Specific Cytoplasmic Glucocorticoid Hormone Receptors in Lactating Mammary Glands†

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ABSTRACT: Specific binding of dexamethasone-*t* by the cytoplasmic fraction of mouse lactating mammary glands was studied. The specific binding component has the property of physiological glucocorticoid receptor by criteria of steroid specificity of binding and saturation of binding sites at low concentrations of the hormone. The specific binding of dexamethasone-*t* to the receptor was inhibited by additions of unlabeled triamcinolone, fluorocortisol, corticosterone, cortisol, and aldosterone and was unaffected by 17β-estradiol, testosterone, androstenedione, and spirolactone SC 14266. Steroid binding activity was also inhibited by pronase and mercurials but not by DNase and RNase. The receptors sediment in sucrose gradients at low ionic strength near 6 S and in sucrose gradients containing 0.4 M KCl near 4 S. The binding reaction had a rate constant for association at 0–4° of 2×10^6 M^{-1} min^{-1}. The equilibrium dissociation constant for the reaction was 6×10^{-9} M and the binding data plotted by the Scatchard technique yielded a straight line indicating that only a single class of specific receptors was detectable. It is suggested that various adrenal corticoids regulate lactogenesis in mammary glands through the specific glucocorticoid receptors.

During pregnancy mammary glands undergo cellular differentiation leading to secretion in the epithelial cells, and the mechanism by which lactation is initiated has been a subject under much discussion. *In vivo* studies have shown that adrenal corticoids may be rate limiting to lactation (Lyons *et al.*, 1958; Nandi, 1959). Similarly mammary gland explants from midpregnant mice cultivated *in vitro* with insulin, cortisol, and prolactin undergo cellular differentiation as indicated by increased rates of synthesis of milk proteins and as well as induction of an alveolar secretory histologic appearance (Stockdale *et al.*, 1966).

Cortisol can be replaced by aldosterone and corticosterone in the culture medium to produce the same effect (Rivera, 1964). Turkington *et al.* (1967) measured the relative activities of various steroid hormones with respect to the synthesis of milk proteins but these studies did not provide any information with regard to specific cellular molecules with which the steroids were interacting. Tucker *et al.* (1971) had reported that bovine mammary cells cultured *in vitro* had a specific mechanism for the binding of cortisol but did not characterize the binding proteins. Several studies show that the interaction of steroid hormones with specific receptor molecules in their target tissues is an early prerequisite in their mechanism of action (Raspe, 1971). Therefore, studies were carried out to examine the nature of binding of those steroids known to be lactogenic in the mammary glands. The present report shows that there are specific glucocorticoid receptors in the cytoplasm of lactating mammary glands and discusses the relation between these receptors and the biological activity of the various steroids with respect to lactation.

Materials and Methods

BALB/c mice used in this study were from our own colony and were between 7–10 days of lactation. In some studies the animals were subjected to bilateral adrenalectomy while under anesthesia. Adrenalectomized animals were maintained on a regular laboratory diet with 0.9% saline in their drinking water.

Dexamethasone-*t* (specific activity 35.2 Ci/mmol) and cortisol-*t* (specific activity 46.3 Ci/mmol) were purchased from New England Nuclear Corp. and were chromatographically pure. Radioactive steroids were prepared in ethanol after evaporation of benzene in which they had been dissolved originally. Nonradioactive steroid solutions were prepared in ethanol. The ethanol content in the final reaction mixture did not generally exceed 2%. However, up to 6% concentration of ethanol in reaction mixtures did not alter the steroid binding reactions.

Buffers. Buffers were as follows: TESH, 0.01 M Tris–0.0015 M EDTA (pH 7.4) containing 0.012 M thioglycerol; TKE, 0.01 M Tris–0.0015 M EDTA–0.1 M KCl; TESH–KCl, 0.01 M Tris–0.0015 M EDTA–0.4 M KCl (pH 7.4) with 0.012 M thioglycerol.

Tissue Homogenization and Fractionation. The animals were sacrificed by cervical dislocation. The mammary glands were removed immediately and chilled. All preparative procedures were carried out at 0–4°. The mammary glands were homogenized in TESH buffer using an all-glass tissue grinder. Generally 1 g of tissue was homogenized in 1 ml of buffer. The homogenate was centrifuged at 105,000*g* for 1 hr. The supernatant was designated the cytoplasmic extract and was used in all the binding studies.

The binding reaction was performed at 0–4° by incubation of aliquots of cytoplasmic extracts (ordinarily in a total volume of 0.4 ml) with known concentrations of the radioactive steroid, either alone or in the presence of competing nonradioactive steroid. After the incubation period (120 min unless stated otherwise), the binding in the cytoplasmic extract was studied by an assay using a Sephadex G-25 filtration technique.

Sephadex G-25 was swollen in TKE buffer for 24 hr at 23°. The swollen gel was cooled at 4°, and fines were removed by aspiration. The gel was packed in the cold in a 20 × 1 cm column using TKE buffer. A 0.4-ml aliquot of the sample was

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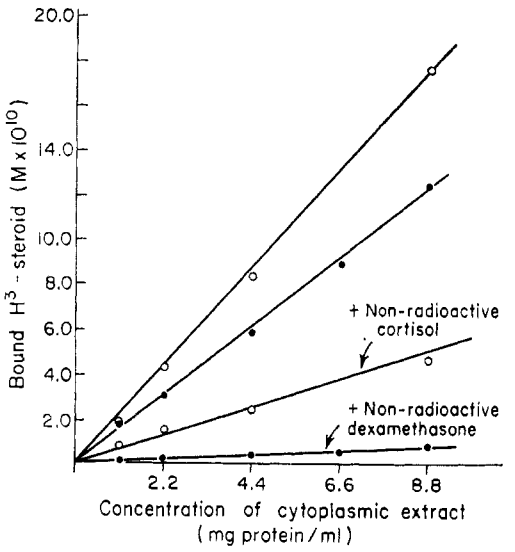

FIGURE 1: Binding of cortisol-*t* and dexamethasone-*t* in the cytoplasm of lactating mammary glands with increasing concentration of protein. Aliquots (0.4 ml) of cytoplasm containing various concentrations of protein were incubated with 1.7×10^{-8} M of dexamethasone-*t* (●) or cortisol-*t* (○) as indicated at 4° for 2 hr. The unlabeled steroids were added at a concentration of 10^{-5} M. Bound radioactivity in each incubation was assayed on Sephadex G-25 columns as described in Materials and Methods.

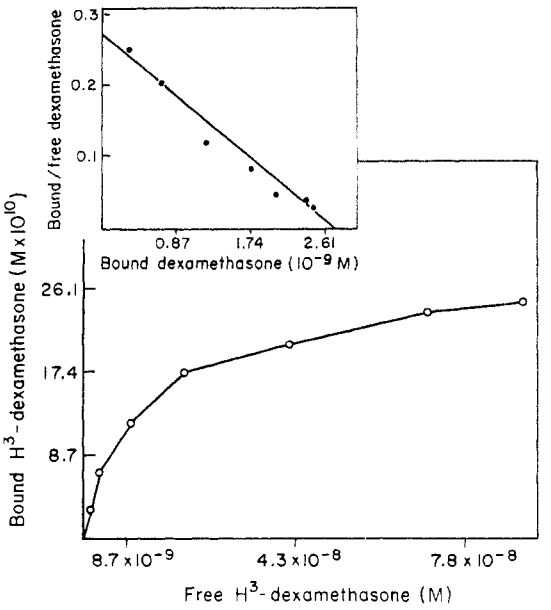

FIGURE 2: Specific cytoplasmic binding of dexamethasone-*t*. Aliquots (0.4 ml) of cytoplasmic extract (22.0 mg of protein/ml) were incubated with various concentrations of dexamethasone-*t* for 2 hr at 0–4°. The data, represented as molar bound steroid in the total incubation, are plotted as a function of free steroid concentration at equilibrium. The free steroid was determined by subtracting the bound steroid from the total steroid present in the incubation mixture. The inset presents the Scatchard plot of the binding data.

applied on the column together with 0.05 ml each of Dextran Blue and Chlorophenol Red solutions to serve as indicator dyes. The samples were eluted with TKE buffer. All operations were performed at 0–4°. Bound steroid was eluted in the volume with the blue dye while the free steroid was retained by the gel and eluted with the red dye. Fractions (0.5 ml) were collected at a flow rate of 50–60 ml/hr. Aliquots (0.2 ml) of the fractions eluted in the void volume were counted to determine the amount of bound steroid.

Sucrose Density Gradient Analysis. The samples to be analyzed (0.2–0.3 ml) were layered on a 4.5-ml gradient of 10–30% sucrose in TESH or TESH–KCl buffer and were centrifuged in a Beckman Model L-2 ultracentrifuge. The tubes were pierced and 32 fractions were collected directly in counting vials. Following the addition of 3 ml of ethanol and 10 ml of scintillation fluid, the radioactivity in each sample was determined. Approximate sedimentation coefficients were determined by the method of Martin and Ames (1961), using yeast alcohol dehydrogenase (7.6 S), liver alcohol dehydrogenase (5 S), and catalase (11 S) as standards.

Extraction and Chromatography of Bound Radioactivity in the Cytoplasmic Extracts. The procedure used to extract and chromatograph bound radioactivity from the cytoplasmic extract was essentially that described by Baxter and Tomkins (1971).

After incubation of cytoplasmic extracts with 10^{-8} M dexamethasone-*t* at 4° for 2 hr, the specifically bound radioactivity was recovered from Sephadex G-25 eluates. The radioactivity from the effluent fractions was extracted with dichloromethane and the extract was subjected to thin-layer chromatography. The solvent systems used were (1) ethyl acetate–chloroform (65:35 or 50:50) and (2) acetone–methylene chloride (30:70). Dexamethasone migrated around 0.25 in system 1 and about 0.5 in system 2 relative to the solvent front.

Protein concentrations of cytoplasmic extracts were determined by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard. DNA contents of the tissue homogenate were measured by the method of Webb and Levy (1955) using calf thymus DNA as the standard.

Radioactivity was measured in a liquid scintillation spectrometer (Beckman, LS 250) with 55% counting efficiency for tritium.

Results

Initial studies carried out using cortisol-*t* or dexamethasone-*t* as radioactive steroids to study the nature of the binding reaction revealed that there was considerably more nonspecific binding with cortisol-*t* than with dexamethasone-*t* (Figure 1). The amount of radioactivity remaining bound to the extract in incubations containing an excess of competing nonradioactive steroid is referred to as the nonspecific binding. Thus the nonspecific binding is due to the steroid which associates with molecules other than the receptor molecules in the cytoplasmic extract. At any given protein concentration, the nonspecific binding with cortisol-*t* (10^{-8} M) was found to be around 26% of the total binding whereas it was only about 7% when dexamethasone-*t* was used as the radioactive steroid. However, as will be shown later, the two radioactive steroids bind to the same receptor in the cytoplasm. Therefore, dexamethasone-*t* was used as the radioactive steroid in most of the binding experiments reported here. Figure 1 also shows that the amount of dexamethasone-*t* bound to the complex was linearly related to the protein concentration of the cytoplasm. All binding assays reported in this study were performed in this linear range of protein concentration.

Assay of Specific Dexamethasone Binding by Cytoplasmic Extracts. The cell-free dexamethasone binding in the cytoplasmic extracts of mammary glands was studied by gel filtration experiments. Figure 2 shows the amount of specifically bound dexamethasone-*t* as a function of the free steroid concentration of the incubation medium. The specific binding

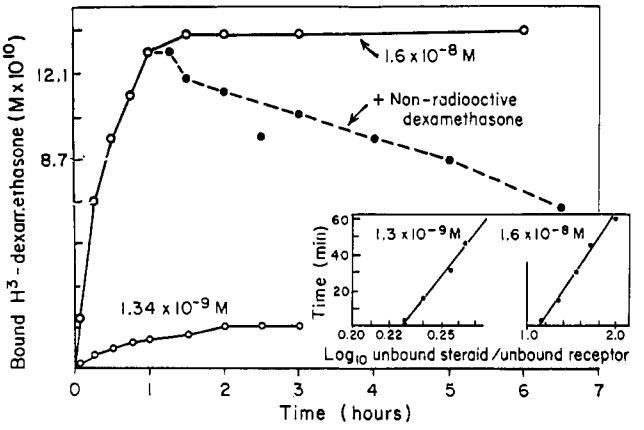

FIGURE 3: Kinetics for dexamethasone association and dissociation with the cytoplasm. Dexamethasone-*t* was added to the cytoplasmic extracts at concentrations as indicated and 0.4-ml aliquots were withdrawn at various time intervals and assayed for binding. Also as indicated, at the end of 1 hr, nonradioactive dexamethasone (1.6×10^{-5} M) was added to a portion of one of the incubations and assayed at various time intervals. The inset represents the analysis of the kinetic data on association of dexamethasone binding to the cytoplasmic receptors. Time is plotted (for the first 60 min) as a function of log (free dexamethasone concentration/free receptor concentration). The free dexamethasone concentration was determined by subtracting the total of the bound steroid from the total dexamethasone concentration. The total receptor concentration was assumed to be equal to the total bound dexamethasone (at equilibrium) with 1.6×10^{-8} M dexamethasone-*t*.

data are also plotted by the Scatchard (1949) technique and yield a single straight line indicating that only a single class of binding sites is present. The dissociation constant of the binding of the steroid to the binding protein was estimated to be 6×10^{-9} M. The concentration of binding sites in this experiment was estimated to be 2.4×10^{-9} M.

On the basis of the Scatchard plot, it can be assumed that one molecule of the steroid is bound to one molecule of receptor. Thus assuming 6.6×10^{-6} μg of DNA/cell nucleus, based on the DNA content of lactating mammary glands (1.15 μg of DNA/mg of tissue) there are about 14000 receptor sites/cell. The average dissociation constant as determined from five experiments using various concentrations of cytoplasmic extract was estimated to be 7.7×10^{-9} M (SD = 0.5×10^{-9} M).

Kinetics and Reversibility of Specific Binding. The time course of association and dissociation at 4° of the dexamethasone receptor complex is shown in Figure 3. The reaction is readily reversible as illustrated by the displacement of bound radioactivity with an excess of nonradioactive dexamethasone. A plot at initial stages of binding, of time *vs.* log (free steroid concentration/free receptor concentration), was linear, thereby indicating a second order kinetics of association. The mean association rate constant as determined in three experiments was 2×10^{6} M^{-1} min^{-1}. The rate of dissociation for the binding reaction obeyed pseudo-first-order kinetics since a plot of log (bound steroid concentration) *vs.* time was linear (Figure 4). The dissociation rate constant was determined from the time of half-dissociation (211 min for the experiment shown in Figure 3) and was estimated to be 3.2×10^{-3} min^{-1}. The mean dissociation rate constant as determined in four experiments was 2.2×10^{-3} min^{-1}.

From the dissociation and association rate constants, the equilibrium (dissociation) constant was calculated to be 9.5×10^{-10} M (1.0×10^{-10}–1.8×10^{-9} M) as compared to 6×10^{-9} M determined from the equilibrium data.

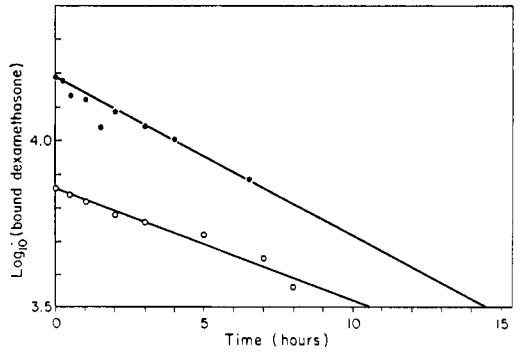

FIGURE 4: Analysis of the kinetic data on dissociation of dexamethasone binding to the cytoplasmic receptors. (●) The dissociation data from Figure 3 was recalculated as log (bound steroid concentration) and plotted as a function of time after adding nonradioactive dexamethasone. (○) The dissociation data from a separate experiment where dexamethasone-*t* was used at a concentration of 6.5×10^{-9} M and nonradioactive dexamethasone was added at 6.5×10^{-6} M. Other experimental conditions were identical with that in Figure 3.

Nature of Bound Radioactive Dexamethasone. After incubation of mammary gland cytoplasm with dexamethasone-*t*, the bound steroid was extracted and chromatographed as described in Materials and Methods. Virtually all of the bound radioactivity migrated with authentic dexamethasone. This indicated that the cytoplasmic receptors primarily bind unaltered steroid.

Sucrose Density Gradient Analyses. To determine the approximate molecular weight of the binding protein, mammary gland cytoplasm was incubated with dexamethasone-*t* and assayed for binding on sucrose density gradients. The results are shown in Figure 5.

In homogenizing buffers of low ionic strength (TESH

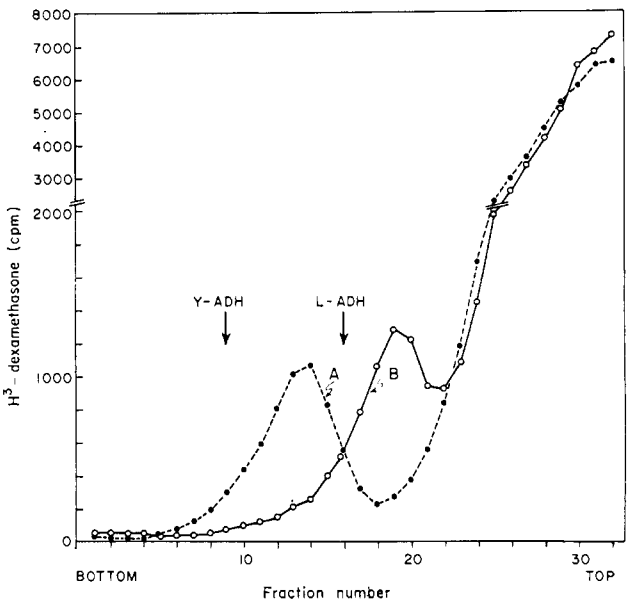

FIGURE 5: Sucrose density gradient patterns of cytoplasmic extract (22.2 mg of protein/ml) of mammary glands incubated at 4° with dexamethasone-*t* (1.5×10^{-8} M): (A) 10–30% gradients made up in TESH buffer; (B) 10–30% gradients made up in TESH-KCl buffer. Yeast alcohol dehydrogenase (Y-ADH) and liver alcohol dehydrogenase (L-ADH) were used as standards to calculate sedimentation coefficients. All gradients were centrifuged at 48K in a Beckman SW 50.1 rotor at 4° for 16 hr. The migration of standards was estimated by enzymatic assays on the effluent fractions.

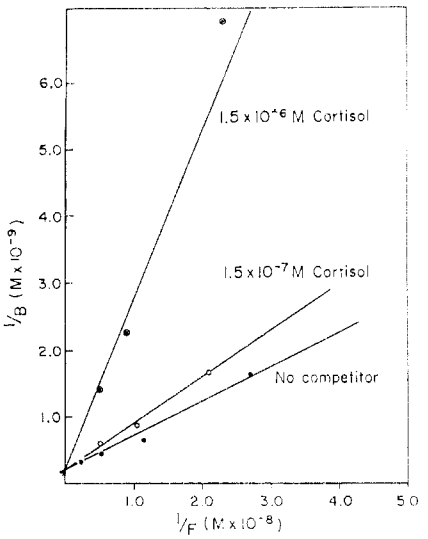

FIGURE 6: Competitive inhibition of dexamethasone binding by hydrocortisone. Binding of dexamethasone-*t* by mammary gland cytoplasmic extracts was determined at various concentrations of dexamethasone-*t* and competing nonradioactive cortisol. The coordinates refer to the reciprocal of the molar concentration of bound dexamethasone (1/(B)) or free dexamethasone (1/(F)).

buffer), the binding protein sediments in the region of 6 S. However, when the extracts are incubated with dexamethasone-*t* and centrifuged on high-salt gradients (TESH-KCl buffer), the specific receptors sediment near 4 S.

Competition of Other Steroids for Specific Binding. Various nonradioactive steroids were tested for their ability to compete with dexamethasone-*t* for the receptor binding sites. The results of these experiments are presented in Table I. The nonradioactive steroids in all experiments were added at the concentrations as indicated. At 10^{-7} M, the ability of various nonradioactive steroids to compete for specific binding of dexamethasone-*t* fell roughly into three classes: inactive, moderately active, and highly active. Estradiol, testosterone, androstenedione, 17α-hydroxyprogesterone, 11α-hydroxyprogesterone, cortisone, and spirolactone SC 14266 were inactive. Aldosterone, cortisol and progesterone were moderately active while triamcinolone, dexamethasone, fluorocortisol, corticosterone, and prednisolone were highly active. At higher concentrations (10^{-6} M or above) of the competing steroids, the moderately active compounds became highly active.

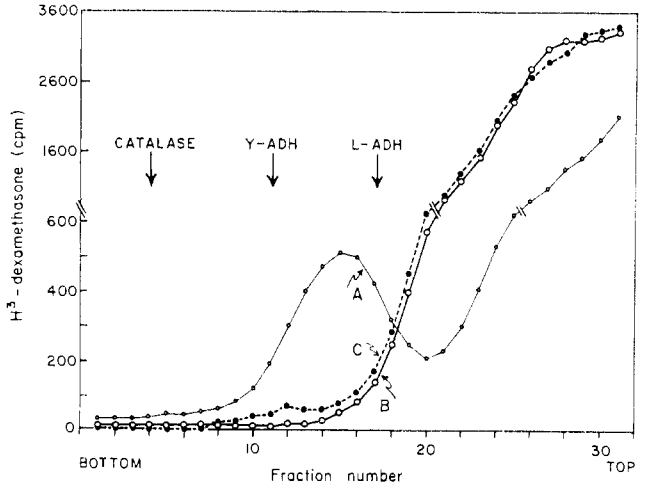

FIGURE 7: Sucrose density gradient patterns of cytoplasmic extracts (labeled with 6.7 × 10^{-9} M dexamethasone-*t*: (A) no addition; (B) 6.7 × 10^{-7} M unlabeled dexamethasone; (C) 7.6 × 10^{-7} M unlabeled cortisol. The gradients were 10–30% sucrose in TESH buffer. Catalase, yeast alcohol dehydrogenase (Y-ADH), and liver alcohol dehydrogenase (L-ADH) were used as standards to calculate sedimentation coefficient. The gradients were centrifuged in Beckman SW 50.1 rotor at 44K for 16 hr at 4°. The migration of standards was determined by enzymatic assays on the effluent fractions.

TABLE I: Effect of Various Unlabeled Steroids on the Binding of Dexamethasone-*t* to the Cytoplasm of the Mammary Gland.

Nonradioactive Steroid	Bound Radioactivity (% of Control)[a]
None	100
17β-Estradiol	100
Androstenedione	100
Spirolactone SC 14266[b]	100
Cortisone	98.9
17α-Hydroxyprogesterone	98.7
11α-Hydroxyprogesterone	93.9
Testosterone	87.0
Deoxycorticosterone	65.3
Aldosterone	58.8
Progesterone	55.0
Cortisol	44.3
11β-Hydroxyprogesterone	28.7
Corticosterone	25.3
Flourocortisol	10.7
Prednisolone	11.0
Triamcinolone	9.68
Dexamethasone	0
Aldosterone	8.83
Progesterone	4.51
Deoxycorticosterone	1.29
Cortisol	1.44

[a] Aliquots of cytoplasmic extracts were incubated at 4° in a total volume of 0.4 ml with 6.2 × 10^{-9} M dexamethasone-*t* alone or with competing nonradioactive steroids at 4° for 2 hr. Nonradioactive steroids were added at 6.2 × 10^{-7} M in all cases except the last four where the concentration was 6.2 × 10^{-6} M. Binding was measured by gel filtration experiments as described in Materials and Methods. Nonspecific binding has been subtracted. The 100% value was 21.5 cpm/mg of tissue or 24.2 cpm/mg of protein. The mean values of the three closely agreeing separate determinations are reported. [b] Spirolactone SC 14266 (Searle Co.) was kindly supplied by Dr. I. S. Edelman.

Nature of Interaction of Competing Steroids with the Binding of Dexamethasone-t to the Receptors. It was of interest to see if the competing steroids were interacting on the receptor directly at the binding site for dexamethasone or due to alterations in receptors elsewhere. The data in Figure 6 show that cortisol behaves as a competitive inhibitor of dexamethasone binding to the mammary gland cytoplasmic extract. This is further verified by sucrose density gradient analyses where the binding of dexamethasone-*t* observed in the 6S region of the gradients was abolished in the presence of unlabeled cortisol (Figure 7). Similarly, progesterone also be-

haves as a competitive inhibitor of dexamethasone binding to the mammary gland cytoplasm (data not shown).

General Properties of the Receptors. Binding activity was totally destroyed by incubating cytoplasmic extracts with trypsin (500 μg/ml) or Pronase (100 μg/ml). Similar treatment with DNase (bovine pancreas, 100 μg/ml) and RNase (bovine pancreas, 100 μg/ml) had no effect on the binding. Therefore the receptors appear to be proteins.

In crude extracts the receptors are more stable when complexed with steroid than in the free state. However, even in the presence of steroids, the receptors were unstable at 4° (approximately 50% activity was lost in 24 hr).

Effects of Adrenalectomy. The dexamethasone-*t* binding capacity of the cytoplasmic fraction of the mammary glands from animals adrenalectomized for 48 hr was consistently higher than the control animals and an average binding capacity was 50% higher than the control animals (range 20–50%).

Cellular Source of the Specific Binding Proteins. Although lactating mammary glands consist predominantly of epithelial cells (Nicoll and Tucker, 1965), it was nevertheless important to check the contribution by fat cells to the specific steroid binding. Parenchymal free fat pads were prepared according to the technique of DeOme *et al.* (1959) and the cytoplasm was assayed for specific binding of dexamethasone-*t*. The fat pad cytoplasm did not have any detectable levels of the specific binding protein (data not shown). Thus the specific binding protein seems to be present only in the epithelial cells.

Discussion

The present studies show that the lactating mouse mammary glands contain a specific protein that reversibly binds certain adrenal steroid hormones. The specific protein satisfies all the general criteria by which hormone receptors are characterized. The steroid–receptor complex sediments in the region of 6 S on low ionic strength sucrose gradients and in the region of 4 S on higher ionic strength sucrose gradients.

In addition to the receptors, mammary gland cytoplasm also contains low-affinity binding sites for cortisol. Most likely, the low-affinity binding sites are at least in part due to the plasma corticoid binding globulin (Seal and Doe, 1966). In addition lactating mammary glands contain several milk proteins and these probably also bind cortisol. Nonspecific binding of estradiol-*t* has been observed with lactating mammary gland cytoplasm (Shyamala and Nandi, 1972).

The estimates of total available binding sites (approximately 14000 sites per cell) is higher than the 7500 binding sites for cortisol present in cultured bovine mammary cells (Tucker *et al.*, 1971). The difference may be either due to culture condition or to the different species of animals used. The estimate reported here is, however, in close agreement with that reported for the glucocorticoid receptors in fetal lung (Ballard and Ballard, 1972).

It should be noted that most of the steroids used in the competition experiments had both glucocorticoid and mineralocorticoid activity. However, the affinity of various steroids to the receptor seems to be related to their potency as a glucocorticoid. This is supported by the fact that spirolactone, an anti-mineralocorticoid, had no affinity for the receptor molecule. Similarly, triamcinolone, which is virtually devoid of mineralocorticoid activity but is a potent glucocorticoid, had very strong affinity to the receptor. However, cortisone is a notable exception. This steroid is known to be a glucocorticoid *in vivo* only by virtue of its conversion to cortisol. Since the binding studies were performed at 4° where there is no significant metabolic transformation, it is likely that the lack of binding of cortisone to the receptor is due to lack of conversion to cortisol. Similar observations have been reported for rat thymus cells (Munck and Wira, 1971).

Although it is clearly established that adrenal steroids play a crucial role in mammogenesis and lactogenesis (Lyons *et al.*, 1958), the exact pathway through which these steroids exert their influences is not clearly understood. Although cortisol is most widely used in organ culture systems as the steroid to promote secretion in mammary gland explants, a variety of other steroids can also be substituted for cortisol (Turkington *et al.*, 1967). Except for cortisone, the data reported here on binding affinity of various steroids to the receptor correlate closely with the ability of various steroids to replace cortisol in causing secretion in mammary explants maintained in organ culture (Turkington *et al.*, 1967). However, it is likely that during organ culture at 37°, there was a significant conversion of cortisone to cortisol in the mammary glands resulting in secretion. Therefore, it is suggested that the ability of adrenal steroids to cause secretion in the mammary glands is a result of their role as glucocorticoids and the specific glucocorticoid receptors present in the tissue are responsible for the mediation of their actions.

Finally, the ability of progesterone to cause inhibition of binding of dexamethasone-*t* to the receptor needs explanation. The exact role of progesterone in mammogenesis and lactogenesis is still not clear. Progesterone has been implicated as an inhibitor of lactogenesis (Wilkman and Davis, 1968; Kuhn, 1969; Davis *et al.*, 1972; Denamur and Delouis, 1972). The data presented here therefore raise the possibility that progesterone may act as an inhibitor of lactation by competing with the glucocorticoids for the receptor sites. Extension of our present studies on the interaction of progesterone with the glucocorticoid binding sites may be helpful in elucidating the role of progesterone during lactogenesis and lactation.

Acknowledgment

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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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[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.