about a significant inhibition of binding. Progesterone, testosterone, dihydrotestosterone, aldosterone, hydrocortisone, or triamcinolone had virtually no effect at the 1 μM level. Interestingly both the Parke-Davis compound, CN-55,945-27, at 10 μM and the cyanosteroid compound, CS-115, at 1 μM, brought about considerable reductions in binding to the 8.6S component probably reflecting their weak estrogenic properties.

Proof of the protein nature of the estrogen-binding moiety in the mammary gland was provided by experiments employing several degradative enzymes, as outlined in Figure 6. In this case, the deoxyribonuclease was pretreated at 35° for 90 min prior to use in order to inactivate contaminating protease activity, as described by Cox *et al.* (1971). Treatment with either deoxyribonuclease or ribonuclease (500 μg of each) did not reduce binding in the 8.6S peak of the gradient. Likewise, 0.1% phenol, which served as a control for the ribonuclease preparation, had no effect on the binding protein.

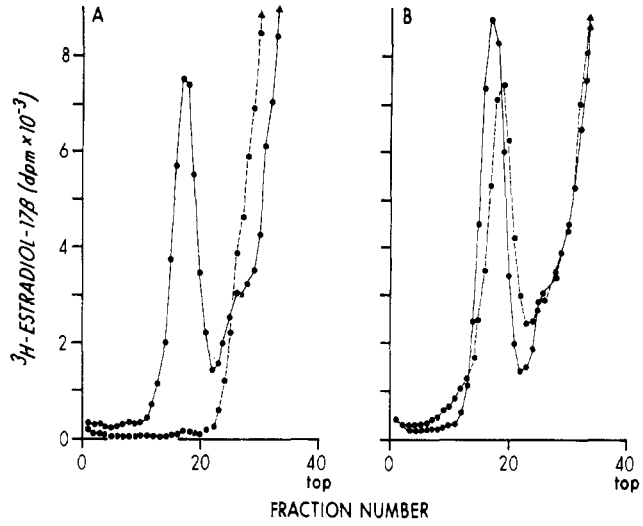

FIGURE 6: Effects of treatment with degradative enzymes on the cytoplasmic estrogen-binding protein. Supernatants from a homogenate of lactating mammary gland (13-days postpartum) were incubated for 1 hr with 2.4 nM [^{3}H]-17β-estradiol. Following the incubation, the mixtures were either left untreated (solid line, panel A) or reacted for 30 min with either Pronase (dashed line, panel A), ribonuclease (solid line, panel B), or deoxyribonuclease (dashed line, panel B) and the binding proteins were separated on sucrose gradients. Concentrations of enzymes given in text.

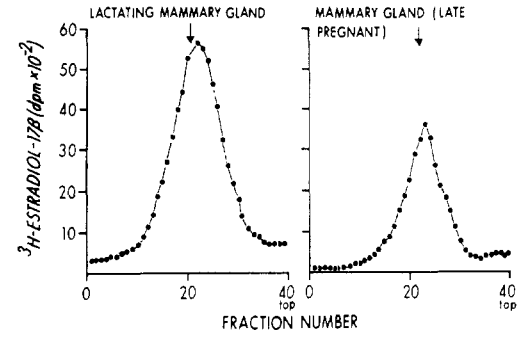

FIGURE 8: Isolation of nuclear bound [^{3}H]-17β-estradiol following administration *in vivo*. Female Fischer rats, either 13-days postpartum or late pregnant, were each injected subcutaneously with 0.26 μg of [^{3}H]-17β-estradiol (0.1 mCi) over the inguinal region (0.05 mCi/side). The rats were sacrificed 2 hr later; the inguinal mammary glands of each and the uterus of the pregnant female were removed, homogenized, and centrifuged at 105,000*g*. The supernatants (200 μl) were layered on 5–40% sucrose gradients. The nuclear pellets were extracted with an equivalent volume of 0.8 M KCl in Tris-EDTA buffer, rehomogenized lightly, and centrifuged at 105,000*g*. Each of the supernatants (400 μl) from this centrifugation was layered on a 5–20% sucrose gradient containing 0.4 M KCl. All of the gradients were then centrifuged at 308,000*g* for 15 hr. The arrows indicate the position of sedimentation of bovine serum albumin (4.6 S) under the same conditions.

p-Chloromercuribenzoate (not shown), a sulfhydryl inactivator, produced a significant, though not striking, reduction in binding (71% of control). The absence of a more complete inhibition remains unexplained. However, treatment of the receptor preparation with Pronase (500 μg/reaction) abolished the binding of 17β-estradiol to the 8.6S protein. The slight increases seen in receptor binding after treatment with deoxyribonuclease or ribonuclease apparently are due to layering of a slightly larger volume of the reaction mixture on each gradient (Figure 6).

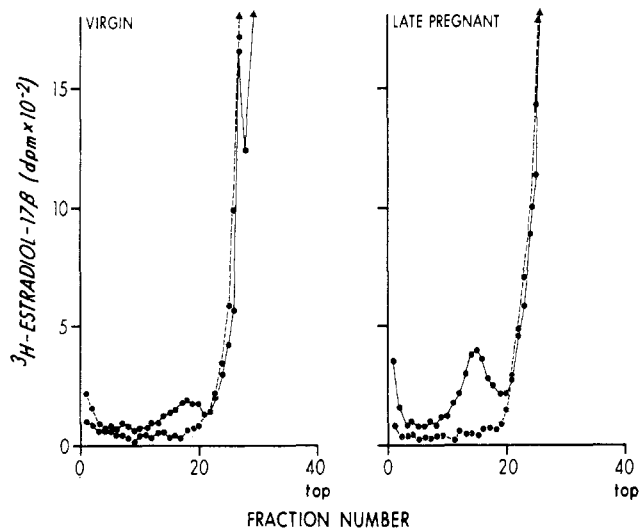

FIGURE 7: Separation of estrogen-binding proteins from the cytosols of mammary gland from pregnant or virgin rats. Supernatants were prepared from the mammary of a late pregnant rat and from the mammary glands of five virgin animals. Following incubation of each of these supernatants with 2.1 nM [^{3}H]17β-estradiol, in the presence (dashed line) or absence (solid line) of 10 μM CN-55,945-27, the reaction mixtures were layered on 5–40% sucrose gradients and centrifuged at 308,000*g* for 15 hr.

Sucrose gradient analyses of cytosol from mammary gland of pregnant animals (Figure 7) revealed a small but definite peak of binding activity in the 8–9S region of the gradient. A lower, but still discernible, peak of radioactivity was observed in the same region of the gradient layered with supernatant from the virgin mammary. The radioactivity profiles of reaction mixtures containing the Parke-Davis inhibitor exhibited a decrease in the amount of [^{3}H]estradiol bound in the 8–9S region, supporting further the specific nature of these receptors.

To assess the physiological significance of the estrogen receptor *in vivo*, animals were injected subcutaneously over each of the inguinal glands with 0.13 μg (50 μCi) of [2,4,6,7-^{3}H]-17β-estradiol. Little binding activity was observed in the 8–9S region of gradients layered with the 105,000*g* supernatant of mammary glands from either lactating or pregnant rats. However, a great deal of binding activity was detected in the 4–5S region of each gradient layered with a 0.4 M KCl extract of the nuclear pellet (Figure 8). A similar peak of binding activity was seen in the nuclear extract of uterine tissue taken from the pregnant animal (data not shown).

Discussion

Early in these investigations, it was determined necessary to establish a method of quickly assessing estrogen-binding capacity in a number of different tissue samples. For this purpose, the dextran-coated charcoal method, as originally described by Korenman (1968) and modified by Mèšter *et al.* (1970), was chosen. While this procedure proved satisfactory for the most part, several modifications were necessary. Some of these have been described in Materials and Methods. The chief concern with the charcoal adsorption assay is the loss of receptor activity due to degradation. To alleviate this concern, one must choose a time and temperature for both the binding reaction and the incubation with charcoal such that valid approximations of binding capacity can be achieved in a reasonable period of time.

The time course for binding *in vitro* of 17β-estradiol to its receptor in the lactating mammary gland is typical of those described for other systems (*e.g.*, uterus and breast carcinoma). Both Mèšter *et al.* (1970) and Feherty *et al.* (1971) reported significant losses of activity at 37° while binding at 0° required several hours to reach completion. The latter report suggested an intermediate temperature (30°) to carry out the incubation.

The conditions of the charcoal incubation (20 min at 25°) employed for the assay of receptors in mammary tissue are similar to those (10 min at 30°) used for measurements of binding capacity by human mammary carcinomas (Feherty *et al.*, 1971). Although higher temperatures sped the removal of nonspecifically bound steroid, they also produced an appreciable loss of binding activity apparently due to degradation of the steroid–receptor complex.

Similar to our observations, heat inactivation of the receptor protein *in vitro* appears to be a general characteristic of many steroid receptor proteins (*e.g.*, Vonderhaar *et al.*, 1970; Puca *et al.*, 1971). This is difficult to reconcile with results obtained *in vivo* (37°) where heat inactivation apparently does not occur to any great extent. Thus, there are rather obvious artificial qualities in the cell-free assay which are not mirrored in the physiological situation. Presumably, this is due to the loss of compartmentalization of intracellular degradative enzymes (*e.g.*, lysosomes) which exists *in vivo*.

The receptor appears to be stabilized when complexed with its ligand at 25°. While there is some degradation both in the presence and in the absence of estradiol, the rate of decay is greater without the steroid. In our experience there was considerable variation in the absolute amount of receptor decay from mammary gland preparation to preparation although there was always *less* decay in the presence of the ligand.

The isotopic profiles of cytosols, which were obtained from lactating mammary gland and labeled with [^{3}H]-17β-estradiol *in vitro*, illustrated a symmetrical peak of bound steroid sedimenting at 8.6S on sucrose gradients. Receptor proteins of similar size, *i.e.*, 250,000 molecular weight, have been reported in the uterus (Toft and Gorski, 1966; Jensen *et al.*, 1967a), anterior pituitary (Notides, 1970), and several mammary tumors of the rat (Jungblut *et al.*, 1967; Kyser, 1970; McGuire *et al.*, 1971; Wittliff *et al.*, 1972a) and human (Jensen *et al.*, 1971; Wittliff *et al.*, 1972b). Our report (Wittliff *et al.*, 1972a) and that of Shyamala and Nandi (1972) described a similar receptor in the cytosol of mammary gland from the rat and mouse. Earlier we (Wittliff *et al.*, 1972a) reported that the dissociation constant of the estradiol–receptor complex in mammary gland was 10^{-9}–10^{-10} M. Similar values have been found for receptors from a variety of estrogen target tissues (*cf.* Jensen and DeSombre, 1972).

A change in the sedimentation properties of the 8S receptor to a 4–5S component was observed under conditions of high salt. Similar results have been noted previously for receptors in other estrogen target organs (*cf.* Jensen and DeSombre, 1972) and have been interpreted as evidence for a subunit structure of the estrogen receptor. We also found no change in the quantity of labeled steroid bound by the 8S receptor when it was converted to the 4–5S form in the presence of KCl. These data suggest that there is a conservation of estrogen-binding sites on these components during the conversion.

The ligand specificity for binding to sites on the estrogen-binding protein in the cytosol of lactating mammary gland indicated a requirement for estrogens. These results are similar to those reported previously for binding to 8S receptor molecules in a number of other organs (*cf.* Jensen and DeSombre, 1972) including the mammary gland of the mouse (Shyamala and Nandi, 1972). Interestingly, a cyanosteroid, CS-115, which has low estrogenicity, competes well for specific estrogen-binding sites on the 8.6S receptor. Comparable effects have been demonstrated for the cytoplasmic receptor from immature rat uterus (J. L. Wittliff, P. C. Beers, R. Hilf, and A. Goldman, unpublished data). Inhibition of specific estrogen binding also was observed following preincubation with the competitive substance, CN-55,945-27. This compound, which is weakly estrogenic, has been used by a number of investigators (Notides, 1970; Jensen *et al.*, 1971; Wittliff *et al.*, 1972a,b; Shyamala and Nandi, 1972) to ensure measurements of absolute binding capacity although use of unlabeled estrogen in the competition assay gave comparable results. Inclusion of either competitive substance in a reaction as a measure of nonspecific binding would appear to be an important control.

A protein nature for the estrogen receptor is suggested by the drastic reduction in binding activity effected by pronase. The lack of effect by either ribonuclease or deoxyribonuclease suggests either that nucleic acid is not a component of the binding-protein structure or that it is not critical for maintaining a viable conformation. This is consistent with data reported for the uterine receptor (Jensen and DeSombre, 1969, 1972). *p*-Chloromercuribenzoate also inhibited the binding of [^{3}H]-17β-estradiol to the 8S receptors in lactating mammary gland. Loss of ligand binding in the presence of this sulfhydryl inhibitor has been reported in the uterine system also (Jensen *et al.*, 1967a,b).

Ovarian and adrenal steroid hormones, as well as several pituitary hormones, are known to play an important role in the development and growth of duct and lobuloalveolar cells of the mammary gland during lactogenesis (Cowie and Tindal, 1971). The formation *in vivo* of nuclear binding complexes (4–5 S) for 17β-estradiol in mammary gland from pregnant and lactating rats lends credibility to the physiological significance of these receptor proteins. Parallel gradients of the cytosols from each tissue, however, revealed low levels of binding in the 8–9S region. This result may be explained in terms of receptor depletion (translocation) under estrogen load (*cf.* Sarff and Gorski, 1971). The fact that significant binding was present in the nuclear fractions following administration of [^{3}H]-17β-estradiol *in vivo* indicates that the rate of turnover of these binding components is significantly less than their cytoplasmic counterparts. These results are consistent with, though not direct proof of, a two-step mechanism of estrogen action in the mammary gland.

Acknowledgments

The authors thank Dr. Elmer H. Stotz and Dr. Russell Hilf for their interest in this study as well as helpful suggestions with the manuscript.

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Cyclic Adenosine 3′,5′-Monophosphate Dependent Phosphorylation of Ribosomal Proteins from Bovine Anterior Pituitary Gland†

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ABSTRACT: Ribosomal proteins were labeled by incubation of pituitary slices with [^{32}P]orthophosphate or by self-phosphorylation of ribosomes with [^{32}P]ATP. Phosphorylation of ribosomal protein was stimulated by cyclic AMP in the cell-free system and by N^6,2′-*O*-dibutyryl cyclic AMP in intact cells. Ribosome-associated protein kinase activity is enhanced after exposure of ribosomes to Triton X-100. The properties of this ribosome-associated protein kinase are similar to those of a protein kinase isolated from the soluble fraction of bovine anterior pituitary gland. The ribosome-associated enzyme catalyzes transfer of the γ-phosphate group of ATP to ribosomal proteins, histones, and other substrates. The enzymatic activity is inhibited by Ca^{2+} and stimulated by cyclic AMP with an apparent K_m for this nucleotide of 1×10^{-7} M. Cyclic IMP and cyclic GMP stimulate protein kinase activity at respective apparent K_m values of 1×10^{-6} and 5×10^{-5} M. Both basal and cyclic AMP-stimulated activities are inhibited by Ca^{2+}. Treatment of unwashed ribosomes with high salt concentrations removed approximately 50% of the phosphorylated proteins, the remaining label being located mainly in one band after polyacrylamide gel electrophoresis of the proteins extracted by 4 M LiCl and 8 M urea. Radioactivity was located in phosphoserine and phosphothreonine residues of ribosomal proteins. The data suggest a possible mechanism by which cyclic AMP could enhance protein synthesis at the translational level in the anterior pituitary gland.

Cyclic AMP[1] has recently been shown to stimulate both total protein synthesis and the release of specific hormones (Labrie *et al.*, 1971a; Adiga *et al.*, 1971; Wilber *et al.*, 1969; Ratner, 1970; Jutisz and de la Llosa, 1970; Fleischer *et al.*, 1969) in the anterior pituitary gland. The stimulatory effect of cyclic AMP on protein synthesis is not blocked by actinomycin D, thus suggesting an effect of the cyclic nucleotide at the translational level (Labrie *et al.*, 1971a).

The presence of a cyclic AMP dependent protein kinase in many tissues in which cyclic AMP is presumably acting as second messenger has led to the proposal that the wide variety of effects elicited by cyclic AMP are mediated by activation of protein kinase (Kuo and Greengard, 1969). In fact, besides the well-known effect of phosphorylation on phosphorylase kinase (De Lange *et al.*, 1968; Walsh *et al.*, 1968) and on glycogen synthetase (Huijing and Larner, 1966; Schlender *et al.*, 1969; Soderling *et al.*, 1970), there is increasing evidence for a cyclic AMP induced phosphorylation of many other important enzymatic and nonenzymatic systems. These data pertain to the hormone-sensitive lipase

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[1] Abbreviations used are: cyclic AMP, cyclic adenosine 3′,5′-monophosphate; dibutyryl cyclic AMP, N^6,2′-*O*-dibutyryladenosine 3′,5′-monophosphate.