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RAT THYMIC ESTROGEN RECEPTOR—II. PHYSIOLOGICAL PROPERTIES

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Specific, high affinity estrogen receptor has been shown to be present in the rat thymus. From *in vivo* and *in vitro* studies as well as competition assays, the receptor possesses specificity for estradiol and the estrogen-like compound diethylstilbestrol, but not for progesterone, testosterone, triamcinolone or cortisol. By sucrose gradient centrifugation studies the thymic estrogen receptor was shown to have a sedimentation value of 7–8s in low salt buffer. Castration resulted in a decrease in thymic estrogen receptor in terms of pmol/g tissue or fmol/mg soluble protein but no change in terms of pmol/mg DNA. Estradiol injection *in vivo* resulted in a rapid decrease of measurable cytoplasmic receptor by 2.5 min, which became undetectable by 20 min and returned to control values by 24 h. Autoradiographic studies show that the radioactive label is concentrated in single cells which may be of the reticulo-epithelial variety.

INTRODUCTION

Removing the source of gonadal steroids by castration results in an increase in thymic weight. Estradiol administration decreases the weight of the thymus in either intact or castrated animals [1]. Estrogens have also been reported to depress host immune responses, related to the T-lymphocyte system [2, 3].

In our previous paper [4], we reported on the presence of high affinity, specific estrogen receptor present in rat and beef thymus. This receptor appears to be located in the reticuloepithelial portion of the thymus and is not present in lymphocytes derived from the thymus. The present paper reports on some additional physiochemical and physiological properties of this thymic estrogen receptor.

MATERIALS AND METHODS

Preparation of tissue and measurement of thymic estrogen receptor. Male rats of the Sprague–Dawley strain were obtained from Harlen Industries of Indiana. The animals were 1–2 months old and weighed approximately 150 g at the time of use. Castration was carried out as described previously [4].

The thymus was removed, freed from extraneous tissue, blotted, weighed and placed in cold saline. To prepare cytosol, the thymus was minced and homogenized in TE buffer (0.01 M Tris, pH 7.5, 1 mM EDTA) at 2°C using a Virtis 45 microhomogenizer. The homogenate was centrifuged in polyallomer tubes at 120,000 *g* for 30 min and the supernatant fraction (termed cytosol) was decanted into a separate container.

To measure estrogen receptor 0.3 ml of cytosol fractions (diluted 1:6, w/v) were incubated with five concentrations of [^{3}H]-estradiol from 8.8×10^{-10} M to 5.4×10^{-11} M with or without the addition of unlabelled estradiol (4×10^{-6} M) for 4 h [5–8]. After incubation each sample was treated with 0.5 ml of dextran coated charcoal to remove free estradiol and after 10 min centrifuged [4]. The supernatant was counted for radioactivity and the specific CPM, affinity of binding and concentration of binding sites calculated as described previously [4].

To determine the sedimentation value of thymic estrogen receptor 0.2 ml cytosol (diluted 1:4, w/v) previously incubated with 8×10^{-6} M [^{3}H]-estradiol and possibly other unlabelled steroids (4×10^{-6} M) was layered onto 5–20% sucrose gradients and centrifuged at 202,000 *g* for 18 h in a Beckman L2-65B ultra centrifuge. The tubes were pierced and 22 drop fractions collected and treated with dextran coated charcoal, centrifuged and counted for radioactivity [4].

Autoradiography. 2, 4, 6, 7, [^{3}H]-estradiol, specific activity 90–110 Ci/mmol, was obtained from New England Nuclear Corporation. This was dried under nitrogen before use and redissolved in 10% ethanol-isotonic saline at a concentration of 1 mCi/3 ml. Rats were injected intravenously with 0.5 ml containing about 166 μCi (0.48 mg) of [^{3}H]-estradiol. Some control rats received excess unlabelled estradiol (15 μg) in oil subcutaneously 30 min before the [^{3}H]-estradiol was injected.

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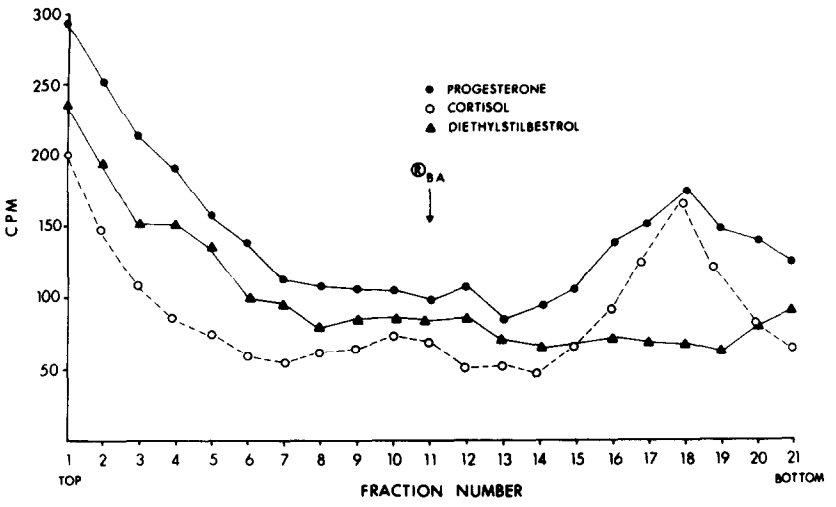

Fig. 1. Sucrose gradient centrifugation study of thymic cytosol estrogen receptors from rats treated *in vivo* with various unlabelled steroids. Intact male rats were injected with 15 μg of unlabelled steroids in oil 30 min before being sacrificed. Aliquots of cytosol were labelled with [H^3]-estrogen and layered on 5–20% sucrose gradients. Centrifugation was for 18 h at 202,000 *g* R_{BA} is Rhodamine Bovine Albumin which sediments at 4.8s.

In all experiments the rats were sacrificed by cervical dislocation 30 min after the injection of [^{3}H]-estradiol. Tissue for autoradiography was pre- methods adapted from Stumpf and Roth [9] and Warembourg [10] modified as follows: small pieces of tissue were quick frozen in isopentane cooled by liquid nitrogen. Frozen sections were cut at 4–6 μm in an AO cryostat at −40°C. Sections were directly mounted onto glass slides which had been pre-coated with Kodak NTB-3 emulsion. Slides were sealed in light-tight boxes containing dessicant and stored at −18°C for 21 to 90 days. They were then photographically processed and stained with hematoxylin and eosin.

RESULTS

Specificity of binding in vitro

The [^{3}H]-estradiol binding is primarily found in the 7–8s fraction (Fig. 1). Diethylstilbestrol (a potent nonsteroidal estrogen) acted as a specific competitor for estradiol and eliminated the 7–8s peak (fractions 16–19). When progesterone or cortisol were the competitor compounds, the 7–8s peak remained intact (Fig. 1). These results are in agreement with those previously reported [4] for the competition assay.

In vivo *steroid treatment*

Male rats were treated with 15 μg of steroid and sacrificed 30 min after injection. It was shown (Fig. 2) that competition by testosterone, dihydrotestosterone, progesterone and cortisol had no effect on the amount of estrogen receptor in the thymus with respect to controls treated with oil only and estradiol and diethylstilbestrol significantly reduced the amount of estrogen receptor present in the thymus. These findings agree with those obtained from the *in vitro* investigation of thymic estrogen receptors.

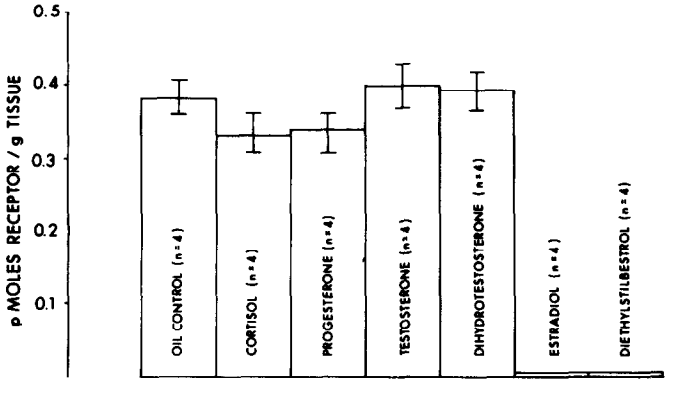

Fig. 2. *In vivo* steroid treatment on rat thymic cytosol estrogen receptor as measured by Scatchard plot analysis. Intact male rats were injected with 15 μg of unlabelled steroids in oil or oil alone 30 min before being sacrificed. $\bar{X} \pm$ SEM. *n* = separate animal preparations.

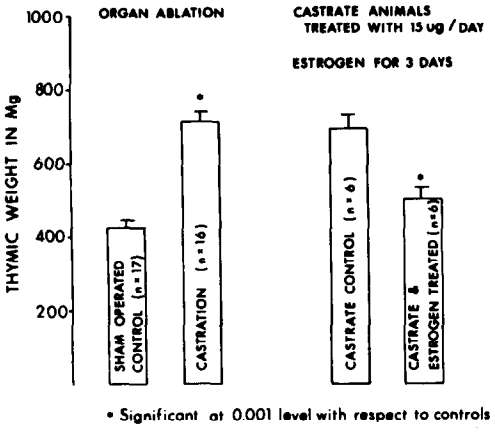

Fig. 3. Thymic weight changes following castration and estradiol treatment. Animals were castrated and 2 weeks later the thymus glands were removed and weighed. In the steroid treated groups, 15 μg estradiol per day was injected subcutaneously for three days and the thymus glands were removed and weighed. The data was analyzed by the unpaired Student's t-test. n = separate animal preparations.

Castration and estradiol treatment: effects on thymic weight

Removal of the testes results in an increased thymic weight. As can be seen from Fig. 3, sham operated controls had a mean thymic weight of 500 mg. In animals castrated for 2 weeks, there was a significant increase ($P < 0.001$) in mean thymic weight as compared to sham operated controls.

Treatment of castrated male rats with estradiol (15 μg/0.3 ml oil subcutaneously/day for 3 days) produced a decrease ($P < 0.001$) in thymic weight with respect to castrate controls injected daily with 0.3 ml of oil only. There was no difference in thymic weight between castrate-estradiol treated animals and intact-sham operated controls. Therefore, the increase in thymic weight following castration can be prevented by estradiol replacement.

Thymic estrogen receptor in intact vs. castrated animals

Castrated animals or sham operated controls were sacrificed 2 weeks after the surgery. As can be seen in Table 1, in sham operated controls the amount of thymic estrogen receptor was approximately 0.4 pmol/g tissue, 10 fmol/mg protein or 0.05 pmol/mg DNA. There was a small but significant decrease ($P < 0.05$) in the castrated groups relative to the control in terms of pmol/g tissue and fmol/mg protein and no changes in pmol/mg DNA.

Time course of the disappearance and reappearance of rat thymic estrogen receptor after a single in vivo *injection of unlabelled estradiol*

Intact male rats were injected subcutaneously with 15 μg estradiol/0.3 ml oil and sacrificed at various times after the injection. It was found (Table 2) by Scatchard plot analysis that the amount of receptor decreased within 2.5 min and by 20 min was undetectable in the cytosol fraction. This decrease probably resulted from translocation of the cytoplasmic thymic estrogen receptor into the nucleus. The free thymic estrogen receptor was detected 12 h after steroid injection, at which time the receptor concentration was approximately half that of control. It returned to nearly normal levels by 24 h.

Autoradiographic studies

Figure 4 is an overview of a rat thymic lobe. The dark outer portion is cortex, and the light inner section is medulla. Although both areas show localization of radioactive estrogen (as measured by the content of silver grains), the cortex appeared to have a higher concentration. Figures 5, 6 and 7 are higher power photographs of the cortex. As can be seen in Fig. 5 although there is an overall nonspecific background of silver grains, areas of increased granule concentration are evident. In animals pretreated with excess unlabelled estradiol 30 min before being injected with [^{3}H]-estradiol, the areas of increased grain localization vanish, but the overall granular background remains (Fig. 6). Because the hematoxylin did not strongly stain the nuclear material in the thymic cells, it is difficult to determine which cells are responsible for this [^{3}H]-estradiol localization. In one instance, however, it can be observed that the stained cell is of a large, oval variety (Fig. 5, top center) and possesses a process at one end. In Plate Fig. 7, two cells of this type are shown. They appear to be approximately 10 μ in length and 5 μ in width and possess processes at the ends that extend outward from the cell body. Since lymphocytes within the thymus do not contain cytoplasmic processes while reticuloepithelial (syncytial epithelial) cells do [11], it is possible that these cells are of the reticuloepithelial type.

Table 1. Estrogen receptor in thymic cytosol of control and castrate rats as measured by Scatchard plot analysis

Treatment	K_A ($\times 10^9$ M^{-1})	pmol/g tissue	fmol/mg protein	pmol/mg DNA
Sham operated controls	4.026	0.395	10.153	0.054
Castrate	3.176	0.304*	8.350*	0.052
Standard error of variance	±0.258	±0.014	±0.393	±0.003

The tissues within each group were pooled and cytosol prepared. Each experiment was run three times, and the data was statistically analyzed by computer using Two Way Anova with unequal cell frequencies; least squares solution.
* Significant at the 0.05 level.
n = 3 pooled groups.

Table 2. Time course of the disappearance and reappearance of rat thymic cytoplasmic estrogen receptor after *in vivo* treatment of the animals with unlabelled estradiol. Receptor measurement was by Scatchard plot analysis

Time after estrogen treatment	pmol/g tissue	fmoles/mg protein
0	0.337 ± 0.030	7.80 ± 0.47
2.5 min	0.221 ± 0.006	6.30 ± 0.12
5 min	0.166 ± 0.042	3.90 ± 0.46
20 min	None	None
45 min	None	None
2 h	None	None
12 h	0.127 ± 0.007	4.93 ± 0.272
24 h	0.243 ± 0.021	7.86 ± 0.693

Intact male rats were injected with 15 μg of unlabelled estradiol and sacrificed at various times after the steroid treatment.
$\bar{X}$ ± SEM.
n = 4 separate animal preparations.

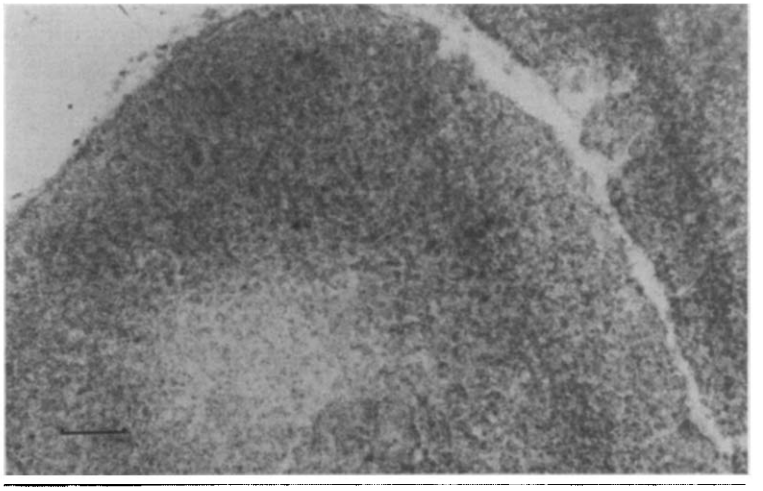

Fig. 4. Low magnification of a section of thymic lobule showing the more darkly stained cortex and lighter medulla. More labelled cells were seen in the cortex, shown in the higher power magnifications. Scale bar equals 100 μm.

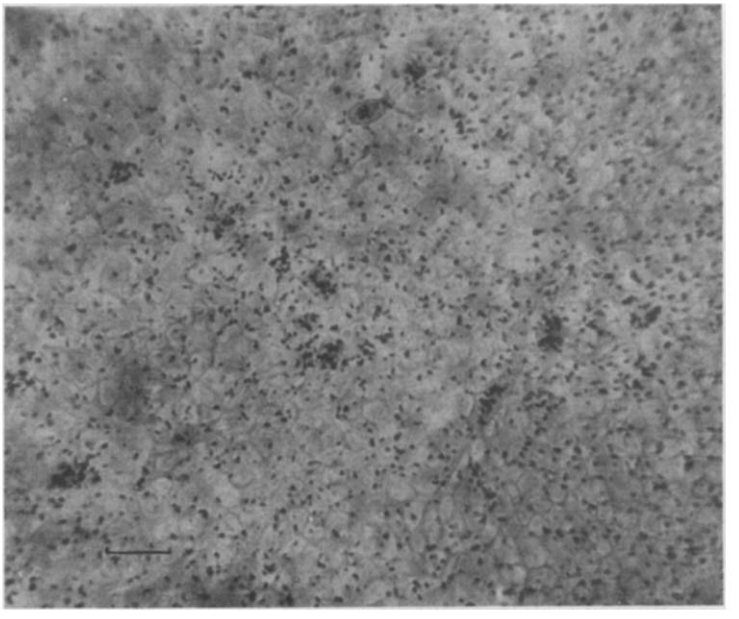

Fig. 5. Medium power magnification of thymic cortex from a [^{3}H]-estradiol injected rat. Most of the heavily labelled cells cannot be easily identified although their apparent configuration resembles reticular cells. Scale bar equals 10 μm.

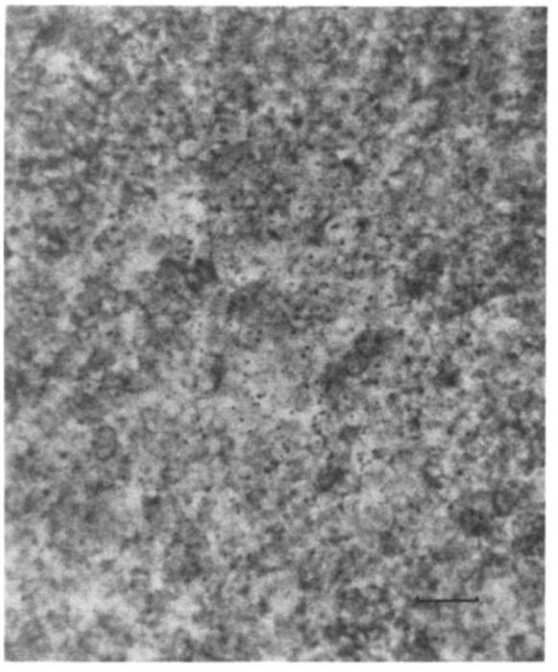

Fig. 6. Medium power magnification of thymic cortex from a rat treated with 15 μg unlabelled estradiol followed 30 min later with [^{3}H]-estradiol. Background grain accumulation is present. Scale bar equals 10 μm.

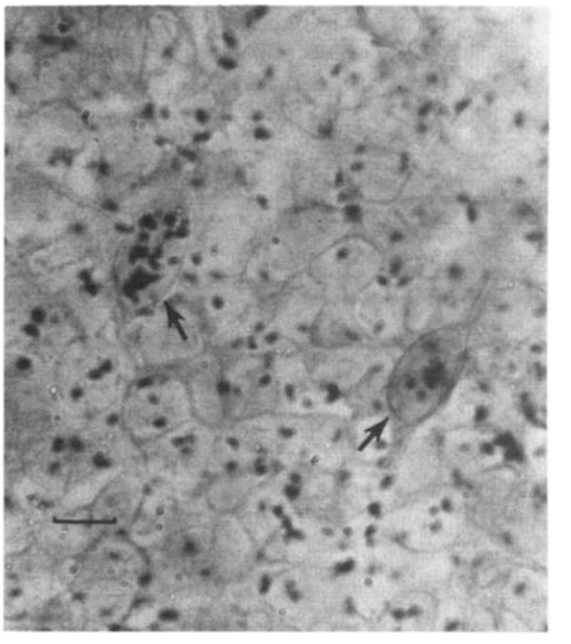

Fig. 7. High power magnification of a similar area as shown in "b" with two spindle shaped cells of reticular appearance, one well labelled by silver grains, and the other, although more clearly delineated, having only about the same number of silver grains as the general background. Scale bar equals 5 μm.

These autoradiographic findings support our results (by Scatchard plot and competition analyses) which indicate that there is specific estrogen binding material within the thymic matrix.

DISCUSSION

It has been reported that castration increases thymic weight by removing the source of gonadal steroids while replacement of these steroids decreases the organ weight [1]. These results have been confirmed in the present study in which estradiol treatment was effective in decreasing thymic weight in castrated animals. This effect is most likely mediated by the specific estrogen receptor present in the thymic reticuloepithelial cell [4]. Thymic estrogen receptor in the rat has a high affinity for estradiol ($K_A = 7 \times 10^9\ M^{-1}$) and is present in limited amounts in the tissue (10 fmol/mg protein or 0.06 pmol/mg DNA) [4, 12–14].

The specificity of thymic estrogen receptor was studied using the *in vitro* techniques of competition analysis [4] and sucrose gradient sedimentation as well as the *in vivo* method of steroid injection and Scatchard plot assay. From the *in vitro* studies [4], it was shown that thymic estrogen receptor is highly specific for estradiol or the estrogen-like compound diethylstilbestrol and does not bind progesterone, testosterone, cortisol, or triamcinolone acetonide. At high substrate concentrations (4×10^{-6} M) it does bind dihydrotestosterone slightly. The present study of the *in vivo* effect of estradiol on thymic estrogen receptor corroborates the *in vitro* results since estradiol and diethylstilbestrol administration deplete the estrogen receptor of thymic cytosol fractions, but other steroids had no effect.

From the time course study of receptor levels in the thymus following injection of estradiol it was demonstrated that cytosol estrogen receptor decreases within 2.5 min following specific steroid injection and was undetectable by 20 min. Estrogen receptor requires about 24 h to return to control levels. This pattern is similar to that reported for uterine estrogen receptor by Clark *et al.* [15, 16] and Morris [17] who showed that there is a very rapid depletion of uterine cytoplasmic estrogen receptor when animals were treated with high doses of estradiol.

It is possible that this depletion and subsequent reappearance of cytoplasmic receptor is a result of translocation and either return or resynthesis of the receptor. However, until the levels of estrogen receptor are measured in the nuclear compartment, this remains purely as speculation. It is, however, quite certain that the disappearance is not due to masking of the cytoplasmic receptor by unlabelled estrogen. In the presence of either endogenous or exogenous unlabelled steroid the K_A at equilibrium as measured by Scatchard plot decreases, but the amount of receptor (i.e., the x-axis intercept) is unchanged [12, 18]. Scatchard plot results from the time course studies show no change in the K_A, but a decrease in the amount of thymic estrogen receptor. This argues for the depletion of the receptor from the cytoplasmic fraction as expected if translocation is taking place.

With regard to the surgically ablated animals, the results indicate that castration causes a significant decrease in the amount of receptor per g tissue or fmol protein with respect to the controls. When receptor per DNA content is studied, it can be shown that there is no significant change. These data suggest that the removal of sex steroids by castration results in an increasing thymic weight due to hypertrophy of thymic cells. These observations are in agreement with microscopic analysis where it has been reported that castration results in thymic cell hypertrophy [19].

Although the mechanism of steroid modulation of T-lymphocytes is still under investigation, our findings tentatively support the conclusion that estrogen receptor may mediate cellular hypertrophy in the reticuloepithelial cells. While an earlier study demonstrated nonspecific thymic incorporation of radioactive estradiol [20] our results [12–14] as well as those of others [21] have shown that thymus contains a high affinity, specific estrogen receptor of limited quantity.

The findings from the autoradiographic studies show that the radioactive label is concentrated in single cells rather than in cell clusters. The labelling of these cells can be prevented by pretreatment with excess unlabelled estradiol before injection with [^{3}H]-estradiol. These observations support our earlier findings (obtained by Scatchard plot and competition analysis) showing the presence of a specific estradiol binding system in thymus tissue.

While some of the cells over which silver grains are localized appear to be of the reticuloepithelial type, due to staining difficulties, identification of these cells remains uncertain. In further studies, we hope to identify these cells and to determine if they are all of the same type.

In summary, we have demonstrated the presence of a high affinity, specific estrogen receptor of limited quantity which is present in the reticuloepithelial portion of thymus. We tentatively conclude that this receptor may mediate certain physiological responses relating to thymus structure and function and might also be indirectly associated with various depressive or activation effects of the cell mediated immune system.

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