Stereospecificity of the Uterine Nuclear Hormone Receptors (33040)

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Early work by Jensen and Jacobson (1) suggested that the specific interaction of certain estrogens with receptors present in the rat uterus was an important step in the biochemical events associated with estrogen action. Further studies by Jensen (2) demonstrated that the uterine receptors interacted strongly with estrogenic compounds such as hexestrol, while weakly estrogenic substances possessed little or no affinity for the tissue. Studies with mouse uteri have yielded similar conclusions (3–6).

Noteboom and Gorski (7) demonstrated the stereospecificity of a receptor, present in the nuclear-myofibrillar fraction of a rat uterine homogenate, and Toft and Gorski (8) reported specificity of a similar nature for a receptor present in the 105,000*g* supernatant fraction. Subsequent *in vitro* studies on the latter receptor yielded similar results (9). Similarly, Maurer and Chalkley (10) have shown that estradiol-17α could not reduce the binding of estradiol-17β to chromatin from calf endometrium.

Work in this laboratory (11,12) has demonstrated the competitive nature of estradiol-17β and estriol for rat uterine receptors. More recently we have developed an *in vitro* technique for studying the binding properties of receptors present in the 700*g* pellet of a uterine homogenate (13). We have already shown the specificity of the *in vitro* binding for several compounds and in this publication we present a more complete picture of the structural requirements necessary for nuclear incorporation.

Methods. The *in vitro* method used was similar to that previously described (13). Female Sprague-Dawley rats, 21–23 days of age were killed, the uteri were removed and homogenized in cold pH 7.2 TMK buffer (0.01 *M* Tris, 0.0015 *M* $MgCl_2 \cdot 7H_2O$, 0.01 *M* KCl) at 25°C. In most experiments 24–26 animals were used with the final homogenate concentration being equivalent to 2 uteri in 1 ml. One-ml milliliter were added to glass-stoppered centrifuge tubes to which 4.5 $\times$ 10^{-5} μg 6,7-^{3}H-estradiol-17β (sp. act. 42.4 C/mmole) plus specified amounts of nonradioactive compounds under investigation had been previously added. The tubes were then incubated at 25°C for 1 hour on a Thomas rotating shaker.

After incubation the tubes and contents were centrifuged at 700*g* for 10 min, supernatant fluids were removed, and the 700*g* pellet was resuspended in 0.5 ml TMK buffer. The suspension was shaken briefly on a Vortex shaker and recentrifuged. The resulting supernatant fraction, designated as "wash," was mixed with 10 ml of Bray's Solution and counted in an Ansitron scintillation counter. The remaining pellet was extracted successively with ethanol, chloroform:ether (2:1), and ether, and the extracts were pooled and evaporated in scintillation vials, redissolved in scintillation fluid (Liqueflor), and counted in the scintillation spectrometer.

The data presented are expressed as "percentage nuclear incorporation" and "percentage in wash," where the former represents the disintegrations per minute (dpm) of 6,7-^{3}H-estradiol-17β (E_2) extracted from the pellet by the organic solvents as a function of the total dpm added to the homogenate, and the latter refers to the dpm of labeled E_2 found in the buffer wash as a percentage of the radioactivity contained in the 700*g* pellet prior to the wash. In all cases, 0.00704 μC of E_2 (4.5 $\times$ 10^{-5} μg) was the total radioactivity added. Thus, % wash = dpm in wash/(dpm in wash + dpm in 700*g* pellet) $\times$ 100; % nuclear = dpm in 700*g* pellet/[total radioactivity added (0.007 μC)] $\times$ 100.

All test compounds used in this study were checked for purity by visual examination of spots produced with a sulfuric acid:ethanol (1:1) spray after thin-layer chromatography using silica gel G with benzene:ethyl acetate

TABLE I. Compounds Effective in Inhibiting Estradiol Incorporation.

Compound	% Nuclear				% Wash			
	1×10^{-3}	5×10^{-3}	1×10^{-2}	5×10^{-2}	1×10^{-3}	5×10^{-3}	1×10^{-2}	5×10^{-2}
Estradiol-17β	54.3	28.4	26.1	21.3	14.1	29.1	34.4	37.2
Ethynyl estradiol	45.5	22.6	18.5	18.5	16.5	35.0	47.3	40.2
Hexestrol	53.5	28.4	23.7		12.3	31.2	36.8	
Diethylstilbestrol	55.2	38.2	31.0	21.5	21.0	31.1	38.7	43.1
6-Keto-estradiol	60.5	46.9	35.0	23.5	10.7	17.7	26.5	40.1
Estriol	65.1	46.3	46.6	22.7	8.0	14.1	18.3	30.7
16-Epiestriol	64.2	49.1	42.0	28.0	7.2	13.1	17.1	29.2
Estradiol-17α	68.7	58.0	49.7	30.0	6.3	8.5	13.2	28.4
Estradiol-16α	71.8	59.5	46.1	32.2	5.6	8.2	15.2	25.7
16-Hydroxyestrone	68.2	61.7	59.7	37.0	5.6	6.3	7.6	19.3
Estrone	68.6	65.3	55.6	41.7	6.1	9.6	10.7	21.6
11β-Hydroxyestradiol			60.6	51.4			9.6	13.3
16-Keto-estradiol		63.8	65.2	53.5		6.4	7.0	11.4
16-Keto-estrone		70.0	66.2	52.3		5.8	7.0	11.2

(50:50) as the developer.

Results and Discussion. Preliminary studies of the effects of several related molecules on estradiol binding by rat uterine nuclei over a wide range of substrate concentrations clearly indicated that strongly competitive substances exerted very significant effects at 1×10^{-3} μg. The next group of compounds was characterized by exerting noticeable inhibition at 1×10^{-2} μg, and the inactive compounds such as testosterone showed no inhibition even at 5×10^{-2} μg. Thus, to limit the extent of the experiment but still collect significant data, nearly all of the compounds examined were used in four concentrations covering the critical points of this range.

It should be mentioned for purposes of comparison that incubation with labeled estradiol alone (4.5×10^{-5} μg) showed nuclear incorporation of 70% and wash of 5% with standard deviations of $\pm$ 2.9% and $\pm$ 1.1%, respectively.

Table I summarizes the data obtained for all compounds that exhibited a significant inhibitory effect at a concentration of 5×10^{-2} μg or less per milliliter on estradiol incorporation. Most values represent the average of two or more determinations.

The best inhibitors are estradiol itself and the potent synthetic estrogens ethynyl estradiol, hexestrol, and diethylstilbestrol. It is encouraging that the latter two nonsteroidal compounds are none the less potent inhibitors in the *in vitro* system, thus giving strong support to the idea that the known *in vivo* effects can be mimicked by the *in vitro* system to a significant extent. All other compounds listed in Table I, while noticeably less effective than the above-mentioned estrogens, show at least some degree of competition for estradiol. The gradations of inhibitory ability of different compounds are well illustrated in the tables, and the important observation that obvious differences between compounds at one concentration may be minimized at another is easily seen. For example, estriol and estradiol have nuclear incorporation values of 46 and 28 per cent, respectively, at 5×10^{-3} μg, but at 5×10^{-2} μg the values are almost identical (about 22%).

Table II summarizes the data obtained on compounds that are essentially ineffective as inhibitors. These compounds either do not possess the estratriene nucleus of the steroidal estrogens or, in the case of compounds with this structure, they all have undergone modification in ring A. Only 1,3,5(10),16-estratetraene-3-ol, which is the most effective of this list of inactive compounds has the intact phenolic ring A. Indeed, it is the only compound in Table II which might be equally well put at the bottom of Table I, based on the slight decrease in 6,7-^{3}H-estradiol incorporation and concomitant slight increase in wash at the 5×10^{-2} μg concentration. As would

TABLE II. Compounds Ineffective in Inhibiting Estradiol Incorporation.

	% Nuclear		% Wash	
	1×10^{-2}	5×10^{-2}	1×10^{-2}	5×10^{-2}
1,3,5,(10),16-Estratetraene-3-ol	72.4	58.2	6.2	9.2
2-Methoxy estradiol	72.1	71.4	5.0	6.1
3-Keto-Δ^4-estren-17β-ol	70.6		5.3	
Δ^4-Estrene-3β,17β-diol	66.8		7.8	
Δ^6-Estrone	69.7		7.5	
2-Hydroxy estrone	67.0	65.0	5.1	6.0
Estradiol-3-methyl ether	70.6		7.6	
Estradiol-3-sulfate	72.6	68.3	3.6	5.8
Trianisylchloroethylene	68.7		6.9	
Vallestril[a]	76.1		4.4	
Testosterone	74.2	69.2	6.0	6.1
2α-Methyl-5α-dihydrotestosterone	70.2		6.0	
Methylcholanthrene	76.1		4.8	

[a] β-Ethyl-6-methoxy-α,α-dimethyl-2-naphthalenepropionic acid.

be expected from the *in vivo* experiments, androgens exert no modifying influence on estrogen binding. Neither do the rather weakly estrogenic nonsteroidal substances such as TACE.

Because of the probable relationship of estrogens and cancer of sex-linked tissues, a known carcinogen was also tested. Under the conditions of the *in vitro* experiment no binding of methylcholanthrene, as expressed by competition with estradiol for nuclear receptors, was observed.

From the data submitted it is not possible to offer a complete explanation of the structural requirements for specific nuclear binding.

Since the estradiol molecule is relatively planar, it is quite possible that its interaction with receptors involves that part of the molecule which is either above or below the plane of the ring structure. Thus substitution at the 2,3,6,11,16, or 17 position might yield information concerning estrogen–receptor interaction.

It is likely that the interatomic distance of the two hydroxyl groups in estradiol-17β is necessary for maximal nuclear binding activity, as can be visualized for estradiol, diethylstilbestrol, hexestrol and ethynyl estradiol. Thus, a further requirement appears to be the proper location of the hydroxyl substituents, along with the phenolic character of the C-3 hydroxyl group.

Any alteration of, or addition to, the phenolic ring or to its proximity, and oxidation of, or vicinal substitution to, the 17-hydroxyl group results in a significant loss of binding. This structural requirement is most clearly demonstrated by the low inhibitory effect of the substances shown in Table II, all of which have undergone molecular alterations of the above-mentioned nature.

The most potent and strongest binding "estrogens" markedly inhibit incorporation of estradiol at 1×10^{-3} μg with single "wash" values in excess of 10%, while all other compounds show wash values below this number. When the amount of steroid is increased fivefold, the next group of inhibitors shows "wash" values well in excess of 10%, and this group consists of all of the "impeded estrogens" (14) used in these experiments.

The fact that estrone has weaker activity in the *in vitro* system than compounds such as estriol or estradiol-17α is to be expected since Jensen has previously shown that estrone has a weak affinity for the uterus under *in vivo* conditions and that this activity is due to conversion of estradiol-17β. Since rat uterine tissue possesses little or no 17β-dehydrogenase activity, the *in vitro* conditions should show even less "binding activity." Thus, compounds such as estradiol-17α, considered physiologically as weaker estrogens than estrone, possess a greater "binding activity" under *in vitro* conditions. These ob-

servations further strengthen the concept that the term "estrogen" can be rather vague and should be redefined in terms of chemical structure and physiological effect.

The inability of testosterone to affect the binding of estradiol is to be expected since competition studies carried out *in vivo* also gave negative results. The compound 2α-methyl-5α-dihydrotestosterone,[1] which is used clinically for suppression of human breast tumors, is also ineffective.

The concept of specificity of nuclear binding must be treated with proper caution. It has been demonstrated that *in vitro* binding is at least a two-step mechanism requiring a supernatant receptor probably followed by enzymatic transfer of the steroid to the nuclear receptor (13). Thus specificity may involve any or all of the receptors and transferring enzyme systems. Moreover, specificity may vary somewhat between these macromolecules.

Summary. The inhibitory effects of several compounds on the *in vitro* binding of estradiol to the rat uterine nuclear pellet were tested. The binding properties of the compounds tested were related to their known physiological effects. Chemical modifications of the estradiol molecule produced marked changes in binding properties, indicating a high degree of specificity for the transfer reactions.

[1] This compound was generously supplied by Dr. A. Segaloff.

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Rare Earth Metals and Soft-Tissue Calcification (33041)

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In the rat certain metallic salts can induce topical calcification at the site of injection (1); this phenomenon is called calcergy. Among these salts (calcergens) are the chlorides of lead, zinc, indium, cerium, and cobalt; numerous other metallic compounds ($CrCl_3$, $FeCl_3$, $SnCl_2$) have proved ineffective in this respect (2). The calcergic deposits show the X-ray diffraction pattern of hydroxylapatite (3). Many calcergens given