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CHARACTERIZATION OF MOUSE MAMMARY TISSUE ESTROGEN RECEPTORS UNDER CONDITIONS OF DIFFERING HORMONAL BACKGROUNDS

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SUMMARY

The receptor-like nature of previously-observed specific 4*S* estrogen binding in adult virgin female mouse mammary tissue cytosol has been further substantiated. The binding component does not arise from a plasma protein contaminant, nor is it an α-fetoprotein-type binder as evidenced by lack of affinity for diethylstilbestrol. The 4*S* protein binds 17β-estradiol with very high affinity ($K_A = 3 \times 10^{10} M^{-1}$), transforms to a 5.5*S* form which translocates to nuclear binding sites under appropriate *in vitro* conditions. *In vivo* cytosol-nuclear binding distribution results are analogous to other estrogen receptor systems. During pregnancy, the 4*S* receptor is gradually replaced by an 8*S* form. This 8*S* complex does not represent a concentration-dependent aggregation of 4*S* subunits, as does the 8*S* receptor encountered in aged mice. The change in receptor concentration throughout pregnancy and lactation reflects the overall increase in tissue weight, and no major changes occur in specific binding activity, expressed on the basis of DNA content, cytosol protein concentration or unit weight of tissue. A single injection of 0.2 mg of prolactin to an adult virgin mouse results in an acute stimulation of receptor number, and a shift in molecular form from 4*S* to 8*S*. The effect is more pronounced if 3 successive daily injections of prolactin are administered, and thyroidectomy enhances the sensitivity of the receptor system to prolactin stimulation. Although the equilibrium binding constants are similar for the 4*S* and 8*S* complexes, analysis of the kinetics of formation and dissociation revealed that the 8*S* complex forms less readily, but has a half-life equal to 3 times that of the 4*S* complex. The differences in the amount, molecular nature and reactivity of the mammary tissue estrogen receptors, and the influence of specific hormonal elements on these parameters, set the stage for analysis of cellular response and responsiveness under diverse physiological and pathological conditions.

INTRODUCTION

In the course of its differentiation and growth, mammary tissue is subjected to the interplay of a complexity of hormones which may act independently or synergistically [1–3]. Thus, at any given time, the cells are exposed to a variety of stimuli, and the task of isolating and describing any one of these becomes formidable. Through the efforts of a large number of investigators, a description of the estrogen receptor system in mammary tissue has emerged and has found widespread application as a diagnostic basis for endocrine therapy of human breast tumors [4].

Although the basic features of estrogen receptor interactions in proliferating mammary tissue have been delineated [5–7], little is known of the endogenous regulation of receptor levels or activity, either in the normal or the neoplastic cell. We have recently reported on some aspects of specific estrogen binding in the normal mouse mammary gland [8]. The observation that diffferent molecular forms of the receptor, as analyzed by sucrose density gradient centrifugation under invariant conditions, were present in quiescent *vs* stimulated mammary tissue prompted a more extensive exploration of this phenomenon. The present study represents our progress to date in this area. Some of these results have been recently presented [9].

MATERIALS AND METHODS

Female mice of the $C3H^+$ or A^+ strain from our own colony were used at various ages as indicated in the text. The animals were killed by cervical dislocation and the second, third and fourth pairs of mammary glands were collected in cold TD buffer (0.01 M Tris, 0.005 M dithiothreitol, pH 8.0) for sucrose gradient studies, or TED buffer (TD buffer supplemented with 0.0015 M Na_2EDTA) for experiments involving initial ammonium sulfate fractionation. All subsequent procedures, except as specifically noted otherwise, were performed at 0–4°C.

For receptor quantification and analysis of equilibrium binding parameters, cytosol (105,000 *g* ultracentrifugal supernatant fraction of tissue homogenate) in TED buffer was brought to 40% saturation with ammonium sulfate and the precipitate was resuspended in buffer. To test the nature of the receptor precipitated in this manner, dialysis against a large

volume of TD buffer was performed and the dialyzed sample was equilibrated with [^{3}H]-17β-estradiol and applied to a 5–20% linear sucrose gradient, with centrifugation at 225,000 *g* for 15.5 h. The dialysis step prevented the partial aggregation of receptor occasioned by the presence of EDTA, which was evidenced by accumulation of radioactivity at the bottom of the tube. The use of EDTA in the original buffer prevented transformation of the cytosol receptor to its nuclear form during ammonium sulfate precipitation, as described by DeSombre *et al.*[10]. Saturation binding curves were constructed from binding levels observed when equal aliquots of cytosol were incubated with increasing amounts of [^{3}H]-17β-estradiol in the presence or absence of a 100-fold molar excess of unlabeled 17β-estradiol. At equilibrium, acidic proteinaceous binding species were precipitated as steroid–protein complexes with protamine sulfate, and the specific radiolabeled binding moieties were measured by liquid scintillation spectrometry (Beckman LS230) with Permablend II (Packard) in toluene (5 g/l) as counting medium. Details of the assay have been published [8, 11]. Binding parameters were determined by the direct linear plot method of graphical analysis [12–14], with cytosol protein levels estimated as according to Lowry *et al.* [15].

Tissue dry weight was determined following 3 successive extractions with acetone and subsequent drying under vacuum, and therefore represents the defatted dry weight. DNA was measured by reaction with *p*-nitrophenylhydrazine, according to Webb and Levy [16].

The *in vitro* nuclear transformation and translocation studies were performed as described by Jensen *et al.* [17], using nuclear preparations purified by the method of Chauveau *et al.* [18]. Association and dissociation rate kinetic experiments were conducted and analyzed according to our previously-published protocol [19, 20].

[2,4,6,7-^{3}H]-Estradiol-17β (100 Ci/mmol) was purchased from New England Nuclear and purified to >98% radiochemical purity by descending paper chromatography. Diethylstilbestrol was obtained from Steraloids. CI-628 was a gift from Dr. Jerry Reel, Parke-Davis Co. Rat prolactin was kindly supplied by NIAMDD, National Institutes of Health.

RESULTS

In a previous study [8], we found that mammary tissue cytosol from adult virgin mice contains pronase-sensitive estrogen-binding components which sediment almost exclusively in the 4*S* region of a sucrose gradient. Steroidal specificity analyses have shown that approximately 50% of this binding is displaceable by unlabeled estradiol-17β (100-fold molar excess over the labeled steroid) or CI-628 (5,000-fold excess), but not by progesterone or testosterone. In all sucrose gradients to be presented in this paper, the term "specific binding" will be used to label the ordinate, indicating that the patterns shown have been corrected for the non-displaceable nonspecific component of the binding.

The question of protein specificity was addressed by eliminating the possibility that the specific 4*S* binding of estradiol-17β represented contamination by residual α-fetoprotein or some other estrogen-binding plasma protein. In panel A of Fig. 1 are shown saturation binding curves of adult virgin cytosol, either alone or with the addition of diluted

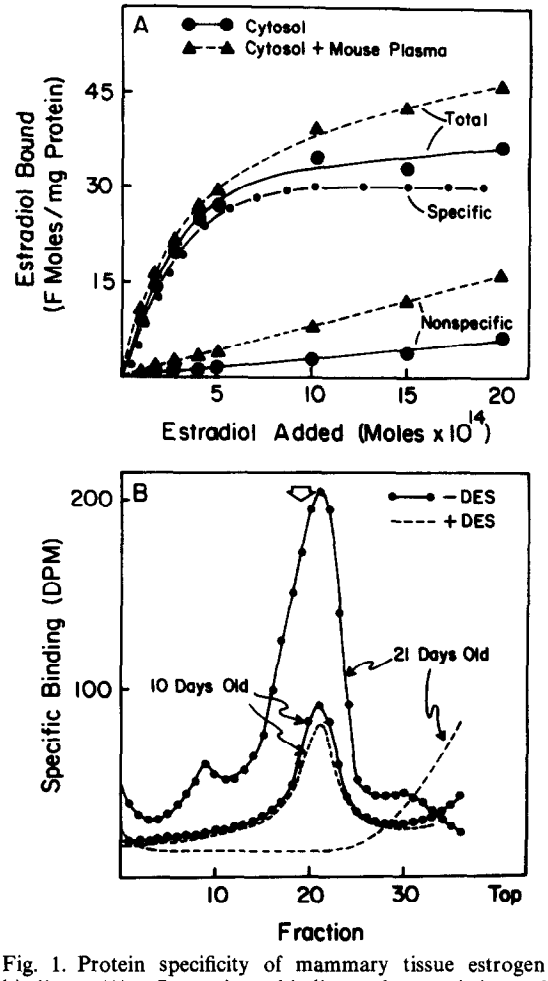

Fig. 1. Protein specificity of mammary tissue estrogen binding. (A). Saturation binding characteristics of ammonium sulfate-fractionated cytosol from virgin adult mice were determined in the presence or absence of a supplemental addition of 1:30 diluted plasma from 7-day-old female mice. Constant amounts of cytosol were incubated with increasing concentrations of [^{3}H]17β-estradiol either with (nonspecific binding) or without (total binding) concomitant addition of excess unlabeled 17β-estradiol. Incubations were for 18 h at 4°C, and the bound species were precipitated with protamine sulfate. Specific binding (dot-dashed line) is the resultant of total minus nonspecific. (B). Specific 17β-estradiol binding (i.e., binding in the presence of [^{3}H]-17β-estradiol minus that in the presence of [^{3}H]-17β-estradiol plus unlabeled 17β-estradiol) was determined in cytosol of 10-day-old or 21-day-old mice by sucrose gradient centrifugation analysis, and is plotted as the solid line. The estrogenic specificity was then further delineated by introduction of diethylstilbestrol (DES) and assessment of its effect on the 17β-estradiol-specific binding.

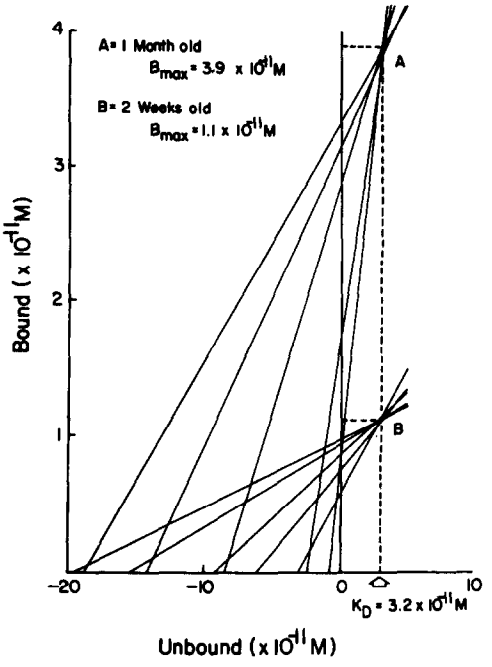

Fig. 2. Direct linear plot analysis of mammary estrogen receptor equilibrium binding parameters. Ammonium sulfate-fractionated cytosol from animals either 2 weeks or 4 weeks of age was incubated to equilibrium with varying amounts of [^{3}H]-17β-estradiol. Specific binding was assessed and molar quantities of unbound and bound steroid were determined at each point. The data were then plotted in K_D,B_{max} parameter space, according to the following arrangement of the Michaelis–Menten equation: $B_{max} = B + B/F \cdot K_D$.

plasma from young mice. The presence of the plasma results in an increased level of total estrogen binding which is matched by an increase in nonspecific binding. Thus, the specific binding curve for the cytosol, representing the algebraic difference between total and nonspecific, is independent of the presence of plasma in the sample. This indicates that the specific binding seen in cytosol cannot be accounted for by the presence of estrogen-binding plasma proteins, and is consistent with the observation that sex steroid-binding globulin is not precipitated in the protamine sulfate receptor assay [21].

In cytosol from 10-day-old mice, diethylstilbestrol did not compete with estradiol-17β for specific binding sites in the 4*S* region (Fig. 1, panel B); in 21-day-old animals, however, diethylstilbestrol was totally effective in this regard. This is additional evidence that the adult binding species are distinct from known non-receptor estrogen-binding proteins, since the latter do not interact with diethylstilbestrol in a high-affinity manner [22, 23].

The data in Fig. 2 represent equilibrium binding parameters of cytosol receptors from animals of different ages, plotted according to the direct linear method [12, 13]. The ontogeny of the receptor was previously studied [8]. Between 2 and 4 weeks of age, a 4-fold increase in receptor binding is seen, with no detectable difference in the affinity of the interaction.

Nuclear translocation of the cytosol receptor was analyzed by *in vivo* and *in vitro* experiments, as further evidence for the true receptor nature of the

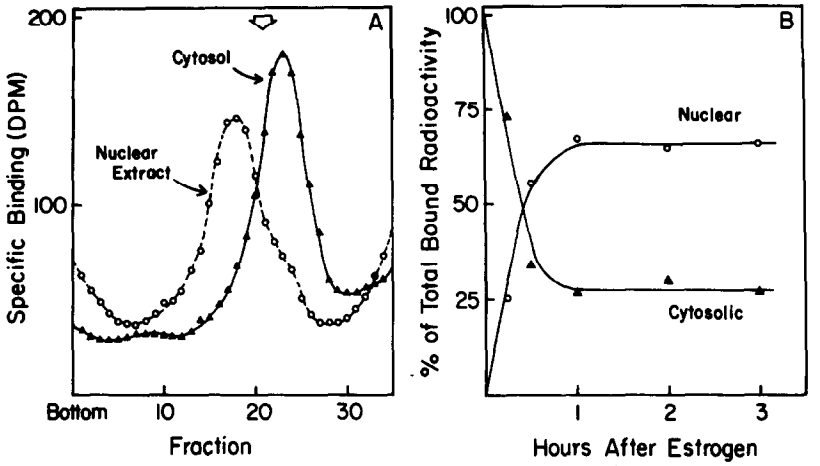

Fig. 3. Transformation and nuclear translocation of the 4*S* mammary tissue estrogen receptor. (A). Cytosol was prepared from adult virgin female mice and incubated with [^{3}H]-17β-estradiol (10^{-9} M) for 1 h at 4°C. One portion was further incubated for 95 min at 4°C (▲). A second portion of the same cytosol was incubated for 45 min at 25°C and then used as suspension medium for purified mammary tissue nuclei. After 60 min at 25°C, the nuclei were sedimented (10 min at 1,000 *g*, 4°C), washed with 0.32 M sucrose and extracted with 0.4 M KCl (○). Sucrose gradient centrifugation of both the cytosol and the nuclear extract was performed in the presence of 0.4 M KCl. The arrow represents the peak fraction of bovine serum albumin sedimentation at 4.6*S*. (B). Mature virgin mice were injected with 0.1 μg of [^{3}H]-17β-estradiol and killed in groups of 4 at the designated intervals thereafter. The tissue homogenate was initially centrifuged at 1,000 *g* for 10 min, and the resulting pellet was washed 3 times with buffer and extracted with 0.4 M KCl in buffer. The extract was isolated by centrifugation for 30 min at 15,000 *g* and submitted to sucrose density gradient centrifugation in high-salt buffer for quantification of 5.5*S* estrogen binding (○). The 105,000 *g*, 60 min supernatant fraction of the homogenate was applied to a low-salt sucrose gradient and the 4*S* radioactivity was measured (▲).

Table 1. Mammary tissue estrogen receptor levels during and following pregnancy

Group	Dry wt tissue per mouse (mg)	Total DNA (mg)	DNA (μg/mg tissue)	Specific estrogen binding (mol/mg protein $\times 10^{-14}$)	(mol/mg DNA $\times 10^{-13}$)	(mol/g tissue $\times 10^{-13}$)
Non-pregnant	42.3	1.75	41.4	4.45	1.77	7.33
Pregnant						
Early (5–9 days)	58.4	2.76	47.3	5.48	1.83	8.65
Mid (10–15 days)	86.2	4.23	49.1	1.02	1.34	6.59
Late (16–19 days)	121.1	6.50	53.7	2.46	1.69	9.09
Postpartum lactating						
3 days	202.0	9.01	44.6	5.82	1.01	4.49
6 days	251.4	9.77	38.9	5.32	0.71	2.76
15 days	335.7	10.20	30.4	3.04	0.49	1.50
30 days	159.9	5.12	32.0	2.28	0.53	1.70
Involuted						
24 days	62.0	2.31	37.3	4.11	1.96	7.32

Animals were killed in groups of 10–15. The body weight of the animals (corrected for weight of fetuses) did not vary by more than 5 g (range of 22–27 g). For studies with postpartum lactating mice, the number of pups was adjusted to 6 on the day of parturition. Lactation normally ceased 21 days postpartum and the glands began to involute.

binding under investigation. Results of recombination studies, where preformed cytosol 17β-estradiol–receptor complexes are combined with purified nuclei under proper conditions to effect transformation and translocation of the complexes, are shown in panel A of Fig. 3. In the high-salt gradient shown, the cytosol receptor sediments at 4*S*, just as it does in a low-salt medium. The receptor complexes extracted from the nuclei following recombination sedimented at 5.5*S*, with a small amount of trailing in the 4*S* region.

Following an injection of [^{3}H]-estradiol-17β (Fig. 3, panel B), the steroid rapidly enters the nucleus at the expense of the cytosol compartment, and a proportional distribution is achieved by 1 h, following which time the relative amount in each fraction remains constant. Giannopoulos and Gorski [24] originally described this distribution pattern for receptor dynamics in the rat uterus.

Our previous measurement of receptor levels during pregnancy [8] indicated that large changes in total tissue receptor content occurred during this period. An evaluation of specific changes in receptor population is presented in Table 1. Throughout pregnancy and lactation, DNA levels rise in approximate proportion to the increasing dry weight of the tissue, as would be expected since virtually all of the mammary gland weight changes during this time are the result of proliferation of parenchymal tissue. Throughout lactation, specific estrogen binding remains constant when expressed on the basis of DNA or cytosol protein, but steadily decreases as a function of unit weight of tissue; this probably reflects appreciable cellular hypertrophy and lipid deposition during this period. There is some indication of a small rise during early pregnancy, a fall in mid-pregnancy, and a second rise toward parturition, paralleling the pattern of serum prolactin levels at these times in the mouse [25]. Clearly, however, the overall change in receptor levels during pregnancy and lactation reflected principally the change in total tissue weight, indicating that the number of receptor-containing cells, rather than the number of receptor molecules per cell, was the determinant of final receptor concentration; this is in agreement with the report of Auricchio *et al.* [26].

The pregnancy-associated shift in molecular form from a 4*S* to an 8*S* moiety [8] is shown in Fig. 4 to be a gradual process. The 8*S* component increases steadily in relation to its 4*S* counterpart as pregnancy proceeds.

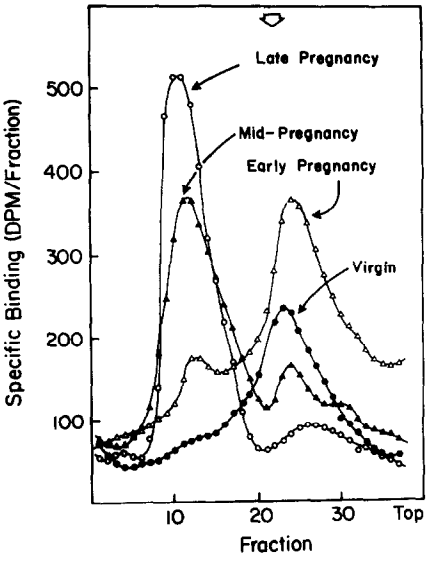

Fig. 4. Sedimentation patterns of specific estrogen binding components in mammary glands throughout pregnancy. Pregnant mice were sacrificed in groups of 4 on either day 6 (△), day 12 (▲), or day 18 (○) of pregnancy. Cytosol preparations of equivalent protein concentrations among groups were incubated with excess [^{3}H]-17β-estradiol, the free steroid was removed by adsorption with dextran-coated charcoal, and the samples were applied to low-salt sucrose gradients and centrifuged. Cytosol from virgin animals was also analyzed (●).

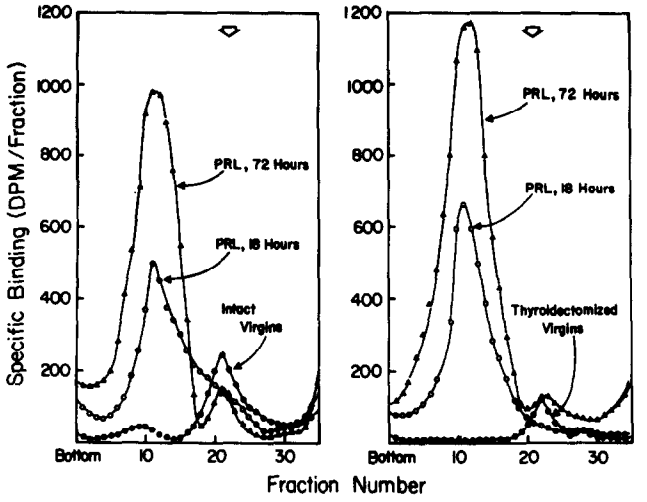

Fig. 5. Effect of prolactin on mammary tissue cytosol estrogen receptor nature and content in the virgin mouse. Virgin animals, either intact (left panel) or 2-weeks' thyroidectomized (right panel), were administered rat prolactin in saline solution in a single intraperitoneal 0.2 mg dosage and sacrificed 18 h later (○), or in 3 daily injections of 0.2 mg each and killed 1 h after the final injection (▲). Cytosol was prepared from each group, including untreated animals (●), and incubated with [^{3}H]-17β-estradiol (10^{-9} M) with or without unlabeled 17β-estradiol (10^{-7} M). Sucrose gradient analysis was performed in low-salt medium. The patterns have been quantitatively normalized to an equivalent concentration of cytosol protein. Arrow = 4.6*S*.

In an attempt to ascertain whether prolactin could directly influence estrogen receptor nature and concentration, adult virgin mice were injected with 0.2 mg of prolactin and sacrificed 18 h later. The dosage was chosen with a prime emphasis on eliciting the desired response and was much higher than would be used to mimic any physiological situation. Under these conditions, prolactin effected a marked enhancement of receptor level, all of the 8*S* variety (Fig. 5, left panel). This acute responsiveness would suggest that the prolactin effect is not mediated through induction of proliferation of a cell type containing different receptor molecules. When administered daily over a 3-day period, prolactin further increased estrogen receptor content. Thyroidectomy was utilized as an index of the specificity of the prolactin effect, since thyroid hormones are known to inhibit the mammotropic effect of prolactin [27]. Ablation of the thyroid allowed a more pronounced effect of prolactin on the mammary tissue receptors (Fig. 5, right panel). Control receptor levels were lower in thyroidectomized than in intact animals; it has been demonstrated previously that this same thyroid effect occurs in the anterior pituitary estrogen receptor system, but not in the uterus or hypothalamus [28].

Dilution of late pregnancy cytosol did not appear to cause a shift of the 8*S* receptor binding peak to lower sedimentation values (Fig. 6, right panel), suggesting that the 8*S* form was not the result of a simple concentration-dependent phenomenon. In contrast, 8*S* receptors which are often encountered in aged mice do tend to de-aggregate to smaller forms. Thus, as exemplified in the left panel of Fig. 6, the ratio of 4*S*:8*S* receptor increases with dilution of the cytosol. This differentiation between 8*S* receptor forms appearing under totally different conditions suggests that the pregnancy 8*S* complex arises from a more complex mechanism than simple aggregation of the 4*S* forms found in the non-pregnant animals.

In equilibrium binding analyses of 5 separate cytosol preparations in each instance, the range of the calculated association constants was 3–5 $\times$ 10^{10} M^{-1} and 2–4 $\times$ 10^{10} M^{-1} for the estradiol-receptor interaction in virgin and late pregnancy cytosol, respectively. This suggested a slightly higher affinity of the 4*S* receptor for the steroid, but the existence of such a difference could not be demonstrated by statistical analysis unless a very large number of determinations were made. Alternatively, measurement of the kinetics of formation and cleavage of the steroid–receptor complexes could be explored with a view toward detection of differences between receptor forms, and this approach was taken in the present case.

When association rate kinetics were investigated under conditions where the concentrations of steroid and receptor were adjusted to permit calculation on the basis of a bimolecular interaction, the results shown in Fig. 7 were obtained. The initial velocity obeyed second-order kinetics for at least 10 min in the case of both the 4*S* virgin receptor form and the 8*S* late pregnancy species. The slopes of the lines, representing the association rate constants, were appreciably different, the rate of formation of the 4*S* complex being 5 times as great as that of the 8*S* complex. These experiments have been performed several times and the results are reproducible from one cytosol preparation to another.

Dissociation rate kinetics were analyzed on pre-

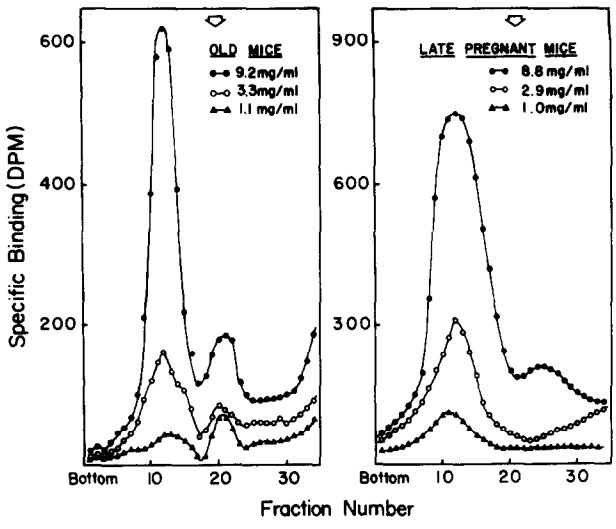

Fig. 6. Protein concentration as a determining factor in sedimentation of 8S forms of mammary tissue estrogen receptor. Normal mammary tissue from aging female virgin mice (11 months old) (left panel) and from 18-day pregnant animals (right panel) was excised and cytosol was prepared at the highest protein concentration shown. Dilutions to the designated concentrations were then made and all samples were equilibrated with [^{3}H]-17β-estradiol $\pm$ unlabeled 17β-estradiol. Following dextran-charcoal treatment, the samples were applied to low-salt sucrose gradients and centrifuged.

formed steroid–receptor complexes at 4°C and 25°C. The data, plotted as an arithmetic temporal function of residual binding, are presented in the upper panels of Figs 8 and 9, for 8S and 4S complexes, respectively. In all instances, attempts to linearize these data as a single-term exponential function were unsuccessful.

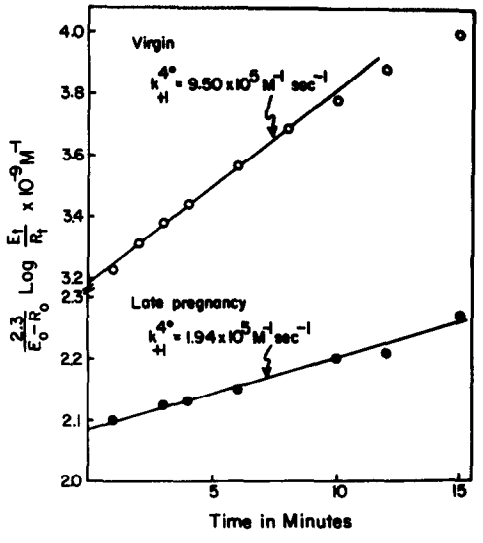

Fig. 7. Association kinetics of estrogen-receptor interactions at 4°C in virgin and pregnancy cytosol. Cytosol was prepared from adult virgin and 18-day pregnant mice and the receptor concentration (R_0) was determined by saturation binding analysis. [^{3}H]-17β-Estradiol was added at a concentration of 0.5 nM (E_0). At intervals up to 15 min, aliquots were taken and delivered into tubes containing excess unlabeled 17β-estradiol. Receptor complexes were precipitated with protamine sulfate and the radioactivity was measured for determination of 17β-estradiol and receptor concentration remaining at each interval (E_t and R_t, respectively). The association rate constant, k_{+1}, was determined as the slope of the lines shown for a second-order kinetic reaction.

The procedures outlined by Rodbard [29] were applied to find the best fit for the data. Two-term or higher multiple-term exponential equations were not appropriate. Considering the presence of an unsaturable level of nonspecific binding, which would contribute as a constant factor into the dissociation equation, the data were best represented according to:

$$[ER]_0 = [ER]_t e^{-k_{-1}t} + N,$$

indicating that the binding at any time, t, was proportional to the initial concentration, $[ER]_0$, as a negative exponential function defined by the dissociation constant, k_{-1}, with the addition of a constant amount of nonspecific binding, N. The value of N for each sample was estimated as the horizontal asymptote of the respective dissociation curve, and subtracted from each point. The resultant single exponential functions were then linearized on semilogarithmic coordinates, and these results are shown in the lower panels of Figs 8 and 9. With the exception of the 4S complex dissociation at 25°C, single-component straight lines were obtained allowing calculation of the dissociation rate constants as the slopes. The very short half-life of the 4S complex at 25°C (approximately 30 min) indicates that the biphasic nature of the curve obtained with this sample was merely a limitation of the amount of bound material remaining, and the dissociation rate constant should represent the slope of the initial segment of the curve.

The dissociation rate data also indicate marked differences between 4S and 8S estrogen–receptor complexes; the findings are summarized in Table 2. At 4°C, the half-life of the 8S complex is 3 times that of the 4S. In spite of large differences in both k_{+1} and k_{-1} for the two receptor forms, it may be seen that the values for the equilibrium association con-

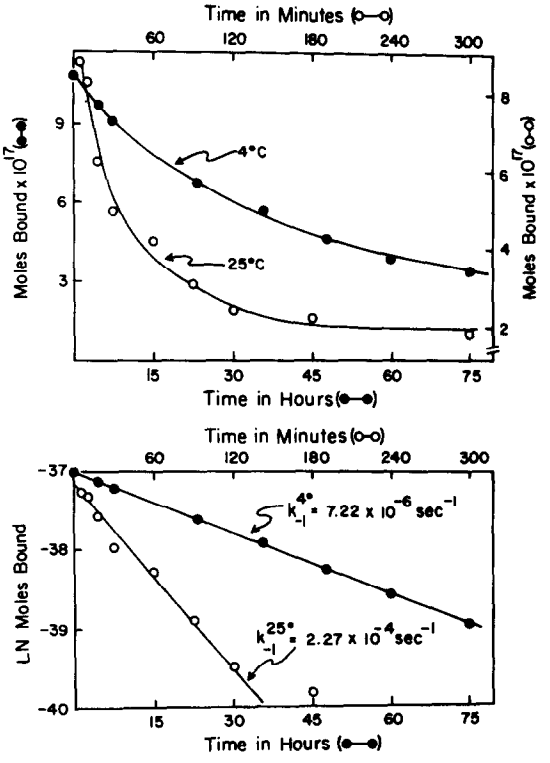

Fig. 8. Dissociation kinetics of 8*S*-receptor–17β-estradiol complexes. Cytosol was prepared from 18-day pregnant animals and equilibrated at 4°C with excess [^{3}H]-17β-estradiol, then reacted with dextran-coated charcoal to remove unbound steroid. A 100-fold molar excess amount of unlabeled 17β-estradiol was then added and dissociation of the complex was allowed to proceed at 4°C or 25°C, with sampling and receptor binding evaluation being performed at various time periods. The arithmetic plots in the upper panel were corrected for nonspecific binding as the asymptote to infinite time, and the corrected curves were then treated as a semi-logarithmic function in the lower panel for determination of the first-order dissociation rate constant, k_{-1}.

stants, K_A, calculated in the Table as the ratio of k_{+1}/k_{-1}, show the same small difference as observed by direct determination of K_A in equilibrium type saturation binding analysis. The kinetic data clearly demonstrate that the formation of the 4*S* complex occurs with greater facility than that of the 8*S* complex. Once formed, however, the dissociation of the 8*S* complex occurs far less readily than that of the 4*S*. A likely explanation would involve the requirement of a steroid-induced conformational change in the 8*S* receptor which is effected less rapidly than a simple interaction, but which confers greater stability to the steroid–receptor complex thus formed.

DISCUSSION

The present data substantiate the previous report [8] that 4*S* estrogen binding proteins in adult virgin mouse mammary tissue cytosol possess properties characteristic of hormone receptors. This binding is mimicked by the receptor-specific synthetic estrogen, diethylstilbestrol; it is separable from plasma binding proteins by virtue of its high affinity of binding; and it is prompted by 17β-estradiol to undergo molecular transformation and translocation to binding sites in the nucleus. Receptor forms sedimenting in the 4*S* region have been previously observed in several systems, but they have generally been treated as interesting oddities. Steggles and King [30] noted a 4*S* estrogen-binding component in rat uterine cytosol from mature, but not from immature or ovariectomized, animals, and this protein had a high affinity for estrogenic steroids and appeared capable of undergoing nuclear translocation. Toft and O'Malley[31] found that the specific progesterone-binding moieties in the non-estrogen-stimulated chick oviduct had 4*S* sedimentation coefficients. Milgrom *et al.* [32] described guinea pig uterine progesterone receptors as fluctuating between 4*S* and 7*S* forms during the estrous cycle. Finally, human breast tumor cytosol may contain 4*S*, 8*S* or any combination of these two receptor forms [33]. In all these analyses, the results were suggestive of a causative role of hormonal stimulation in manifestation of a particular receptor form; however, it appears that the nature and degree of this change in one receptor system may be quite different from that in another.

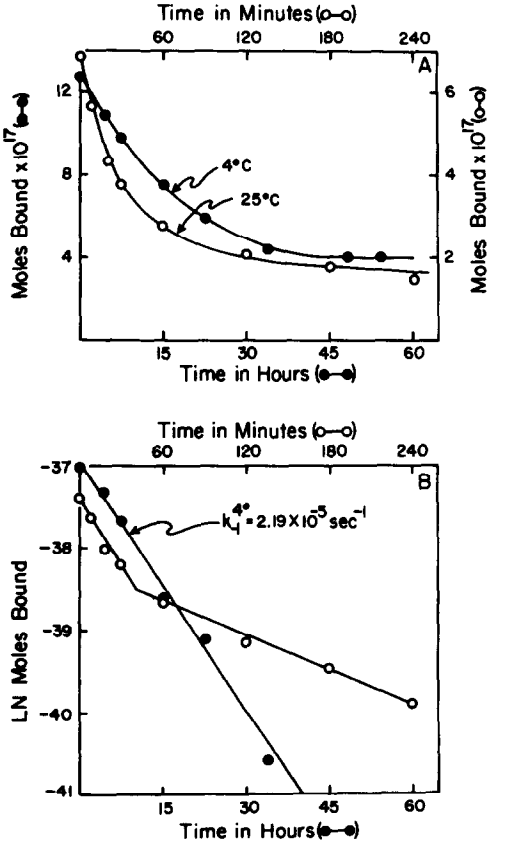

Fig. 9. Dissociation kinetics of 4*S*-receptor–17β-estradiol complexes. Cytosol from mature virgin mice mammary glands was prepared and treated as described in the legend to Fig. 8.

Table 2. Kinetic parameters of 4S and 8S receptor binding

Animal source	Temperature	k_{+1} ($M^{-1}s^{-1}$)	k_{-1} (s^{-1})	$t_{\frac{1}{2}}$ (h)	K_A (M^{-1})
Virgin (4S)	4°C	9.5×10^5	2.2×10^{-5}	9.0	4.3×10^{10}
	25°C	—	4.5×10^{-4}	0.45	—
Late pregnant (8S)	4°C	1.9×10^5	7.2×10^{-6}	26.7	2.7×10^{10}
	25°C	—	2.3×10^{-4}	0.85	—

Values for the association and dissociation rate constants, k_{+1} and k_{-1}, are summarized from Figs 7–9. The equilibrium association constants, K_A, were calculated as the ratio k_{+1}/k_{-1}. The half-life of the complex was determined as $\ln 2/k_{-1}$. The sedimentation positions of the samples were verified by sucrose density gradient centrifugation.

While it can be argued effectively that neither 4S nor 8S receptor species actually exist within the cell *in vivo* [34], this convenient method of presentation does at least indicate that there are different native forms. Our findings of indistinguishable equilibrium constants for the two families of estrogen–receptor complexes are consistent with most of the reported observations of others [30–32]. The present communication represents the first evidence for differences in binding behavior between 4S and 8S receptors, namely a significant discrepancy between the rates of both complex formation and dissociation. Kinetic measurements can prove to be very valuable adjuncts to equilibrium binding analyses because rate constants can be determined with a high degree of accuracy and reproducibility. This stems partly from the fact that nonspecific binding is virtually eliminated from consideration of association rate kinetics because the very high affinity sites determine the initial velocity; moreover, correction for nonspecific binding in the dissociation reaction is easily made because the labeled steroid which dissociates from the receptor enters into a constant pattern of interaction with unsaturable nonspecific binding sites, permitting accurate determination of this reaction as a constant factor. Thus, kinetic constants are sensitive indicators of the actual dynamics of a ligand–protein interaction when two binding systems of widely-variant affinity are present simultaneously in the sample.

The ability of prolactin to induce the 4S–8S conversion *in vitro* has also been observed in the rat [35], and permits some degree of speculation about the implications of this phenomenon *in vivo*; however, such suggestions must be tempered by our total ignorance of the role of other hormones in the process, especially progesterone during pregnancy. Shortly prior to parturition, prolactin levels in mouse serum are very high, but the animals do not lactate. It has been presumed that the female sex hormones, the levels of which fall precipitously at parturition, hold the lactation response in abeyance during late pregnancy [36, 37]. Estrogen action in the uterus is characterized by short-term actions (hyperemia, water imbibition) and long-term actions (tissue growth), and correlations have been made between the duration of response and the length of time during which estrogenic substances remain bound within the cell nucleus [38, 39]. If an analogous situation is extant within the mammary gland, one would expect the 4S receptor complex to be more effective in controlling transient acute responses, and the 8S complex to be a better regulator of sustained action, on the basis of their respective kinetic parameters. Alternatively, the situation might be viewed as a function of the circulating level of estrogen, whereby the 4S species would be more efficient when the estrogen titer is high, and the 8S would conserve estrogen action when the endogenous levels fall. Simplistically, then, the 4S receptor form would be present in states of relatively low hormonal stimulation, where the sensitivity of the tissue response to estrogen is essentially a reflection of the serum level of estradiol-17β. The steady conversion from 4S to 8S during pregnancy could prepare the tissue for the sudden drop in the amount of estrogen available to the cytoplasmic receptors at a time when parenchymal tissue proliferation is still required, by "storing" active estrogen in the nucleus as a receptor-bound species, thus permitting uninterrupted continuation of the mitogenic action of estradiol-17β on the tissue.

Neoplastic cells behave as though they were in a constantly hyperstimulated state. If such cells contained a heterogeneous population of 4S and 8S receptor forms, regulatory control by the 4S receptors would presumably be lost before that of the 8S receptors because the capacity of the 4S receptor system, by virtue of its more rapid kinetic binding properties, would become overloaded first. The 8S system reponds less acutely and would therefore be less readily susceptible than the 4S system to the overriding effects of excessive hormonal influence. Wittliff and Savlov [33] have examined the correlation between endocrine therapy responsiveness and the relative concentration of 4S and 8S receptor forms in human breast tumor samples; their results strongly suggest that the 8S receptors alone are functionally responsive to estrogen under these conditions. This would be consistent with the hypothesis that the 4S forms may be saturated in these cells, and therefore unable to respond to superimposed fluctuations in

hormonal levels. Although attempts to extrapolate normal intracellular relationships to those existing in a grossly abnormal environment must be purely speculative, the possibility that the molecular form of the receptor dictates the nature and extent of its responsiveness would appear to be provocative enough to deserve further investigation.

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