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ESTRADIOL BINDING BY RAT THYMUS CYTOSOL

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SUMMARY

Preparations of rat thymus cytosol are shown to specifically bind [^{3}H]-estradiol. Binding is stable during overnight incubations at 4°C and increases linearly with increasing protein concentration in the incubation up to at least 2.2 mg/ml. There are two components with binding activity, one of high affinity, and one of lower affinity. The high affinity component has a $K_a = 1.16 \pm 0.38 \times 10^{11}\ M^{-1}$ and a binding capacity of $7.32 \pm 1.65 \times 10^{-15}$ mol/mg cytosol protein. On sucrose density gradients binding appears in two regions, 6.6–7.6 *S* and 2.5–4 *S*. Binding of [^{3}H]-estradiol in the 6.6–7.6 *S* region is competed for by a 100-fold excess of nonradioactive estradiol while this level of nonradioactive estradiol only partially competes with [^{3}H]-estradiol in the 2.5–4 *S* region.

INTRODUCTION

The thymus is instrumental in maturation, and to some extent in maintenance of cell-mediated immune functions. It has been shown to be influenced by steroid hormones. Glucocorticoids cause an inhibition of glucose uptake and phosphorylation by thymus cells over short time periods, and cytolysis over longer periods [1, 3]. Adrenalectomy, on the other hand, leads to thymic hypertrophy [4]. A glucocorticoid receptor has been demonstrated in thymus cells, peripheral lymphocytes, and lymphosarcomas [5–8]. Physiological levels of glucocorticoids appear to be involved in controlling lymphopoiesis and thymic size [9].

Estrogens and androgens also appear to be involved in thymic function. Thymic involution begins around puberty. Endogenous estrogens or androgens may not be causal factors in this naturally occurring involution but large exogenous doses of estrogens or androgens cause involution [9–13]. Reversible involution occurs during pregnancy and lactation [14]. On the other hand, gonadectomy, like adrenalectomy, leads to thymic hypertrophy [10–12]. This evidence for an association between estrogens, androgens, and the thymus prompted us to look for estrogen receptors in thymus cytosol.

Studies described here demonstrate cytosol estrogen binding activity in the thymus of gonadectomized rats between 23 and 95 days of age. The binding activity has two components, one of high affinity, and one of lower affinity.

EXPERIMENTAL

Animals. Sprague–Dawley CD rats were gonadectomized, or gonadectomized and adrenalectomized, 2–10 days prior to experiments.

Tissue preparation and incubation. Animals were sacrificed by exposure to an ether atmosphere. Thymus tissue was removed, trimmed, washed in TED buffer (10 mM Tris pH 7.4, 1.5 mM EDTA, 1 mM DTT), weighed, and homogenized in 8 volumes w/v of TEDG buffer (10 mM Tris, pH 7.4, 1.5 mM EDTA, 1 mM DTT, 10% glycerol) except where indicated. Homogenates were centrifuged at 105,000 *g* in a Beckman 50 Ti rotor in a Spinco Model L ultracentrifuge. Cytosol was incubated overnight (16–22 h) at 4°C in 1 ml at approximately 1 mg/ml protein except where indicated, with indicated concentrations of [2,4,6,7-^{3}H (N)]-17β estradiol (New England Nuclear, sp. act. = 105 Ci/mmol) and non-radioactive steroids (Sigma). Free steroid was removed with dextran coated charcoal (final concentrations, 0.27% charcoal (Sigma), 0.027% dextran (Sigma)) followed by centrifugation in an IEC PRJ centrifuge 259 head at 2000 rev./min for 12 min. Aliquots of supernatant (bound steroid) were removed, liquid scintillation fluid (toluene:triton x:liquifluor 500:917:83) added, and radioactivity measured in a Packard tricarb Scintillation Counter, Model 3002 with 30.3% efficiency for tritium. Total radioactivity was similarly determined from aliquots taken before addition of charcoal-dextran. The protein concentration in the incubation was determined by the method of Lowry[15] using bovine serum albumin as a standard.

Specific binding is determined by subtracting binding in the presence of [^{3}H]-estradiol and 100-fold excess nonradioactive estradiol from binding in the presence of [^{3}H]-estradiol alone.

Sucrose density gradients. Linear 5–20% sucrose density gradients (4.8 mls) were prepared in TEDG buffer. One half ml of cytosol incubation (which was preincubated as described above, at 4°C for 1 1/2 h) was layered on each gradient. The gradients were centrifuged at 45,000 rev./min for 17 h in a Beckman SW 50.1 rotor in a Beckman model L-2 ultracentrifuge

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Table 1. Steroid specificity of binding

Steroids in incubation media		[^{3}H]-Estradiol bound (d.p.m./ml)	Binding competed for by other steroids (d.p.m./ml)	% Inhibition of binding by other steroids
[^{3}H]-Estradiol	1×10^{-9} M	8704	—	—
[^{3}H]-Estradiol and estradiol	1×10^{-9} M 1×10^{-7} M	4891	3812	44
[^{3}H]-Estradiol and testosterone	1×10^{-9} M 1×10^{-7} M	8525	179	2.0
[^{3}H]-Estradiol and cortisol	1×10^{-9} M 1×10^{-7} M	8285	419	4.8
[^{3}H]-Estradiol and progesterone	1×10^{-9} M 1×10^{-7} M	8314	390	4.5
[^{3}H]-Estradiol and dihydrotestosterone	1×10^{-9} M 1×10^{-7} M	8096	608	7.0

Thymus tissue from female rats, 50 days of age, ovariectomized at 40 days (incubation protein concentrations were 1 mg/ml). Values shown are means of duplicate determinations.

at 46°C. Three drop fractions were collected and radioactivity determined as described above. The sedimentation coefficients of binding components were estimated by comparison with a [^{14}C]-BSA standard (~9000 c.p.m./5 μl) which was subjected to centrifugation on a parallel gradient.

Analysis of Scatchard curves. As described in the Results section Scatchard plots of cytosol estradiol binding display hyperbolic characteristics. Using the two-component binding model, one can estimate relevant parameters through computer optimization methods. Briefly, data points at both ends of the curve are used to obtain two 'starting' slopes via linear regression. A set of normal equations is then formed to solve for two ordinate intercepts such that the least mean squares fit of the radial sum of the two lines to the experimental points is best. Values of slopes are then altered by a certain amount and the process of intercept estimation is repeated. This iteration is continued for a prefixed number of times or until no significant reduction in the sum of squares of radial deviation can be observed. The final set of two slopes and two ordinate intercepts specify the two binding components involved. When the curve is well-defined the optimization process is quite stable and the convergence is reasonably fast [16].

RESULTS

The specificity of the binding of [^{3}H]-estradiol by thymus cytosol was investigated by incubating cytosol with [^{3}H]-estradiol in the presence or absence of 100-fold excess of nonradioactive steroids (Table 1). Non-radioactive estradiol substantially competes with binding of [^{3}H]-estradiol. The other steroids tested show little if any competition.

Table 2 compares the binding of [^{3}H]-estradiol by thymus cytosol, lung cytosol, blood serum, and blood cell cytosol. Specific binding is determined by subtracting binding in the presence of [^{3}H]-estradiol and 100-fold excess non-radioactive estradiol from binding in the presence of [^{3}H]-estradiol alone. There is little, if any, specific binding by the non-thymic tissues.

It is important to establish that binding of estradiol by thymus cytosol is linear with protein concentration, particularly in the region in which the incubations are performed (1 mg/ml). This is shown in Fig. 1. Binding of estradiol increases linearly with protein concentration over the range included in the experiment.

The stability of binding during the course of an overnight incubation at 4°C is shown in Fig. 2. Bind-

Table 2. Tissue specificity of binding

Tissue	Conc'n [^{3}H]-estradiol in incubation	Total binding (d.p.m./ml)	Non-saturable binding (d.p.m./ml)	Specific binding (d.p.m./ml)
Thymus	7.5×10^{-10} M	5107	2632	2475
	1×10^{-9} M	6616	3050	3565
Blood-Serum	7.5×10^{-10} M	3499	3125	374
Blood-Cells	1×10^{-9} M	921	971	−50
Lung	7.5×10^{-10} M	3796	3524	272
	1×10^{-9} M	5091	4884	206

Thymus tissue from female rats, 49 days of age, ovariectomized at 40 d. Blood was centrifuged in a Sorvall RC-2 centrifuge at 3000 rev./min for 15 min to separate serum and pellet of "blood cells". Blood cells and lung were treated as described for thymus. Protein concentrations were adjusted to 1 mg/ml. Values shown are means of duplicate determinations.

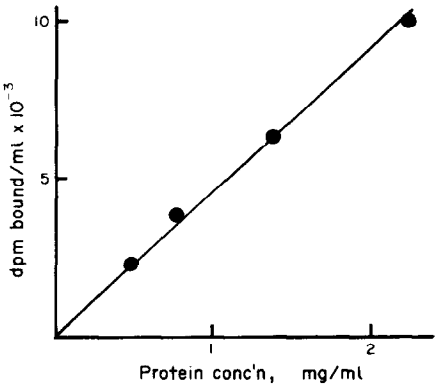

Fig. 1. Variation of specific binding of [^{3}H]-estradiol with protein concentration of incubation. Thymus tissue from male rats, 47 days old, gonadectomized at 45 days. Tissue preparation as described in Experimental except homogenization was in 2 vols. TEDG buffer and incubation was at protein concentrations indicated. Concentration of [^{3}H]-estradiol in incubations was 2×10^{-9} M.

ing reaches a plateau at approximately 4 h and is constant for the duration of the experiment.

The association constant, K_a and the binding capacity of thymus cytosol for estradiol were determined by Scatchard analysis [17]. Scatchard plots of specific binding yield curves indicating the presence of more than one component. Curves were resolved into linear components by computer analysis as described in Experimental. Figure 3 shows a representative experiment along with the linear components and the theoretical curve resulting from radial addition of these components. Data from seven experiments on animals between 23 and 70 days of age were combined to obtain a $K_a = 1.16 \pm 0.38 \times 10^{11}\,M^{-1}$ and a binding capacity of $7.32 \pm 1.65 \times 10^{-15}$ mol/mg cytosol protein. No difference was found between male and female rats, nor were age dependent variations between 23 and 70 days observed. Preliminary studies of androgen binding showed little, if any, specific binding. The value obtained for K_a is higher than values obtained with conventional estrogen target tissues, such as uterus ($K_a = 0.3 - 3 \times 10^{10}\,M^{-1}$) [18–20], pituitary ($K_a = 3.2 - 5.9 \times 10^{10}\,M^{-1}$) [21]

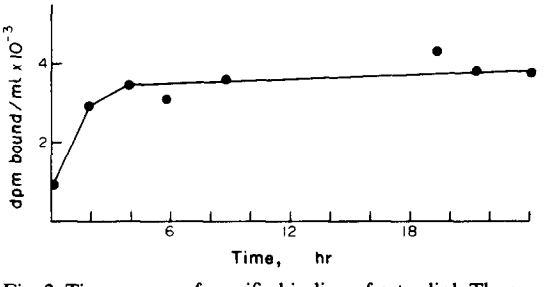

Fig. 2. Time course of specific binding of estradiol. Thymus tissue from female rats, 50 days of age, ovariectomized at 40 days. Concentration of [^{3}H]-estradiol = 1×10^{-9} M. Each point is the mean of duplicate determinations. The sample values for specific binding differ from the mean

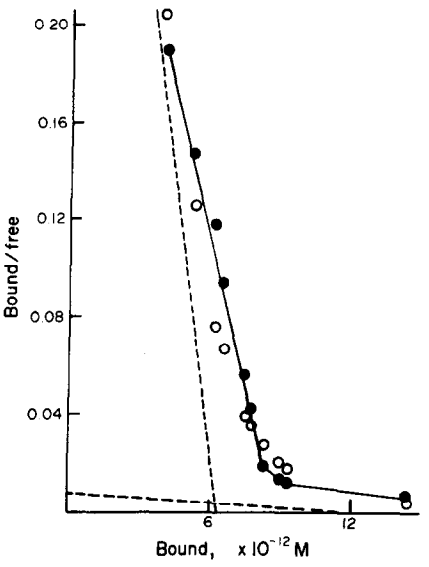

Fig. 3. Scatchard analysis of cytosol estradiol binding. Thymus tissue from female rats, 69 days old, ovariectomized at 64 days. Concentrations of [^{3}H]-estradiol in incubation media ranged from 2.5×10^{-11} M to 5×10^{-9} M. ● experimental points, mean of duplicate determinations; O theoretical points resulting from computer resolution into linear components (also shown) followed by radial addition to obtain curve. K_a of steep portion of curve $= 7.72 \times 10^{10}\,M^{-1}$. The sample values of B/F differ from the appropriate mean by less than 6% in all cases, with an average difference of 2%.

and hypothalamus ($K_a = 4 - 6.7 \times 10^{10}\,M^{-1}$) [22]. In an experiment utilizing both uterine and thymic tissue from rats ovariectomized for three days the thymic tissue behaved as described here. The uterine tissue gave a linear Scatchard plot with a K_a of $3 \times 10^{10}\,M^{-1}$ consistent with reports from other groups referenced above. The binding capacity of thymus cytosol for estradiol is less than that found with uterus ($1–8 \times 10^{-13}$ mol/mg cytosol protein) [18–20], pituitary (2×10^{-13} mol/mg cytosol protein) [21] or hypothalamus ($1.3–2.0 \times 10^{-14}$ mol/mg cytosol protein) [22]. The particular methods used to analyze curved Scatchard plots may give slight differences in values obtained for K_a and binding capacity. Nonetheless, figures reported here may indicate a different binding molecule. The lower affinity component has a K_a in the range of $5 \times 10^8\,M^{-1}$ to $1.5 \times 10^9\,M^{-1}$, and has not been studied in detail.

In conventional estrogen target organs the cytosol receptor has an 8 S sedimentation coefficient in sucrose density gradients in low salt buffer [23, 24]. Binding of [^{3}H]-estradiol in this region is totally competed for by an excess of non-radioactive estradiol. Thymus cytosol exhibits specific binding of [^{3}H]-estradiol in two regions of a 5–20% sucrose gradient (Fig. 4). One peak occurs between 6.6 and 7.6 S. In the presence of a 100-fold excess of nonradioactive estradiol this peak does not occur. Thus it behaves

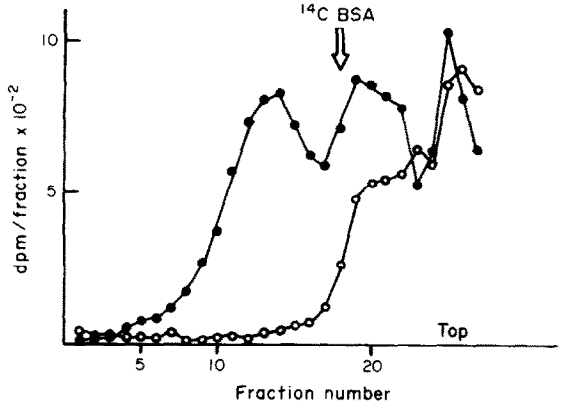

Fig. 4. Sedimentation pattern of cytosol estradiol binding. Thymus tissue from female rats, 95 days old, ovariectomized at 90 days. Tissue preparation as in Experimental except homogenization was in 2 vols. TEDG buffer. Protein conc'n in incubation was approximately 5 mg/ml. ● incubation in presence of [^{3}H]-estradiol 1×10^{-9} M; ○, incubation in presence of [^{3}H]-estradiol 1×10^{-9} M and non-radioactive estradiol 1×10^{-7} M.

competed for by non-radioactive estradiol. This is characteristic of lower affinity binding. Michel *et al.*[25] show the existence of two components binding estrogens in uterine cytosol from immature rats. The smaller of these (4–5 *S*) originates, at least partly, from the serum. Animals used in many experiments shown here were above an age (2 months) at which Michel *et al.*[25] no longer observed the smaller component. Serum from 49d animals showed no substantial specific binding of estradiol by charcoal-dextran techniques (Table 2). This rules out serum contamination in results reported here.

DISCUSSION

The results shown here indicate, for the first time, the presence in the thymus tissue of the rat of a macromolecular component capable of specific binding of estradiol. Results shown in Table 2 make contamination an unlikely explanation of these results. The thymus is a complex organ composed of a variety of cell types. The experiments presented here do not distinguish between cell types. Thymic lymphocytes and/or epithelial cells are the most attractive candidates for cell types which are capable of estradiol binding. A partial explanation for the low binding capacity of thymic cytosol may be that only a small percentage of thymic cells contain the binding component.

The K_a found for binding of estradiol by thymic cytosol is approximately 4–10 times higher than that found for uterus and about two times higher than that found for pituitary and hypothalamus. Since values vary somewhat between groups is it difficult to determine the significance of this. It is possible that the binding component in the thymus cytosol is different from that found in other tissues.

The finding of estrogen binding in the thymus raises the question of the possible role(s) of estrogen receptors in this tissue. This cannot be answered at present, although the thymic involution caused by large amounts of estrogen [9–13] and the hypertrophy that accompanies gonadectomy [10–12] indicate that thymus size is controlled in part by estrogens. The present findings indicate that this control may be mediated by an estrogen receptor system similar to that present in more traditional estrogen target tissues.

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REFERENCES

1. Dougherty T. F., Berliner M. L., Schneebeli G. L. and Berliner D. L.: Hormonal control of lymphatic structure and function. *Ann. N.Y. Acad. Sci.* **113** (1964) 825–843.
2. Hallahan C., Young D. A. and Munck A.: Time course of early events in the action of glucocorticoids on rat thymus cells in vitro: Synthesis and turnover of a hypothetical cortisol-induced protein inhibitor of glucose metabolism and of presumed ribonucleic acid. *J. biol. Chem.* **248**, (1973) 2922–2927.
3. Makman M. H., Dvorkin B. and White A.: Evidence for induction by cortisol in vitro of a protein inhibitor of transport and phosphorylation processes in rat thymocytes. *Proc. natn. Acad. Sci. U.S.A.* **68** (1971) 1269–1273.
4. Ishidate M. and Metcalf D.: The pattern of lymphopoiesis in the mouse thymus after cortisone administration or adrenalectomy. *Aust. J. exp. Biol. Med. Sci.* **41** (1963) 637–649.
5. Munck A. and Brinck-Johnsen T.: Specific and nonspecific physiochemical interactions of glucocorticoids and related steroids with rat thymus cells *in vitro*. *J. biol. Chem.* **243** (1968) 5556–5565.
6. Munck A., Wira C., Young D. A., Mosher K. M., Hallahan, C. and Bell P. A.: Glucocorticoid–receptor complexes and the earliest steps in the action of glucocorticoids on thymus cells. *J. steroid Biochem.* **3** (1972) 567–578.
7. Kirkpatrick A. F., Kaiser N., Milholland R. J. and Rosen F.: Glucocorticoid-binding macromolecules in normal tissues and tumors. Stabilization of the specific binding component. *J. biol. Chem.* **247** (1972) 70–74.
8. Lippman M. E., Halterman R. H., Leventhal B. C., Perry S. and Thompson E. B.: Glucocorticoid-binding proteins in human acute lymphoplastic leukemic blast cells. *J. clin. Invest.* **52** (1973) 1715–1725.
9. Goldstein G. and Mackay I. R.: *The Human Thymus.* William Heinemann Medical Books, London (1969) p. 128.
10. Chiodi H.: The relationship between the thymus and the sexual organs. *Endocrinology* **26** (1940) 107–116.
11. Ross M. A. and Korenchevsky V.: The thymus of the rat and sex hormones. *J. Pathol. Bacteriol.* **52** (1941) 349–360.
12. Plagge J. C.: The thymus gland in relation to sex hormones and reproductive processes in the Albino rat. *J. Morphol.* **68** (1941) 519–545.
13. Schacher J., Browne J. S. L. and Selye H.: Effect of various sterols on thymus in the adrenalectomized rat. *Proc. Soc. exptl. Biol. Med.* **36** (1937) 488–491.
14. Brouha L. and Collin R.: Equilibre hormonal et gravidite. *Ann. Physiol. Physiocochem. Biol.* **11** (1935) 773–880.

15. Lowry D. H., Rosebrough A. J., Farr A. L. and Randall R. J.: Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193** (1951) 265–275.
16. Lee E.: (personal communication).
17. Scatchard G.: The attractions of proteins for small molecules and ions. *Ann. N.Y. Acad. Sci.* **51** (1949) 660–672.
18. King R. J. B. and Mainwaring W. I. P.: *Steroid–Cell Interactions.* University Park Press, Baltimore (1974) p. 190.
19. Steggles A. W. and King R. J. B.: The use of protamine to study [6,7-^{3}H]-oestradiol-17β binding in rat uterus. *Biochem. J.* **118** (1970) 695–701.
20. Sanborn B. M., Rao B. R. and Korenman S. G.: Interaction of 17β-estradiol and its specific uterine receptor. Evidence for complex kinetic and equilibrium behavior. *Biochemistry* **10**, (1971) 4955–4961.
21. Korach K. S. and Muldoon T. G.: Characterization of the interaction between 17β-estradiol and its cytoplasmic receptor in the rat anterior pituitary gland. *Biochemistry* **13** (1974) 1932–1938.
22. Korach K. S. and Muldoon T. G.: Studies on the nature of the hypothalamic estradiol-concentrating mechanism in the male and female rat. *Endocrinology* **94** (1974) 785–793.
23. Jensen E. V. and DeSombre E. R.: Mechanism of action of the the female sex hormones, *Ann. Rev. Biochem.* **41** (1972) 203–230.
24. Chan L. and O'Malley B. W.: Mechanism of action of the sex steroid hormones (first of three parts). *N. Engl. J. Med.* **294** (1976) 1322–1328.
25. Michel G., Jung I., Baulieu E-E., Aussel C. and Uriel J.: Two high affinity estrogen binding proteins of different specificity in the immature rat uterine cytosol, *Steroids* **24** (1974) 437–449.