Differential Inhibition *in Vitro* of 17β-Estradiol Binding in the Mouse Uterus and Vagina by Optical Antipodes of Estrogens

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(Received November 10, 1967, and in revised form February 15, 1968)

SUMMARY

On incubation *in vitro*, tritium-labeled 17β-estradiol is taken up and retained by the uterus and vagina, but not by the diaphragm, of the immature mouse. The uptake of labeled 17β-estradiol by the uterus and vagina was inhibited by unlabeled optical antipodes of some potent estrogens: 17β-estradiol and the demethylated analogs corresponding to Fenocyclin and to methallenestril. The more estrogenic of the two antipodes of these estrogens was also the more potent inhibitor of uptake. The (−)-antipode of 17β-estradiol was very active in inhibiting uptake and was also found to be antiestrogenic. The (−)-antipode of the Fenocyclin phenol was about 200 times as active in inhibiting uptake, and also about 200 times as estrogenic, as the (+)-antipode. The correlation in activity between the two tests was somewhat less for the methallenestril phenol antipodes.

The two antipodes of an estrogen thus have a relative binding affinity for the target organs which is fairly well correlated with their relative estrogenic activities. This is evidence that the studied binding sites in the target organs really are related to receptors of the effector cells.

INTRODUCTION

Estrogenic substances in some way selectively promote growth of the reproductive organs. This tissue selectivity indicates that the responsive organs differ in some fundamental respect from the unresponsive organs. It is also well known that estrogenic substances are selectively taken up only by the tissues which they affect and not by others (1–7). Possibly the selective uptake is related to the estrogenic effect. To test this the uptake of estrogenic substances *in vitro* by the uterus and vagina of the immature mouse has been compared with their estrogenic effects *in vivo*. It is known that under proper conditions the *in vitro* interaction between estrogen and target tissue is a reasonably good model for *in vivo* conditions (8–10). To avoid complications from differences in physicochemical properties, pairs of optical isomers were tested. Their physicochemical properties, other than optical activity, are identical. The compounds tested were the antipodes of 17β-estradiol[1] and of some estrogenic carboxylic acids which are closely related chemically (Fenocyclin and methallenestril, which are methyl ethers, and the corresponding free phenols). Because these compounds could not be obtained labeled with a high specific activity they were tested for their ability to inhibit the uptake of tritium-labeled 17β-estradiol.

[1] Abbreviations: 17β-estradiol, estra-1,3,5(10)-triene-3,17β-diol; Fenocyclin, 1-ethyl-1,2,3,4-tetrahydro-7-methoxy-2-methyl-2-phenanthrenecarboxylic acid; Fenocyclin phenol, 1-ethyl-1,2,3,4-tetrahydro-7-hydroxy-2-methyl-2-phenanthrenecarboxylic acid; methallenestril, 3-(6-methoxy-2-naphthyl)-2,2-dimethylpentanoic acid; methallenestril phenol, 3-(6-hydroxy-2-naphthyl)-2,2-dimethylpentanoic acid; PPO, 2,5-diphenyloxazole; dimethyl POPOP, 1,4-bis-2-(4-methyl-5-phenyloxazolyl)benzene.

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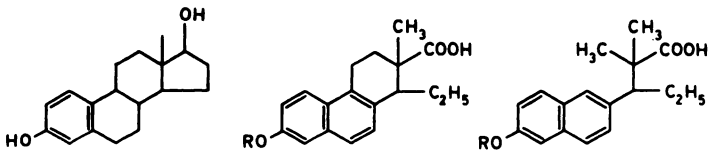

17β-Estradiol

R = H (Fenocyclinphenol)
R = CH_3 (Fenocyclin)

R = H (Methallenestrilphenol)
R = CH_3 (Methallenestril)

MATERIAL AND METHODS

Compounds. Tritium-labeled 17β-estradiol (labeled with tritium at the 6 and 7 positions) was purchased from New England Nuclear Corporation, Boston, Massachusetts. This radioactive 17β-estradiol is the natural (+)-antipode (Dr. N. Silberman, New England Nuclear Corp.; personal communication). Its specific activity was 140 μc/μg. The radiochemical purity was controlled by thin-layer chromatography at intervals. More than 98% of the total recovered radioactivity moved with the 17β-estradiol zone on the chromatograms. The nonradioactive (+)-antipode of 17β-estradiol (m.p. 173–174°) was purchased from Sigma Chemical Company, St. Louis, Missouri. A sample of the (—)-antipode of 17β-estradiol (Lot. No. I-1830-138A) was kindly donated by Dr. Gordon A. Hughes at the Wyeth Laboratories Inc., Philadelphia, Pennsylvania. Racemic Fenocyclin and methallenestril were kindly donated by Ciba AB, Vällingby, Sweden and Erco AB, Stockholm, Sweden, respectively. The optical resolution of Fenocyclin and methallenestril was carried out by the author according to (11) and (12), respectively. The resulting optical antipodes gave identical IR-spectra. They were demethylated by hydrobromic acid according to conventional methods. Chemical data on the antipodes of Fenocyclin, methallenestril and on the corresponding free phenols are summarized in Table 1. Optical rotations were recorded on a Perkin-Elmer polarimeter model 141. The recorded rotations for the demethylated compounds were measured on a little material and in micro cells and must be considered with reservation. The chemical data of the (—)-antipode of methallenestril used in this communication were also found to be identical with those of a specimen of the same compound kindly donated by Dr. Jean Jacques at Collège de France, Paris.

Animals. Immature female mice of the NMRI albino strain were utilized when they were 14–17 days old and weighed 8–10 g. They were fed on water and a commercial diet which was available ad libitum.

TABLE 1

Physicochemical properties of the optical antipodes of Fenocyclin and methallenestril and their corresponding free phenols

Optical rotations were recorded in absolute ethanol.

Substance	$[\alpha]_D^{22°}$	Melting point (°C)
(−)-Fenocyclin	−97.9	211–214
(+)-Fenocyclin	101.2	214–216
(−)-Fenocyclin phenol	−101	—
(+)-Fenocyclin phenol	109	—
(−)-Methallenestril	−24.83	141–143
(+)-Methallenestril	24.27	141–143
(−)-Methallenestril phenol	−24.85	—
(+)-Methallenestril phenol	24.27	—

Uterotropic and antiuterotropic assays. Uterotropic activity was measured as the increase in uterine dry weight. The estrogen, in 0.1 ml of olive oil, was administered by the subcutaneous route 3 times at 24-hour intervals. The controls received only oil. At 24 hours after the last injection the animals were killed. Their uteri were dissected free and dried for 24 hours at 100°.

Finally the uteri were weighed. Uteri were taken from at least 5 animals per group.

The antiuterotropic activity of (—)-17β-estradiol was measured similarly. The (—)-antipode was injected subcutaneously in 0.05 ml olive oil simultaneously with the subcutaneous injection of 0.01 μg (+)-17β-estradiol in 0.05 ml oil. The (—)-antipode was injected at one flank of the mouse and the (+)-antipode at the other flank. Three injections were given and the uteri were then handled as described for the uterotropic assay.

Principle of the in vitro method. The conditions for *in vitro* experiments in this communication were adopted from reference 13. The tissues were first incubated with radioactive 17β-estradiol in the absence (controls) or in the presence of the nonradioactive antipode to be studied. Under the conditions used the target tissues accumulate estrogens partly in a specific way but there is also a considerable nonspecific accumulation. Material taken up nonspecifically can be largely reduced by a subsequent washing in the presence of a large excess of nonradioactive estrogen. Most of the estrogen taken up by the nontarget tissue, the diaphragm, is washed out while most of the estrogen taken up by the target tissues, the uterus and vagina, is retained. The tissue specificity of the *in vitro* uptake of estrogens is thus markedly improved by this procedure. The 17β-estradiol concentration ratios of controls for uterus/diaphragm increase from 4, before, to 25–50, after the washing.

Experimental details of the in vitro method. The incubation medium was Krebs-Ringer phosphate buffer (14), pH 7.4, prepared from reagent grade chemicals and redistilled water. Two percent (w/v) bovine serum albumin (Cohn, fraction V Sigma B grade) was added. Radioactive and nonradioactive estrogens were kept in ethanolic stock solutions. Aliquots of these solutions were taken to dryness *in vacuo* and redissolved in the buffer.

The uterus, vagina, and diaphragm were dissected out immediately after the animals had been killed. The uterus was divided at the cervix into two equal parts, and the vagina was longitudinally slit into two equal parts. Strips of about 3 mg were cut out from the diaphragm. One half-uterus, and in many cases also one half-vagina and one piece of diaphragm, from each of two animals were added to a stoppered flask. The flask contained 3 ml of buffer and had air as the gas phase. It was shaken at constant temperature in a Warburg apparatus. In many experiments one half-uterus and one half-vagina were incubated with one antipode while the other half-uterus and half-vagina of the same animal were incubated with the other antipode.

The tissues were first incubated at 37° for 1 hr with 0.0005 μg of radioactive 17β-estradiol per milliliter (controls); the experimental flasks also contained the nonradioactive test antipode. Then, the tissues were transferred to other flasks which contained 0.15 μg of nonradioactive 17β-estradiol in 3 ml of buffer and were incubated for 1 hr at 25°. After these two incubations the tissues were gently blotted between two filter papers and weighed wet on a torsion balance. The tissues were individually put into scintillation vials and digested with Hyamine (Packard Co.). One milliliter of a mixture of 1 M Hyamine in methanol and toluene (1:3 v/v) was added to each vial, and the vials were shaken for 3–4 hr at 70°. Then 5 ml of scintillation solution (0.5% PPO and 0.03% dimethyl POPOP, Packard Co., in A.R. grade toluene) was added. The radioactivity was measured in a Tri-Carb liquid scintillation spectrometer Model 3324 (Packard Co.). At least 10,000 counts were recorded, but no sample was measured for a longer period than 50 min. The counting efficiency was determined by external standardization and was found to be around 25%.

The chemical nature of the radioactivity accumulated under these *in vitro* conditions has been analyzed by partition chromatography and crystallization methods according to reference 15. By these methods, it has been found that most of the accumulated radioactivity in the uterus and vagina behaves as the original 17β-estradiol.

RESULTS

The uterotropic activities of the optical antipodes of 17β-estradiol are illustrated in Fig. 1. The (+)-antipode is the naturally occurring one. Because the (—)-antipode has a very low potency and was available only in limited amounts, a full dose-response curve could not be obtained.

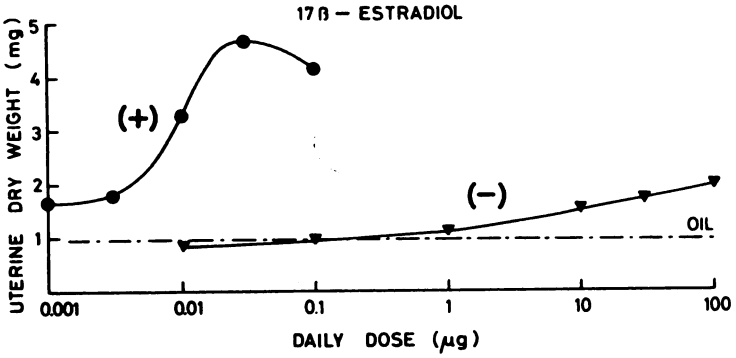

FIG. 1. *Uterotropic activities of 17β-estradiol antipodes*

It is evident from the figure, however, that there is a great activity difference between the two isomers. The (—)-antipode gives a very flat log dose-response curve, and its uterotropic potency is therefore not strictly comparable with that of the (+)-antipode. However, the dose of the (—)-antipode necessary for a 2-fold increase in uterine dry weight is at least 10,000 times that of the (+)-antipode.

Figure 2 shows the inhibitory effect of the nonradioactive 17β-estradiol antipodes on the uptake of the (+)-antipode of tritium-labeled 17β-estradiol by the uterus and vagina of the mouse. The uptake is expressed as percentage of an uninhibited control. (In the conditions used the final level of radioactivity per unit wet weight in the control vagina is about 100–120% and the final level in the control diaphragm is less than 4% of that of the control uterus.) Both antipodes of 17β-estradiol in sufficient amounts depressed the level of radioactivity in the uterus and vagina to very low values. The effect on the vagina was greater than on the uterus. The (+)-antipode had about 80 times the inhibitory activity of the (—)-antipode both on the uterus and on the vagina. The very low content in the diaphragm was only slightly reduced by the antipodes (not shown in the figure). The inhibitory activity of the

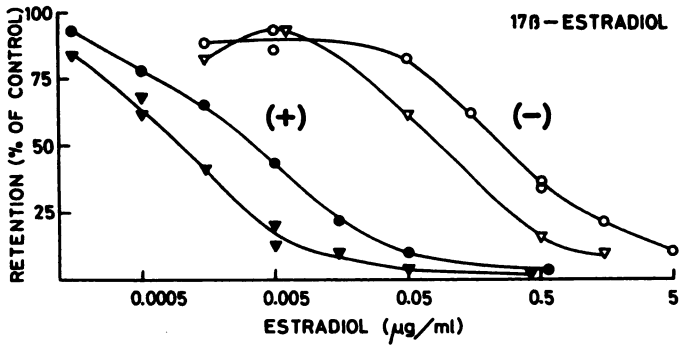

FIG. 2. *Effect of unlabeled 17β-estradiol antipodes on the in vitro uptake of labeled 17β-estradiol in the uterus and vagina*

The circles represent the uterus and the triangles represent the vagina. There were tissues from 4 animals per group.

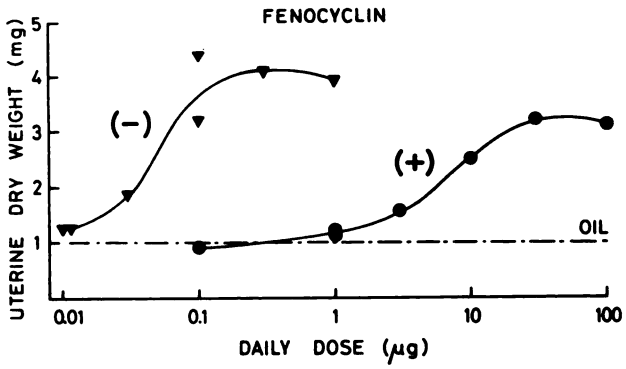

FIG. 3. *Uterotropic activities of Fenocyclin® antipodes*

(—)-antipode of 17β-estradiol is high in relation to its relative estrogenic activity (cf. Fig. 1). Therefore it was tested if the (—)-antipode was antiestrogenic. This was indeed found to be so (Table 2).

TABLE 2
Antiuterotropic activity of (−)-17β-*estradiol*
There were 10 or 11 mice per group.

(−)-17β-Estradiol, daily dose (μg)	Uterine dry weight (mg)
None (oil)	3.46 ± 0.10
1	3.27 ± 0.18
10	2.51 ± 0.12[a]

[a] $P < 0.001$.

The estrogenic carboxylic acid Fenocyclin has 2 asymmetric carbons and is the potent one of the two possible racemates. The (—)-antipode of Fenocyclin was about 200 times as uterotropic as the (+)-antipode (Fig. 3). It has been found previously that the methyl ether group has to be removed from Fenocyclin before it can influence the uptake of 17β-estradiol *in vitro* (16). In that communication evidence was also presented that the demethylation to the corresponding free phenol was a prerequisite for the biological effect. In Fig. 4 the uterotropic activities of the racemate and of the antipodes of the Fenocyclin phenol are shown. The (—)-antipode was about 300 times as potent as the (+)-antipode and about twice as potent as the racemate. The effect of the antipodes of the Fenocyclin phenol on the uptake of radioactive 17β-estradiol by the uterus and vagina is shown in Fig. 5. The (—)-antipode was about 200 times as active as the (+)-antipode. Again, the uptake of 17β-estradiol by the vagina was more strongly inhibited than the uptake by the uterus. The very

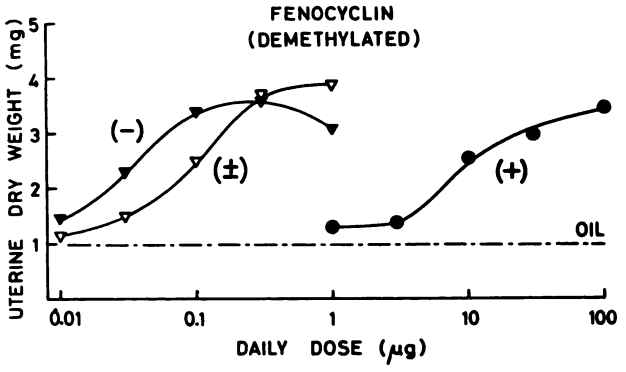

FIG. 4. *Uterotropic activities of the racemate and the antipodes of the free phenol corresponding to Fenocyclin*
Open symbols represent the racemate, and closed symbols represent the antipodes.

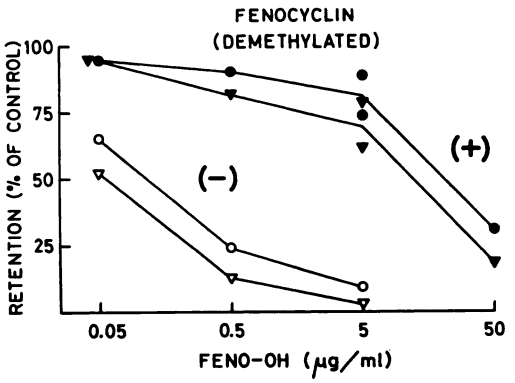

FIG. 5. *Effect of the antipodes of the free phenol corresponding to Fenocyclin on the in vitro uptake of labeled 17β-estradiol in the uterus and vagina*

The circles represent the uterus, and the triangles represent the vagina. There were tissues from 4–6 animals per point.

low diaphragm uptake was also slightly lowered, and there was a probable activity difference between the antipodes (not shown in the figure). Figure 6 shows a comparison of the effects of the (—)-antipode and the racemate of the Fenocyclin phenol on the uptake of 17β-estradiol by the uterus and the vagina. The (—)-antipode was about twice as active as the racemate, a result that again shows that the racemate derives its biological activity from the (—)-antipode.

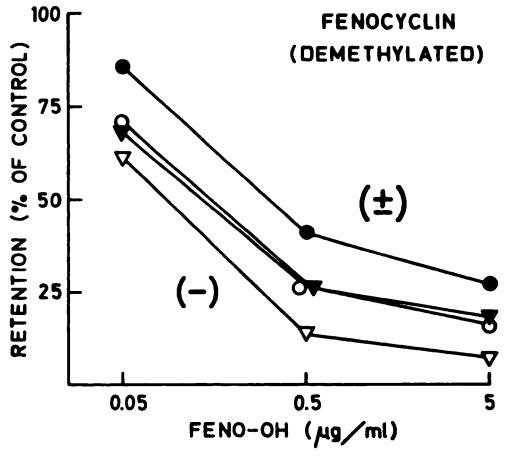

FIG. 6. *Effect of the racemate and the (−)-antipode of the free phenol corresponding to Fenocyclin on the in vitro uptake of labeled 17β-estradiol in the uterus and vagina*

The circles represent the uterus while the triangles represent the vagina. Closed symbols represent the racemate, and open symbols the (—)-antipode. There were tissues from 4 animals per point.

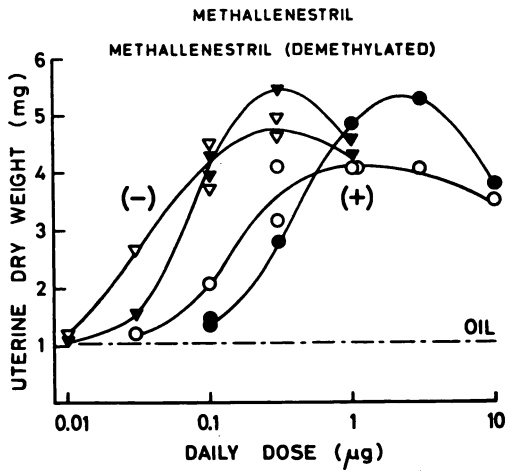

FIG. 7. *Uterotropic activities of the antipodes of methallenestril and of the corresponding free phenol*

Closed symbols represent methallenestril antipodes, and open symbols represent the antipodes of the corresponding free phenol.

The estrogenic carboxylic acid methallenestril has one asymmetric carbon atom and its two antipodes were found to have different estrogenic activities. The antipodes of the corresponding free phenol had a similar

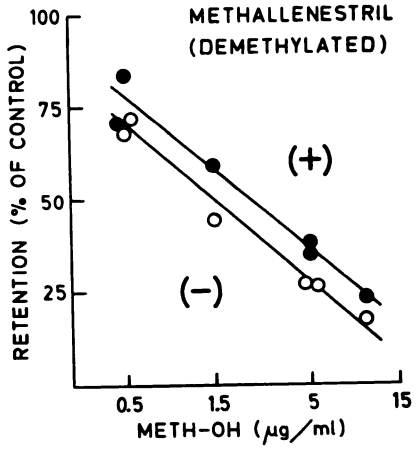

FIG. 8. *Effect of the antipodes of the free phenol corresponding to methallenestril on the uptake of 17β-estradiol in the uterus*

There were tissues from 4 animals per group.

TABLE 3

Comparison between the effects of antipodal estrogens on the in vitro uptake of (+)-17β-estradiol in the uterus and vagina and their uterotropic activities

The data are calculated from the figures.

Substance	*In vitro* Concentration (μg/ml) for 50% inhibition				*In vivo* Daily dose (μg) for half-maximum tropic effect		*In vivo/ in vitro*
	Uterus I	Vagina	Uterus / vagina	Antipode ratio, uterus	Uterus II	Antipode ratio	Uterus II / Uterus I
(+)-17β-Estradiol	0.0035	0.001	3.5	70	0.0075	(>10,000)	2.1
(−)-17β-Estradiol	0.25	0.085	2.9		>100		>400
(+)-Fenocyclin phenol	20.0	12.0	1.7	180	12	300	0.6
(−)-Fenocyclin phenol	0.11	0.055	2.0		0.036		0.3
(+)-Methallenestril phenol	2.4	—	—	1.6	0.15	4	0.06
(−)-Methallenestril phenol	1.5	—	—		0.04		0.03

ilar difference in estrogenic potency (Fig. 7). The two (−)-antipodes were 4–5 times as uterotropic as the (+)-antipodes. From the previous investigation (16) it is known that methallenestril also has to be demethylated in order to affect 17β-estradiol uptake in the uterus, i.e., presumably to elicit biological effect. The effect of the two antipodes of the methallenestril phenol on the uptake of radioactive 17β-estradiol by the uterus is shown in Fig. 8. Also in this assay the (−)-antipode was the most potent one, but the activity ratio between the two antipodes was only about 1.6.

The observed biological activities of the antipodal estrogens are summarized in Table 3.

DISCUSSION

The amount of radioactive 17β-estradiol taken up by the uterus and vagina of the uninhibited controls under the present *in vitro* conditions is about 10^{-5} μg/mg wet weight. This is well below the saturation level *in vitro* and corresponds to the amount taken up by the uterus after the subcutaneous injection of 0.01 μg 17β-estradiol (7). This dose, given daily for 3 days, elicits a submaximal growth stimulation on the uterus (cf. Fig. 1). The accumulation studied *in vitro* can thus be considered to be within a "physiological" range.

In the standard uterotropic assay many events besides the interaction of estrogen with the target organ affect the response, e.g., the rate of absorption from the injection site, metabolism, excretion. The *in vitro* system is much simpler since it contains only the target organ and the estrogen.

In Table 3, last column, it is shown that there are considerable differences in the ratios of uterotropic activity to *in vitro* activity between different estrogens. These differences can be explained by the complexity of the *in vivo* test. The carboxylic acids, Fenocyclin and methallenestril, are physicochemically quite different from 17β-estradiol e.g., they are much more polar and hydrophilic (16). Such factors probably affect the distribution and turnover time in the body. The comparison of activities between the two optical antipodes of

an estrogen is more applicable because the two antipodes will distribute identically in the body and behave differently only in contact with other asymmetric areas. Since the "detoxifying" enzymes which convert the estrogens to glucuronides and sulfates are quite unspecific, it is possible that they are also metabolized and excreted identically.

The two antipodes of 17β-estradiol were found to have very different uterotropic properties (Fig. 1). The (—)-antipode was not only very much less potent but also gave a very shallow log dose-response curve. In Fig. 2, however, it is shown that the (—)-antipode is a comparatively potent inhibitor of the *in vitro* uptake of the (+)-antipode. If uptake by the binding sites is necessary for the tropic effect of the (+)-antipode one would expect the (—)-antipode to inhibit the response of the uterus to injected (+)-antipode. This was found to be so (Table 2). A shallow log dose-response curve for uterotropic activity, similar to that found for (—)-17β-estradiol, is characteristic for the so-called impeded estrogens. The natural estrogen estriol is one further member of this group (17). Estriol is also antiuterotropic and a potent inhibitor of 17β-estradiol uptake in the present *in vitro* system (unpublished results). Thus, both (—)-17β-estradiol and estriol retain some affinity for estrogen binding sites but have a very low efficacy.

It is interesting to note that Buzby *et al.* (18) found that the (—)-antipode of 17β-estradiol has only slightly less antilipemic activity than estrone in the adult rat while it has about 1700 times less uterotropic activity than estrone in the immature mouse. The structural requirements for antilipemic activity are apparently much less stringent than those for estrogenic activity and also for uptake in the uterus.

The estrogenic activities of the optical antipodes of Fenocyclin and its corresponding free phenol have been tested by others in a modified Allen-Doisy test (criterion: vaginal cornification) in the spayed rat. The antipodes were injected subcutaneously in oil on a single occasion (11). The (—)-antipodes of both Fenocyclin and its corresponding free phenol were found to be about 200 times as active as the (+)-antipodes. This activity difference is in good agreement with the present findings. The estrogenic activities of the Fenocyclin antipodes were also close to those of the corresponding antipodes of the free phenol (11, present findings) which indicates that the necessary demethylation step (16) does not show preference for either antipode. *In vitro,* the (—)-antipode was about 200 times as active as the (+)-antipode. Thus, the difference between the antipodes in uterotropic and vaginotropic potency and the difference in potency in the *in vitro* system are similar. The estrogenic activity of the methallenestril antipodes has been tested by others in an Allen-Doisy technique which was identical with that used for Fenocyclin (12). The (—)-antipode of methallenestril was found to be 3–4 times as active in the rat as the (+)-antipode (however, no full dose-response curve seems to have been recorded). In the present strain of mice, a similar difference in uterotropic potency was found between the antipodes of methallenestril and between the antipodes of the corresponding free phenol. *In vitro* the (—)-antipode of the methallenestril phenol was only slightly more active as uptake inhibitor than the (+)-antipode. In this case there is thus less agreement between the two tests than in the case of Fenocyclin.

In vitro the two antipodes of 17β-estradiol and of the Fenocyclin phenol were found to inhibit the vaginal uptake of (+)-17β-estradiol more strongly than the uterine uptake. The concentrations necessary for 50% reduction of the uterine retention were 2–4 times higher than those required for a similar reduction of the vaginal retention (Table 3, *in vitro* columns). In similar experiments, estriol and estrone have also been found to be 2–3 times as active against the vagina as against the uterus (unpublished results). All these estrogens thus appear to have a greater affinity for binding sites in the vagina than in the uterus, but the constancy of the effective concentration ratios, uterus/

vagina, indicate that the structural requirements for fixation to the binding sites are similar both for the uterus and the vagina.

It has been claimed by Pfeiffer (19) that optical antipodes of potent drugs differ greatly in potency and that the activity difference between the antipodes decreases with decreasing potencies of the drugs. In the present investigation, the more potent antipode of 17β-estradiol and of the Fenocyclin phenol were found to be, respectively, about 100 and 200 times as active as the less potent antipode in the *in vitro* test. The antipodes of the methallenestril phenol, on the other hand, were almost equipotent with a potency ratio of about 1.6. It should be noted that *in vivo* the more potent antipode of methallenestril is about as uterotropic as the more potent Fenocyclin antipode. The Pfeiffer relationship thus appears to be invalid for these compounds. A probable reason could be the fact that, while in 17β-estradiol and Fenocyclin the asymmetric carbon atoms are fixed in rigid molecules, the asymmetric carbon of methallenestril is a part of a long, flexible side chain. A flexible molecule has a greater conformational freedom and can be expected to assume the critical conformation necessary for fixation to the receptor more easily than a rigid molecule.

Although the estrogenic acids Fenocyclin and methallenestril differ markedly in structure from 17β-estradiol, it is interesting to note that they have similar crystallographic characters. Horeau and Jacques (20) used thermal analysis on mixtures of racemic methallenestril and the synthetic estrogen *meso*-hexestrol and found them to be isomorphic. In a similar investigation it has been found that *meso*-hexestrol and 17β-estradiol also are isomorphic (21). Further evidence for crystal similarity between these estrogens is the formation of so-called quasi-racemates between two antipodal compounds of opposite configurations. It was found that the methyl ether of estrone and (+)-methallenestril as well as (+)-methallenestril and (+)-Fenocyclin could form such racemates (22). The formation of quasi-racemates occurs only between two compounds of antipodal configurations which have very similar crystals, although isomorphism is not absolutely necessary (23).

ACKNOWLEDGMENTS

I would like to thank Dr. Gordon A. Hughes, who kindly donated (−)-17β-estradiol. Erco AB donated racemic methallenestril and Ciba AB donated racemic Fenocyclin. Mrs. Carola Engström offered competent technical assistance. The work was supported by the Swedish Cancer Society.

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