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Endogenously Blocked High Affinity Estradiol Receptors in the Immature and Mature Rat Uterus[1,2] (38062)

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(Introduced by J. Kieler)
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The traditional methods of determining high affinity steroid receptors (1, 2) will only measure free receptor sites (R). However, it is conceivable that a portion of the high affinity receptors may be blocked by endogenous hormone. If this is so, then conventional quantitations may have a built-in error which cannot be neglected in comparative and other studies.

Two approaches were followed in an attempt to clarify the presence of receptors blocked by endogenous hormone (R-E) in rat uteri. According to Puca and Bresciani (3) it is possible to split R-E by heat treatment. R-E therefore might be evaluated by assaying heat induced changes in the quantity of R. An increase in binding capacity would indicate that R measured before heat treatment represents only the "apparent binding capacity" (ABC), while R measured after heat treatment might represent the "total binding capacity" (TBC), provided that R-E is totally split.

TBC might also be estimated by an exchange labeling of supernatant aliquots in which all high affinity receptor sites are saturated with nonlabeled estradiol before incubation with radioactive hormone. If the half life of the receptor-hormone complex ($R\text{-}E_{t/2}$) is known, TBC can be calculated.

Materials and Methods. Uteri were obtained from inbred immature female rats (Wistar, SPF, Moellgaard, Ltd., Denmark) less than 30 days old, weighing about 50 g. The absence of macroscopic follicles in the ovaries was taken as additional evidence of immaturity.

Uteri were also obtained from inbred mature female rats (Wistar, Fibiger colony) 7–8 weeks old, weighing 150 g. The estrous state of the animals was not recorded, but the presence of macroscopic follicles in all animals was noted.

The animals were killed by cervical dislocation and the uteri immediately removed and cooled on melting ice. The tissue was homogenized in an Ultra-Turrax homogenizer (Janke and Kunkel, Staufen, West Germany) by three 10 sec cycles separated by 30–40 sec cooling in melting ice. The homogenization was completed in a Potter-Elvehjem glass–glass homogenizer with 5–10 strokes. Three immature (about 0.15 g) or two mature uteri (0.3 g) were combined. Tris 10 m*M*, pH 7.4, EDTA 1.5 m*M*, and NaN_3 1.0 m*M* (TE buffer) (4) served as homogenization buffer which was added to immature and mature uteri in the respective proportions of 1:5 and 1:3.

The homogenates were spun at 100,000*g* av. at 4° for 1 hr and the supernatants either were assayed immediately or stored at −70°. Frozen supernatants were meticulously thawed in melting ice ensuring a temperature of 0° in the supernatant. With this technique reproducible receptor capacities were obtained after up to 5 days of storage, while the activities in frozen intact organs were found to decrease after storage at −70°.

The free high affinity receptors were as-

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sayed according to the method described by Feherty *et al.* (1). The supernatants were incubated at 0° with 5 concentrations of 3H-estradiol, ranging from 7.2×10^{-9} *M* to 2.9×10^{-10} *M*, for 2 hr. 3H-estradiol dissolved in 5 μl absolute ethanol was added to 25–50 μl of supernatant (30–200 μg protein). The total volume was raised to 80 μl by adding TKE buffer (4) containing tris 10 m*M*, pH 7.4, KCl 50 m*M*, EDTA 1.0 m*M*, and NaN_3 1.0 m*M*.

Incubation with 3H-estradiol was terminated by adding 250 μl dextrancoated charcoal suspension. After adsorption for 10 min at 0° the charcoal was spun down at 800*g* for 10 min at 4°. Finally, 200 μl of the supernatant was counted for radioactivity in a Beckman LS-255 scintillation counter. Quench correction was performed by the channels' ratio method.

To correct for deficient adsorption, blanks were prepared from 5 μl 3H-estradiol and 75 μl TKE buffer, and the counts from these subtracted from experimental values.

The binding capacities were read from a Scatchard plot, which makes possible a correction for unspecific binding of 3H-estradiol as well as a determination of the K_d for the receptor-hormone complex. This binding capacity represents apparent binding capacity (ABC), not including R-E complexes.

3H-estradiol (specific activity 85 Ci/mmol) was purchased from the Radiochemical Center, Amersham, UK. Unlabeled estradiol was generously supplied by Leo Pharmaceuticals, Copenhagen.

Protein was determined according to Lowry *et al.* (5). Statistical evaluation was performed with Wilcoxon's signed rank test.

Influence of heat treatment on binding capacity. Supernatants prepared from mature rat uteri homogenates, as described, were incubated respectively at 40° and 25° for periods of 28–82 sec and 120–960 sec. Using a Vario-Perpex pump (LKB, Bromma, Sweden), 100 μl of supernatant were pumped through a chromatographic tube wound around a copper cylinder immersed in a waterbath at 40°. Increasing incubation times were obtained by stepwise decrease of the speed of the pump. Incubations were carried out at 25° in test tubes placed in a waterbath. Samples were withdrawn at various intervals, as shown in Fig. 1. They were immediately cooled to 0°, and the binding capacity was determined as already described.

TBC determination by exchange labeling. TBC was measured by an exchange labeling of a supernatant aliquot in which all high affinity sites were saturated by incubation for 1 hr with unlabeled estradiol at 1.8×10^{-7} *M*. Unbound hormone was separated from bound hormone by gel filtration using a column of Sephadex G-25 swollen in TKE buffer, 1 × 17 cm. The contaminating hemoglobin indicated the position of the protein fraction containing the high affinity estradiol receptors. The binding capacity in this saturated preparation was then determined by incubation with 5 3H-estradiol concentrations for 4–6 hr, exactly as for determination of ABC. This binding capacity was designated ABC_{sat}. It was assumed that only estradiol receptor sites vacant after

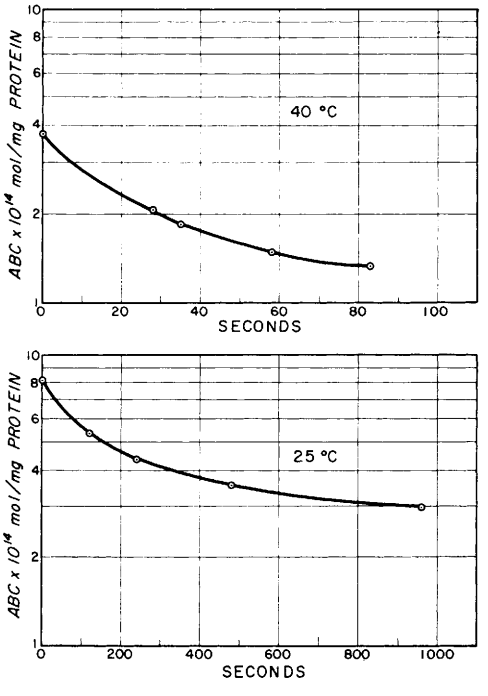

FIG. 1. Heat Inactivation of High Affinity Estradiol Binding Protein in Supernatants from Mature Rat Uterus. Dimensions of ABC: mol estradiol bound/mg supernatant protein.

dissociation of the R-E complex could bind radioactive estradiol. The fraction being dissociated was calculated from a determination of the half life of the receptor-hormone complex.

A supernatant preparation was incubated with 3H-estradiol at 7.2×10^{-9} *M* for 2 hr. The receptor-hormone complexes formed were isolated by gel filtration on Sephadex G-25 as stated above and kept at 0°. Samples of 100 μl were withdrawn from 0 hr–18 hr and rechromatographed on small unswollen Sephadex G-25 columns 0.5 × 4.0 cm (250 mg dry Sephadex G-25) in a disposable Pasteur pipette. The protein fraction was counted for radioactivity and the protein content determined. The time-radioactivity/mg protein relationship obtained was linear in a semilogarithmic plot. The half life of the receptor-hormone complex ($R\text{-}E_{t/2}$) was found to be 1400 min (range 1100–1700 min).

With an incubation time t, TBC was then calculated by the equation: $TBC = ABC_{sat} \times f(t/R\text{-}E_{t/2})$; $f(t/R\text{-}E_{t/2})$ was determined from a semilogarithmic half life plot for $t/2 = 1400$ min.

Results and Discussion. The role of steroid receptor proteins in the biochemical mechanism of action of steroid hormones is well established (6, 7). At the same time, increasing understanding of the effects of steroid hormones on growth and differentiation in target tissues has called new attention to neoplastic lesions in tissues under hormonal regulation.

If determinations of steroid receptor capacities shall serve as useful biochemical parameters in an investigation of hormonal response in tumor tissue, it is essential that the quantitation not be hampered by the different endocrinological states encountered in such a study.

To determine the presence of preformed R-E in rat uterus we tried to heat split the complex. A very rapid decrease in binding capacity was recorded at both 40° and 25°. In the semilogarithmic plot (Fig. 1) the decrease was not linear with time but leveled off. Half values were reached after 30 sec and 300 sec of heat treatment respectively at 40° and 25°. Thus even if R-E had been split at these temperatures, the experiment only revealed the thermolability of the system, but failed to measure TBC. However, with these findings in hand, incubation was then carried out at 0° in the manner described, instead of at 30° as recommended by Feherty *et al.* (1).

Using the exchange labeling in supernatants saturated with unlabeled estradiol, the ratio ABC/TBC and ABC was found to be significantly higher in immature compared to mature uteri, while there was no significant difference in TBC (Table I).

The technique reported here for demonstrating the presence of endogenously blocked high affinity receptors is relatively simple. The assumptions mentioned in the experimental section, however, cannot be directly assessed, but the results for ABC/TBC = 0.95 in immature uterine tissue do support the validity of the approach to determination of TBC.

TABLE I. Median Values of Apparent and Total Binding Capacity in Immature and Mature Rat Uterine Supernatants.[a,b]

	n	ABC/TBC	ABC × 10^{13}	TBC × 10^{13}	$K_d \times 10^9$
Immature rat uterus	7	0.95	4.5	7.8	2.39
Mature rat uterus	6	0.36	1.4	4.3	2.68
Immature compared to mature rat uterus (Wilcoxon's signed rank test)		$P < 0.01$	$P < 0.01$	$P > 0.1$	$P > 0.1$

[a] *n*: number of uterine tissue pools investigated.

[b] Dimensions of ABC and TBC: mol estradiol bound/mg supernatant protein.

The differences demonstrated between immature and mature uterine tissue cannot be accounted for by differences in hormone binding itself, as the K_d values for the two groups are identical. K_d as well as ABC values are somewhat higher than those reported by Feherty *et al.* (3). But this may be explained by the lower incubation temperature used in this study.

Summary. The presence of endogenously blocked high affinity estradiol receptors in rat uterine tissue was determined by an exchange binding assay in supernatants previously saturated with unlabeled estradiol. Compared to determinations of free receptor sites, this technique indicated that up to 60% of receptor capacity in mature rat uterine tissue might be blocked by endogenous estrogen.

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