Molecular Parameters Involved in the Estrogenicity of Mestranol and Ethynylestradiol (35751)

D. W. HAHN, J. L. MCGUIRE, F. C. GREENSLADE, AND G. D. TURNER
(Introduced by J. P. DaVanzo)
Division of Pharmacology, Ortho Research Foundation, Raritan, New Jersey 08869

Although all of the oral contraceptives available in the United States employ either ethynylestradiol (EE) or mestranol (3-methyl ether of 17α-ethynylestradiol) (MEE) as the estrogen component, little is published about the nature of the biological or biochemical responses to these compounds. Two current concepts are (a) only estrogens bind to estrogen receptors, and (b) one of the earliest responses to estrogens in the female reproductive tract is the acceleration of RNA synthesis. We used these two molecular parameters of estrogenicity to corroborate biological assays showing that ethynylestradiol is relatively more potent as an estrogen than is mestranol.

Materials and Methods. Unlabeled estradiol-17β, cortisol, progesterone, and testosterone were purchased from Sigma. 6,7-^{3}H-Estradiol-17β (40 Ci/mmole) and 5-^{3}H-uridine (21.2 Ci/mmole) were purchased from New England Nuclear Corporation. All compounds were found to be pure by thin-layer or paper chromatography. 6,7-^{3}H-Mestranol (142 μCi/μmole) was obtained from Syntex Laboratories, Inc. This compound was purified by celite partition chromatography according to the method of Abdel-Aziz and Williams (3) and found to be pure by thin-layer chromatography using a solvent system of hexane:ethyl acetate (7:5).

New Zealand white rabbits (2 kg) and immature mice were used in cytosol incubation studies. In both sucrose density gradient isolation experiments and tissue distribution studies Long Evans female rats (200–225 g), ovariectomized 2 weeks previously, were used. Female mice (25–50 g), ovariectomized for 14–18 days, were used in the RNA synthesis experiments. All animals were killed by cervical dislocation.

Sucrose gradients were formed by a Beckman gradient maker. A Packard Tricarb oxidizer was used for determining radioactivity both in uterine samples and in the PCA-precipitate in the RNA studies. A Beckman model L-265-B ultracentrifuge was used for centrifugation; both a Packard Model 3375 Tri-Carb liquid scintillation spectrophotometer and an Intertechnique Model SL-30 spectrometer were used for radioactivity determinations. External standards were used to correct for quenching.

In tissue distribution studies, 5 μCi of ^{3}H-mestranol was injected iv into ovariectomized rats, and the animals were killed 4 hr after injection. Uteri were removed, weighed, and homogenized in chloroform:methanol (2:1). Steroids were extracted with methylene chloride and analyzed by thin-layer chromatography using a solvent system of hexane:ethyl acetate (7:2). Identification of labeled compounds was made based on known standards. In a second study, experimental animals were dosed with 1 mg of unlabeled estradiol-17β 15 min prior to injection of labeled mestranol.

In cytosol incubation experiments, uteri from either 10 rabbits or 100 immature mice were minced and washed for 1 hr in Tris–HCl buffer (pH 7.5 at 4°) before homogenization in 2/5 vol of the same buffer (0.01 *M* Tris–HCl buffer, pH 8.0, containing 0.001 *M* EDTA, 0.25 *M* sucrose) at 4°. The homogenate was first centrifuged at 12,000*g* for 15 min and the resulting supernatant was then centrifuged at 273,000*g* for 1 hr. Reaction mixtures consisting of (a) 0.2 ml of buffer (0.01 *M* Tris–HCl buffer, pH 8.0, containing 0.001 *M* EDTA, 0.25 *M* sucrose, and 25,000 cpm of ^{3}H-estradiol-17β/ml); (b) nonradioactive steroids where called for; and (c) 50

μl of uterine supernatant were incubated at 4° for 16 hr. After incubation, 1.0 ml of activated charcoal suspension (0.25 Norite A and 0.0025% dextran w/v buffer) was added. The samples were vortexed gently, incubated for 15 min at 4°; and centrifuged at 2500 rpm for 10 min. The supernatants were quantitatively extracted with toluene phosphor, and the radioactivity was measured.

In sucrose density gradient centrifugation experiments, rat uteri were washed at 4° for 1 hr in buffer (including several washes for 1 hr) to rid the tissue of blood. Tissue was then homogenized in 5 vol of phosphate buffer (0.2 *M* Na_2HPO_4; 0.2 *M* NaH_2PO_4; 0.001 *M* EDTA, pH 7.5) at 4°. The homogenates were first centrifuged at 12,000*g* for 30 min and the resulting supernatants were then centrifuged at 273,000*g* for 1 hr. Five tenths ml of the final supernatants were incubated at 4° with 0.9 μl of 6,7-^{3}H-estradiol-17β or ^{3}H-mestranol (100 μCi/ml of ethanol) plus competitors where noted at a concentration of 2×10^{-6} *M* or multiples thereof for 1 hr. A 200-μl sample of the incubation media was then layered on a 5–20% linear sucrose gradient and centrifuged at 273,000*g* for 14 hr using an SW-50-L-rotor. The tubes were then pierced at the bottom and the eluted fractions (0.2 ml) were collected in counting vials, extracted in toluene phosphor, and the radioactivity in the fractions was determined. All results have been reproducible.

In the RNA studies either ethynylestradiol or mestranol was injected subcutaneously. At various times after injection and 2 hr before killing, the mice received 6 μCi of ^{3}H-uridine in 0.2 ml of 0.9% NaCl via the lateral tail vein.

Uteri were removed, pooled by treatment group, and immediately frozen in ice. In the control group, the animals received 0.1 ml of 0.9% NaCl solution followed by tritiated uridine.

Total RNA synthesis was measured essentially by the method of Miller and Emmens (4). Each sample was homogenized in distilled water and the homogenate was made 0.2 *N* with respect to perchloric acid (PCA). The PCA-precipitate was allowed to form overnight and was washed in 0.2 *N* PCA by filtration. It was collected on a tared Whatman No. 2 filter paper. The filter paper plus the PCA-precipitate was then air dried, weighed, and combusted in a Packard sample oxidizer.

RNA was isolated for fractionation by homogenization of tissues in a mixture containing sodium aminosalicylate (6%), sodium dodecyl sulfate (5%), and sodium magnesium-litho fluor-silicate (0.01%). Homogenates were deproteinized at 60° in a mixture of phenol (70%), *m*-cresol (12%), and 8-hydroxyquinoline (0.1%). Nucleic acids were precipitated from the aqueous phase with ethanol–*m*-cresol (9:1; v/v), purified by ethanol precipitation, and fractionated by methylated albumin kieselguhr (MAK) ion exchange chromatography (5).

MAK was prepared by the method of Mandell and Hershey (6). 100–400 mg of RNA in 0.2 *M* buffered saline was mixed with 1 g of MAK. Nucleic acid weight classes bound to the MAK were eluted sequentially with 15 ml of 0.2, 0.5, 0.65, and 1.2 *M* buffered saline and 1.5 *M* ammonium hydroxide.

In each step, eluted nucleic acids were separated from those still bound to the MAK by centrifugation at 9000 rpm for 5 min. Nucleic acids in 5-ml aliquots of each elution were precipitated by the addition of 15 ml of nonlabeled yeast RNA (carrier) and 1 ml of ethyltrimethylammonium bromide (7). Precipitated nucleic acids were collected by filtration on Whatman GFA filters and solubilized in Cellosolve for determination of radioactivity.

Results. In uteri from ovariectomized rats injected with ^{3}H-mestranol, radioactivity was found in the uterus identifiable on the basis of the analysis as both mestranol and ethynylestradiol. Ethynylestradiol predominated. In uteri from experimental animals pretreated with unlabeled estradiol-17β 15 min prior to injection of ^{3}H-mestranol, two very small peaks of radioactivity were again detected by TLC analysis; these again migrated with mestranol and ethynylestradiol standards, but there was approximately a tenfold reduction in the quantity of each.

Incubation experiments using either imma-

ture mouse or rabbit uterine cytosol fraction also indicated competitive inhibition of ^{3}H-estradiol-17β binding by both ethynylestradiol and mestranol. However, in Table I note that mestranol competitively inhibits binding of estradiol-17β to a lesser degree than does ethynylestradiol. Similar data were found in sucrose density ultracentrifugation analysis of rat uterine cytosol fractions (Fig. 1). It was found that only ethynylestradiol competed for the estrogen receptor peak when each was tested as a competitor at concentrations of 2×10^{-6} M. However, when the competing concentration of unlabeled mestranol was raised to 10^{-5} M, mestranol was found to competitively inhibit binding of estradiol-17β. In a third set of experiments, high concentrations of ^{3}H-MEE were found to bind in the same region where ^{3}H-estradiol-17β is found to bind. Unlabeled estradiol-17β competitively inhibits this binding.

Figure 2 compares the incorporation of

TABLE I. Effect of Nonradioactive Steroids on Binding of ^{3}H-Estradiol-17β by the Cytosol Fraction of Rabbit and Mice Uteri Following Incubation for 16 hr at 4°.

Data are based on 10 and 5 trials, respectively, for each compound and are expressed as the Mean ± SE. Two known estrogens, estradiol-17β and ethynylestradiol, strongly competed. Mestranol also weakly competed, but competition increased as the concentration of MEE increased.

Potential competitors	Uterus (cpm)	
	Rabbit	Mouse
None	1650 ± 83	3346 ± 148
Cholesterol	1621 ± 71	3424 ± 84
Cortisol	1689 ± 102	3382 ± 93
Estradiol-17β	45 ± 12	74 ± 18
1X Mestranol	1380 ± 77	1929 ± 132
2X Mestranol	—	1948 ± 73
5X Mestranol	—	1375 ± 30
Ethynylestradiol	34 ± 11	63 ± 23
Progesterone	1592 ± 93	3201 ± 155
Testosterone	1570 ± 104	3275 ± 87

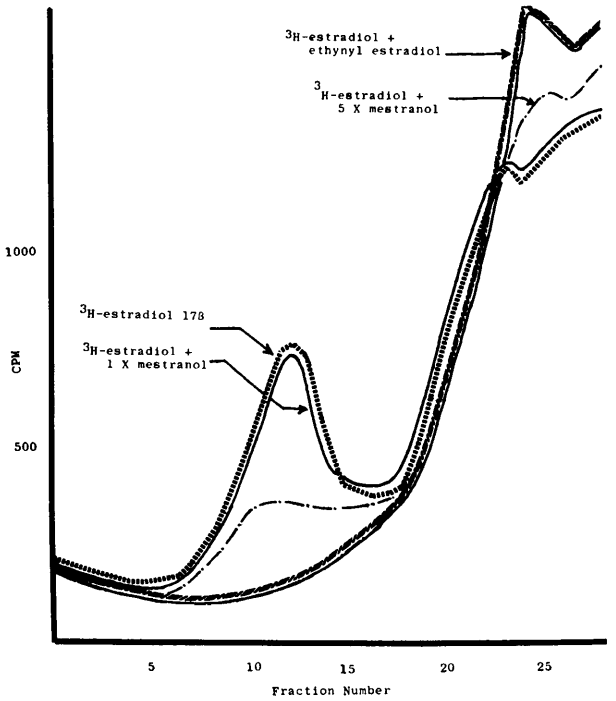

FIG. 1. Representative sucrose density gradient patterns of rat uterine-cytosol showing: (a) the standard profile of ^{3}H-estradiol-17β binding; (b) competition for binding by ethynyl estradiol; (c) lack of competition by mestranol at a concentration of 2×10^{-6} M; and (d) competition by mestranol for estradiol binding to its receptor at a concentration of 10^{-5} M. Two-tenths ml of cytosol containing isotope and potential competitors were layered on a 5–20% sucrose density gradient and centrifuged for 16 hr at 205,000g at 4°.

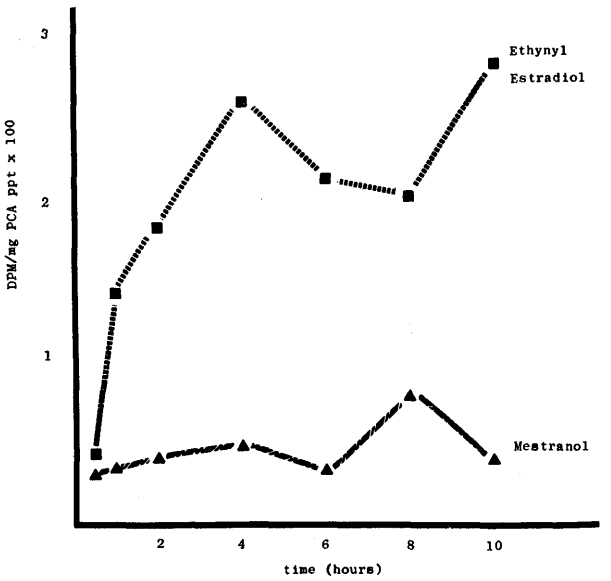

FIG. 2. Incorporation of tritiated uridine into uterine RNA at various times after a 0.1-μg injection of either mestranol or ethynylestradiol. Each point is a single determination; the experiment was repeated twice with similar results.

^{3}H-uridine into uterine RNA during a 2-hr pulse at various times after injection of equal doses (0.1 μg/animal) of ethynylestradiol and mestranol. The RNA synthetic rate increases rapidly after administration of ethynylestradiol. A similar relationship between the rate of RNA synthesis and time after estrogen treatment is reported for estradiol-17β (4). On the other hand, the RNA synthetic rate increases very little from the "unstimulated" level after the injection of mestranol. This experiment was repeated twice

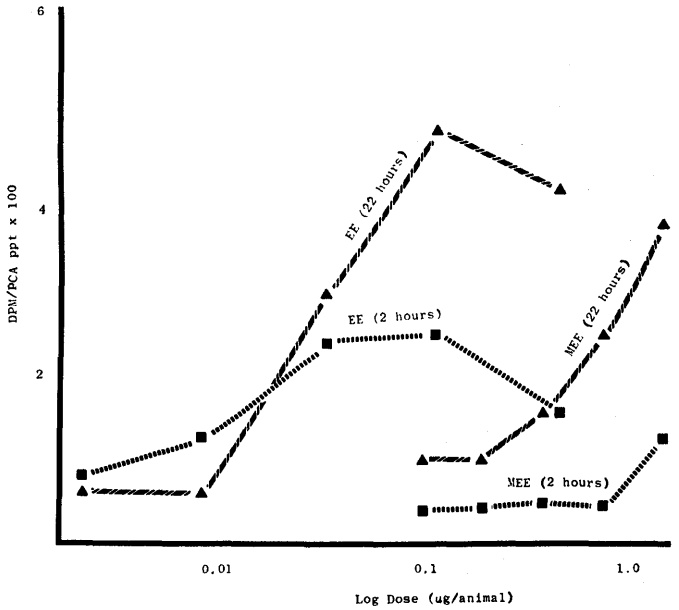

FIG. 3. Relationship between dose of mestranol or ethynylestradiol and uterine RNA synthesis at 2 and 22 hr after drug administration. Each point is the average of duplicate determinations.

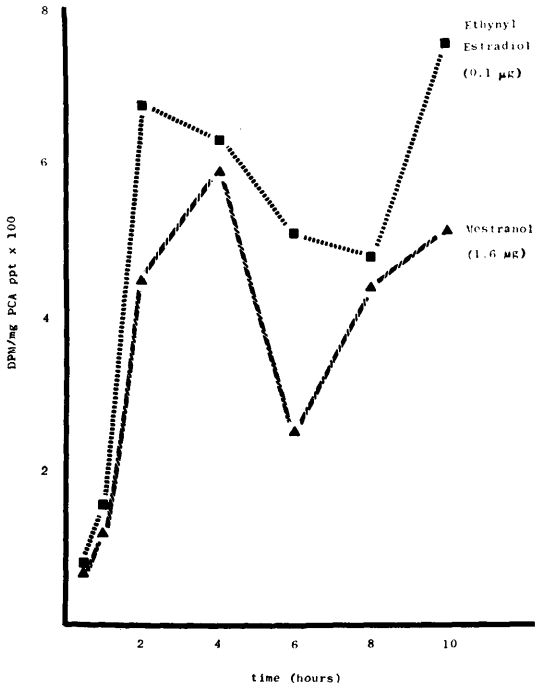

FIG. 4. Incorporation of tritiated uridine into uterine RNA at various times after injection of 0.1 μg of ethynylestradiol or 1.6 μg of mestranol. Each point is the average of duplicate samples.

with essentially the same results.

Figure 3 relates increasing dose (μg/animal) of mestranol or ethynylestradiol with the RNA synthetic response immediately (2 hr) and considerably (22 hr) after drug administration. The 22-hr response shows ethynylestradiol approximately 30 times more potent than mestranol in terms of stimulation of uterine RNA synthesis. The 2-hr response, which presumably would allow less time for metabolism, indicates at least a 100-fold difference in potencies. No response was noted at 2 hr postmestranol administration at a dose level less than 1.6 μg/animal.

The experiment shown in Fig. 4 reveals that when the dose of mestranol is increased to 1.6 μg/animal, the early uterine RNA synthetic rate increases to a pattern much like that induced by ethynylestradiol at 0.1 μg/animal. RNA induced by the above doses was fractionated by methylated albumin kiesulguhr (MAK) ion exchange chromatography into sRNA, rRNA, and TD-RNA (8). The proportion of RNA synthesized as each fraction was essentially identical after mestranol or ethynyl estradiol treatment.

Discussion. Published biological studies (9–11) indicate that mestranol is 30–50% less estrogenic than ethynylestradiol. We investigated two molecular parameters of target tissue responses to estrogens. The first was binding to estrogen receptors, a parameter earlier used by McGuire *et al.* (12), and the second was induction of uterine RNA synthesis used by Miller and Emmens (4) as a measure of estrogenic activity.

Our studies using molecular end points, substantiate the difference in potency between EE and MEE. EE binds to the estrogen receptor more strongly than does MEE, and at a dose of 0.1 μg/animal, only EE induces rapid synthesis of uterine RNA *in vivo*.

However, our data also raise a question which we are unable to answer at this time. Abdel-Aziz and Williams (3) and Williams (13) reported that one of the urinary metabolites of mestranol is ethynylestradiol. Jensen *et al.* (14) found that whereas both radioactive EE and MEE accumulated in the

uterus following injection of tritiated mestranol, EE strongly predominated. These results suggested to Jensen that in order to act as an estrogen, MEE must first be demethylated to furnish EE which is taken up and retained by receptors in target cells. He interpreted the small amount of labeled mestranol found in the uterus simply to result from background levels of labeled mestranol and not from target cell receptor binding. Our data confirm his findings, but we feel there is another possible interpretation of this data. We found that a large dose of unlabeled estradiol-17β caused a tenfold reduction of both radiolabeled EE and MEE present in the uterus after injection of ^{3}H-MEE. This procedure has commonly been used to demonstrate *in vivo* competition for estrogen receptors. Competitive inhibition of uterine uptake of MEE *in vivo* indicated to us that mestranol itself binds to uterine estrogen receptors.

In vitro studies also demonstrated that both MEE and EE bind to the estrogen receptor though again EE bound more strongly. In the RNA studies at a dose of 0.1 μg/animal, only EE induced rapid synthesis of uterine RNA *in vivo*. However, dose–response curves indicate that higher doses of MEE generate equivalent and identical RNA synthetic responses as does EE. Unfortunately in these *in vivo* studies we cannot eliminate all possibility of tissue metabolism. However, our data showing that mestranol binds to an estrogen receptor both *in vitro* and *in vivo* suggest that mestranol itself may be weakly estrogenic without conversion to ethynylestradiol.

Summary. Two molecular parameters of estrogenicity, binding to estrogen receptors and induction of uterine RNA synthesis, were used to compare the estrogenicity of mestranol and ethynylestradiol. Following iv injection of tritiated mestranol, both mestranol and ethynylestradiol accumulated in the uterus with a predominance of the latter. Unlabeled estradiol-17β depressed uptake of both drugs by the uterus *in vivo*, and both compounds bound *in vitro* to a component of high speed uterine supernatant believed to be the estrogen receptor. Ethynylestradiol has a greater binding affinity for its receptor than does mestranol. At a dose of 0.1 μg/animal only ethynylestradiol induced significant RNA synthesis. However, at a markedly elevated dose, mestranol generated equivalent and identical RNA synthesis.

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