ASSESSMENT OF "ACTIVATION" OF ESTROGEN RECEPTORS IN LACTATING MAMMARY GLAND USING DNA-CELLULOSE BINDING

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SUMMARY. To circumvent the need for isolated nuclei in studies on activation of estrogen-receptor complexes in mammary gland of the rat, a DNA-cellulose binding assay was employed using a cell-free system. Incubation at 28°C for 30 min of receptors previously charged with [^{3}H]estradiol markedly enhanced their association with DNA-cellulose. Once activated, estrogen-receptor complexes bound maximally to DNA-cellulose within 20-30 min. The temperature optimum for activation was 28 $\pm$ 2°C using cytosol preparations. The temperature-induced activation required the presence of both steroid and cytosolic receptors simultaneously. Density gradient centrifugation revealed that, unlike those of uterus, both activated and charged estrogen-receptor complexes of lactating mammary tissue sedimented as a 4 S species in sucrose gradients containing 0.4 M KCl.

INTRODUCTION

Tissues responsive to steroid hormones contain specific binding proteins which appear to be a prerequisite for their action in target cells. These cytosolic steroid-receptor complexes undergo translocation into the nuclei where they are thought to stimulate molecular events required for development and differentiation (e.g., 1, 2). At temperatures less than physiologic, translocation takes place slowly *in vitro* (3, 4). However, the rate of nuclear association of steroid-receptor complexes was enhanced by elevating the temperature (3, 4). Jensen and coworkers (5) characterized a temperature-dependent activation *in vitro* for the estrogen receptor in rat uterus, in which a 4 S species was transformed into a 5 S component. Only the latter complex exhibited high affinity for the target tissue nuclei (5).

Presence of cytosolic and nuclear binding proteins for estrogens, glucocorticoids, and progestogens in normal and neoplastic mammary gland has been

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investigated extensively by our laboratory (6-10), as well as by other workers (11-13). Recently we described a cell-free system in which 28°C induced an activation of estrogen-receptor complexes resulting in elevated binding to nuclei purified from mammary tissue (14). DNA coupled to cellulose may substitute for purified nuclei in measuring the binding of hormone-receptor complexes in other tissues (15). In this study we characterized the activation of the estrogen receptors from lactating mammary gland of the rat using a modification of the procedure described by Alberts *et al.* (16).

MATERIALS AND METHODS: Sprague-Dawley rats, lactating 18-21 days were sacrificed and the inguinal pair of mammary glands removed and used immediately or kept frozen at -80°C until used. Minced tissue was homogenized with a Polytron^{R} using 1-2 vol (w/v) of either 10 mM Tris·HCl buffer pH 7.4 containing 1 mM EDTA or 40 mM Tris buffer alone. The homogenate was centrifuged at 105,000 x g for 30 min at 1-3°C. Aliquots of cytosol fractions were charged with [^{3}H]estradiol-17β at a final concentration of 10 nM in the presence or absence of diethylstilbestrol of final concentration of 2 μM to correct for nonspecific binding. After a 2 hr incubation at 0°C which was sufficient to reach equilibrium aliquots of the charged cytosol were incubated at 28°C for 30 min either in the presence or absence of 1 M urea depending upon the condition examined. As a control of charged receptors, aliquots of the cytosol were incubated further for 30 min at 0°C which did not bring about significant activation.

For DNA-cellulose binding studies, native calf thymus DNA (Sigma) was coupled to cellulose (Whatman CF-11) by the procedure described by Alberts and Herrick (17), suspended in 20 volumes (w/v) of 10 mM Tris·HCl buffer containing 0.15 M NaCl and stored as a frozen slurry at -20°C. For the binding assay, 2.0-2.5 ml of the thoroughly mixed DNA-cellulose slurry was placed in each test tube and washed with an excess of the Tris buffer. The binding of 500-600 μl of activated or charged cytosol to packed DNA-cellulose (300-450 μg DNA) was determined after mixing and incubating at 0°C for 30 min with occasional shaking. At the end of the incubation, 3 ml of ice cold 10 mM Tris·HCl buffer was added, mixed, and the contents centrifuged for 5 min at 2500 x g. The DNA-cellulose pellet obtained after several washings was extracted with 600 μl of the Tris·HCl buffer pH 7.4 containing 1 mM EDTA and 0.6 M KCl for 30 min at 1-3°C. At the end of the extraction, 200 μl of 3% dextran-coated charcoal suspension made 0.6 M with regard to [KCl] prepared according to Boylan and Wittliff (7), were added directly to the extraction mixture, suspended, and incubated for 10 min at 0-3°C. The mixture was then centrifuged at 2500 x g for 5 min at 1-3°C, and aliquots of the supernatant were analyzed for the amount of [^{3}H]estradiol-receptor complexes by counting in a Triton-toluene fluor (9).

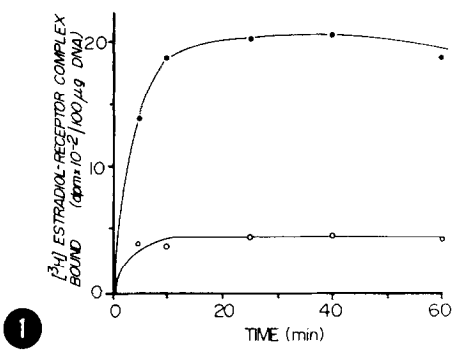
For sedimentation analyses, aliquots of either activated or charged cytosols first were mixed with a pellet of dextran-coated charcoal (7) and incubated 10 min at 0°C, then these reaction mixtures were centrifuged at 2500 x g for 5 min 1-3°C to sediment the charcoal. Aliquots (0.2 ml) of the clear supernatant were layered on linear 5 to 20% sucrose gradients prepared in 10 mM Tris·HCl buffer, pH 7.4 containing 1 mM EDTA and 0.4 M KCl. The gradients were centrifuged at 246,000 x g for 16 hr at 1-3°C using a Spinco SW-60 Ti rotor.

RESULTS AND DISCUSSION: One of the principal goals of our laboratory is to

determine if there is a defective event in the response mechanism in certain mammary tumors which contain cytosolic estrogen receptors but are unresponsive to hormone manipulation (18). We have reported earlier that incubation of [^{3}H]estradiol-receptor complexes from mammary gland at 28°C for 30 min brought about their "activation" such that enhanced binding to isolated mammary gland nuclei was observed (14). However, the use of isolated nuclei is complicated by 1) the requirement of considerable quantities of tissue, 2) lack of a good method for purifying mammary gland nuclei and 3) poor reproducibility of nuclear uptake. Using DNA-cellulose binding (17), we developed a rapid, sensitive and reproducible method of assessing activation *in vitro* of estrogen receptors in mammary tissue and further, used this procedure to characterize the nature of the activation process.

A time course study of estrogen receptor association with DNA-cellulose was conducted to establish the conditions of the assay (Fig. 1). As seen, preincubation of the complexes in cytosol at 28°C resulted in a markedly enhanced binding to DNA-cellulose at 0°C reaching a maximum 3-5 fold above that of the unincubated control within 20-30 min. In this discussion we shall refer to estrogen receptors which show increased affinity for DNA-cellulose after warming as *activated* complexes. Those which have associated with [^{3}H]estradiol at 0°C and do not bind readily to DNA-cellulose are termed *charged* complexes. The results in Table I indicate that binding of these estrogen receptors to DNA-cellulose was due to their association with the DNA and not the cellulose matrices.

In order to determine the optimum activation temperature, the cytosol-hormone complexes were incubated at various temperatures between 0° and 35°C. The enhancement in the steroid-receptor complexes' ability to bind to DNA-cellulose occurred with elevated temperature, reaching a maximum at 28°C (Fig. 2). The decline was observed at temperatures greater than 30°C due presumably to thermal denaturation or dissociation of the steroid-receptor complexes. Fig. 2 also illustrates that activation of the steroid receptor



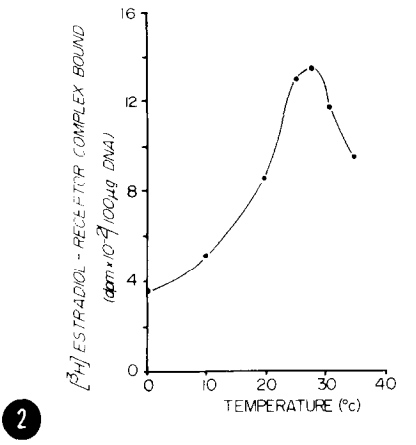


Figure 1. Time Course of Binding of [^{3}H]Estradiol-Receptor Complex to DNA-Cellulose.

Cytosol prepared in 10 mM Tris·HCl buffer, pH 7.4, containing 1 mM EDTA was charged with tritium-labeled steroid as described under Materials and Methods. One half of the reactions containing charged cytosol were activated by incubating at 28°C for 30 min (●), while the others were kept at 0°C (charged only) (○). Then cytosols were cooled to 0°C. Constant volumes (0.5 ml) of above cytosol were incubated with DNA-cellulose for the indicated times at 0°C. The DNA-cellulose binding assay was as described under Materials and Methods. Values reported were corrected for the amount of [^{3}H]estradiol-receptor complexes present in charged cytosol.

Figure 2. Temperature Dependence of Activation of [^{3}H]Estradiol-Receptor Complexes.

Cytosol prepared in 10 mM Tris·HCl buffer was charged with 10 nM [^{3}H]estradiol-17β as described under Materials and Methods. Aliquots (0.6 ml) were removed and incubated further in the presence of labeled steroid at the temperature indicated for 30 min. After cooling to 0°C, the extent of activation of steroid-receptor complexes in each fraction was evaluated by incubation with DNA-cellulose for 30 min at 0°C. The DNA-cellulose assay was as described in the text. All reactions were corrected for nonspecific binding using diethylstilbestrol.

occurred at lower temperatures but to a lesser extent. A similar observation has been reported by Kalimi *et al.* (19) for the binding of glucocorticoid-receptor to DNA-cellulose.

Using the conditions of time and temperature established thus far, we examined the hormonal requirement of the activation process (Table II). Several different reaction mixtures containing estrogen receptors in different states were prepared according to the conditions outlined in Table II. Briefly,

Table I. DNA DEPENDENCE OF BINDING BY ESTRADIOL-RECEPTOR COMPLEXES

ESTRADIOL-RECEPTOR COMPLEXES[a]	SPECIFIC BINDING (dpm/reaction)[b]	
	DNA-Cellulose	Cellulose
Charged	2072 ± 75	1 ± 1
Activated	7113 ± 110	1 ± 1

[a]Cytosol prepared in 10 mM Tris·HCl buffer, pH 7.4 containing 1 mM EDTA was charged with [^{3}H]estradiol-17β as described under Materials and Methods. These receptors were then either activated at 28°C for 30 min or kept at 0°C for 30 min (charged only).

[b]Constant volumes (0.5 ml) were incubated with either cellulose or DNA-cellulose for 30 min at 0°C. The amount of steroid-receptor complex bound to cellulose or DNA-cellulose was evaluated as described under Materials and Methods. Values reported are mean ± standard error of the mean of 3 determinations.

Table II. REQUIREMENT OF ESTRADIOL-17β FOR ACTIVATION OF ESTROGEN RECEPTORS

	REACTION CONDITIONS[a]	SPECIFIC BINDING[b] (dpm/100 µg DNA)
1)	R_c · $\xrightarrow[28°C,\ 30\ min]{-E}$ $\xrightarrow[0°C,\ 2\ hr]{+E}$ $E \cdot R_c$	265 ± 20
2)	R_c · $\xrightarrow[0°C,\ 2\ hr]{+E}$ $\xrightarrow[0°C,\ 30\ min]{+E}$ $E \cdot R_c$	460 ± 17
3)	R_c · $\xrightarrow[0°C,\ 2\ hr]{+E}$ $\xrightarrow[28°C,\ 30\ min]{+E}$ $E \cdot R'_c$	1577 ± 25

[a]Receptors prepared in 10 mM Tris·HCl buffer, pH 7.4 containing 1 mM EDTA, were incubated with 10 nM [^{3}H]estradiol-17β at the temperatures and for the times indicated. Separate reactions were conducted with 200 fold excess of diethylstilbestrol as a measure of nonspecific binding. $E \cdot R_c$ represents charged receptors where $E \cdot R'_c$ represents receptors which have been charged and activated.

[b]All reactions involving association with DNA-cellulose were conducted at 0°C, 30 min. Data represent the mean ± S.E.M. of 3 determinations.

in reaction 1) uncharged receptors were incubated at 28°C, 30 min prior to their association with [^{3}H]estradiol-17β to determine the extent of activation in the absence of steroid. In reaction 2), receptors were charged with [^{3}H]estradiol but not activated. The estrogen receptors in reaction 3) were first charged and then activated using the conditions established previously. As shown in Table II, receptor preincubated at 28°C in the *absence* of estradiol did not bind readily to DNA-cellulose. Charged receptors bound twice as well as receptors which were first warmed and then charged while the estrogen receptors which were *both* charged and activated exhibited the greatest affinity for DNA-cellulose. Our finding that estradiol was required for activation is similar to the observation of Jensen *et al.* (5) using estrogen receptors in rat uterus.

It has been reported that estrogen receptors in the immature rat uterus undergo a shift in their sedimentation behavior from approximately 4 S to 5 S when heated to 25-28°C either in the absence (5, 20) or presence of 1 M urea (21). Analysis by sucrose gradient centrifugation of the charged and heat-activated estrogen receptors from uteri of lactating rats confirmed these findings (Fig. 3A). In contrast with those of the uterus, estrogen receptors from mammary glands, activated under identical conditions, did not exhibit an alteration in their sedimentation characteristics (Fig. 3B). Estrogen receptors of rat mammary gland which either had been charged only or charged and activated sedimented as a 4 S species. Essentially the same sedimentation behavior was observed when the estrogen receptors were heat-activated in the absence of urea (data not shown). Thus, if temperature activation altered the conformation of estrogen receptors from mammary tissue, the change was of a smaller magnitude than that induced in the uterine estrogen receptor.

In summary, employing a cell-free system, we have shown that incubation of newly formed steroid-receptor complexes at 28°C brings about their activation characterized by rapid association with DNA. This event was temperature dependent and required the interaction of estradiol with its receptor. The

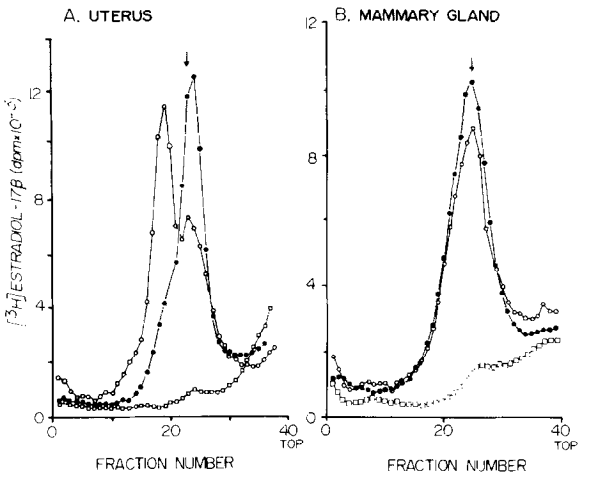


Figure 3. Influence of Activation Conditions on Sedimentation Profile of Estrogen Receptors in Uterus (A) and Mammary Gland (B) of the Rat.

Uteri and mammary gland from 18-day lactating rats were homogenized separately in 2 and in 3 volumes (w/v) respectively of 40 mM Tris•HCl buffer. Aliquots of the cytosols were charged with steroids as described in Materials and Methods for 2 hr at 0°C. A portion of the reactions containing charged receptors were made 1 M with respect to [urea] and incubated at 28°C for 30 min, then cooled to 0°C (O) while others were incubated further at 0°C for 30 min in the absence of urea (●). Nonspecific binding (□) was measured in the presence of unlabeled diethylstilbestrol as described in the text. The unbound [^{3}H]estradiol-17β in each reaction was adsorbed to dextran-coated charcoal pellets; then the cytosol preparations which did not receive urea earlier were made 1 M with respect to [urea] prior to separation by sucrose gradient centrifugation at 246,000 x g for 16 hr at 1-3°C. The arrows indicate the sedimentation of a 4.3 S marker protein.

molecular nature of the activation process of the estrogen-receptor complexes in cytosol of lactating mammary gland remains obscure. Our finding that there was no change in the sedimentation behavior of estrogen-receptor complexes in mammary gland during activation suggests that this mechanism in mammary gland is *unlike* that proposed for estrogen receptors in rat uterus.

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