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# Induction of uterine peroxidase by hexestrol analogues

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The rapid increase in the activity of peroxidase (EC 1.11.1.7) in the uterus following treatment of ovariectomized or immature rats with estrogen [1, 2] has led to the proposal that this enzyme may be a useful specific marker for those tissues which respond to estrogen [3–5]. Recently, we described a biochemical assay for estrogens based on the correlation between the estrogenic activity of a number of steroids and their abilities to induce peroxidase in the immature rat uterus *in vivo* [6]. The results gave a more appropriate measure of the physiological characteristics of the steroids than those based on binding affinity to specific receptors. This system has also been used to determine whether the catechol estrogens have any antiestrogenic properties [7]. It was therefore considered of interest to extend these studies to hexestrol [meso-3:4-di(*p*-hydroxyphenyl) hexane] and its derivatives and to look for antagonism of peroxidase induction by a structural analogue of hexestrol.

*Materials.* Most of the compounds tested were from the collection of estrogens synthesized by Mr. W. Lawson at the Courtauld Institute of Biochemistry, London [8–10] and were provided by Dr. W. Coulson. 2-Hydroxyestradiol-17$\beta$ was a gift of the Cancer Chemotherapy National Service Center, Bethesda, MD. Hexestrol was purchased from the Sigma Chemical Co., St. Louis, MO. [4-$^{14}$C]Estradiol (52 mCi/mmole) from Amersham, Oakville, Ontario, was shown by chromatography and autoradiography to be free of radioactive impurities. It was diluted with carrier to a specific radioactivity of 2–3 mCi/mmole and kept at 4° in the dark as a stock solution in ethanol (1 mg/ml). The diethyl ether used for extraction was free of peroxides.

*Preparation of uterine extracts.* Immature female Holtzman rats weighing 70–95 g were given a priming dose of estradiol (5 $\mu$g in 0.2 ml sesame oil) subcutaneously on day 1 and the test compounds in oil on day 5, before killing the animals 18 hr later. The uteri were dissected free of adhering fat, blotted, and weighed. The tissue was then cut into small pieces and homogenized in 5 ml of 0.1 M sodium phosphate buffer, pH 7.0, in a Potter–Elvehjem homogenizer with a Teflon pestle. The homogenate was diluted to 10 ml with buffer and centrifuged at 100,000 *g* (4°) for 30 min. After washing with buffer, the sediment was rehomogenized in the appropriate amount of 1.2 M NaCl to give a 5% (w/v) homogenate. It was centrifuged again at 100,000 *g* for 30 min, and portions of the supernatant fraction were used for the subsequent enzyme assays.

*Determination of peroxidase activity.* The uterine extract (1 ml), derived from 25 mg wet wt of tissue, was incubated for 30 min or 1 hr at 38° with [4-$^{14}$C]estradiol (1.8 $\mu$M), 2,4-dichlorophenol (0.25 mM), $H_2O_2$ (0.25 mM) and bovine serum albumin (10 mg) in 0.1 M sodium phosphate buffer, pH 7.4; total volume was 4 ml. After incubation, the medium was extracted with ether (3 × 1 vol.) and the radioactivity in the aqueous fraction was determined by scintillation counting as described previously [11]. Peroxidase activity was also determined by a more direct method using guaiacol as substrate [12]. The reaction mixture (3 ml) contained guaiacol (13 mM) and $H_2O_2$ (0.33 mM) in 0.01 M sodium phosphate buffer (pH 7.0) and 1.0 ml of the fraction containing peroxidase. The linear increase in absorbance at 470 nm resulting from the oxidation of guaiacol was then followed at 25° in a Beckman model 25 recording spectrophotometer.

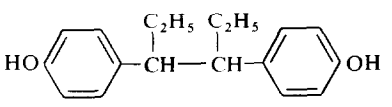
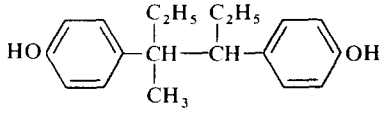
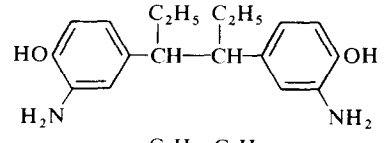
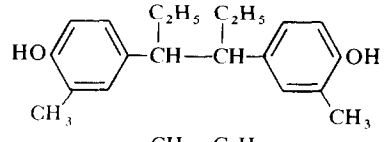
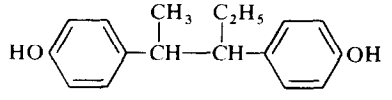
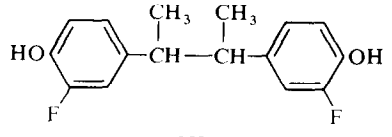
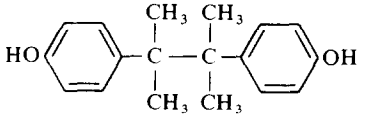
*Results and discussion.* A good correlation was obtained between the estrogenic activity of a series of hexestrol analogues and their ability to induce peroxidase in immature rat uteri (Table 1). This extends our previous studies with steroids related to estradiol [6]. The compounds to be tested were arranged in order of decreasing potency in bringing about vaginal cornification in ovariectomized rats [8–10]; their uterotrophic effect was also determined. Peroxidase activity was measured by the conversion of [4-$^{14}$C]estradiol to water-soluble products and by the oxidation of guaiacol, two methods that have been shown previously [6] to give comparable results.

With the exception of XII, the compounds chosen for testing had intact *p*-hydroxyl groups in the aromatic ring because their replacement by other groups is known to cause a marked decrease in estrogenic potency [9], as well as in binding affinity to receptors in the uterus [13]. Polar substituents in the side-chain abolished estrogenic activity (Table 2). Diethylstilbestrol epoxide, however, had been found previously to retain much of the activity of its parent compound [14, 15].

One of the objectives of these studies was to determine whether a natural estrogen such as 2-hydroxyestradiol-17$\beta$, which has been shown previously [7] to inhibit the action of estradiol, would show similar antagonism toward a synthetic estrogen and whether hexestrol analogues might act as anti-estrogens. For the latter purpose, 3,3′-difluorobutestrol (VI), having weak estrogenic activity, appeared to be the best candidate by analogy with anti-estrogens such as nafoxidine (U-11,/100A) and CI-628 which, in high doses, are estrogenic and able to induce uterine peroxidase [16, 17]. Analogue VI, however, showed no anti-estrogenic activity at any of the doses tested, even though 2-hydroxyestradiol-17$\beta$ at a dose of 100 $\mu$g/rat decreased significantly the response of the animals to hexestrol (1 $\mu$g/rat) (Table 3). Neither compound affected uterine weight, but this dissociation of effects on weight increase and peroxidase induction in the uterus was also observed when 2-hydroxyestradiol was administered concurrently with estradiol [7].

The increase in peroxidase activity in the immature rat uterus is a slightly less sensitive index of estrogenic potency than the increase in uterine weight, but nevertheless, it provides a useful alternative assay. It has the potential to yield additional information about the mechanism of estrogen action, particularly in distinguishing between the early inductive phase and the later phase of true growth [18, 19]. It is possible that the lack of an inhibitory effect on the estradiol-induced uterine weight increase by 2-hydroxyes-

Table 1. Structure–activity relationship of hexestrol and its derivatives*

| | Compound | Dose ($\mu$g) | Uterine wt (mg) | Percent of added [4-$^{14}$C]estradiol converted to water-soluble products | Rate of oxidation of guaiacol [$\Delta E_{470} \cdot \min^{-1}$ (g tissue)$^{-1}$] |
|---|---|---|---|---|---|
| |  | | 41.4 ± 1.2 | 11.9 ± 2.1 | 0 |
| I | | 1 | 87.8 ± 1.7 | 37.7 ± 2.7 | 8.2 ± 1.2 |
| | | 5 | 95.5 ± 4.6 | 47.4 | 15.2 |
| II |  | 1 | 83.6 ± 3.3 | 42.6 | 8.4 |
| | | 5 | 99.2 ± 6.6 | 47.3 | 9.2 |
| | | 10 | 95.4 ± 9.5 | 52.2 | 23.0 |
| III |  | 1 | 82.0 ± 2.6 | 34.1 | 4.0 |
| | | 5 | 89.5 ± 2.4 | 43.2 | 9.2 |
| | | 10 | 90.3 ± 3.0 | 50.3 | 11.5 |
| IV |  | 1 | 82.9 ± 8.9 | 20.0 | 2.6 |
| | | 5 | 92.8 ± 5.4 | 38.6 | 8.8 |
| | | 10 | 94.0 ± 4.1 | 43.0 | 10.6 |
| V |  | 1 | 72.3 ± 5.5 | 14.6 | 0 |
| | | 5 | 82.6 ± 6.3 | 24.5 | 1.6 |
| | | 10 | 88.2 ± 6.5 | 43.8 | 7.7 |
| VI |  | 5 | 65.2 ± 2.9 | 7.4 | 0 |
| | | 10 | 67.7 ± 2.8 | 17.5 | 1.4 |
| | | 25 | 75.2 ± 3.1 | 23.9 | 4.5 |
| | | 50 | 81.6 ± 4.1 | 37.7 | 7.6 |
| VII |  | 10 | 57.0 ± 3.5 | 16.5 | 1.6 |

* Estrogen-primed immature rats were given a single subcutaneous injection of the test compound in oil (0.2 ml) and killed 18 hr later. Uterine extracts from 25 mg of tissue were incubated with [4-$^{14}$C]estradiol or with guaiacol as described in the text. Values are the means of two experiments using uteri pooled from groups of five rats, except for the control and hexestrol groups (eighteen experiments) in which the S.E.M. for peroxidase activity is also given.

tradiol, while peroxidase activity was decreased, was due to the catechol estrogen affecting only certain reactions in the first of these two phases, thus producing a dissociation of estrogenic effects. Under normal conditions, the relative increases in peroxidase content brought about by estrogen have been shown to parallel the relative increases in uterine weights [4, 20].

In summary, we have compared the ability of eleven analogues of hexestrol with that of the parent compound to produce an increase in uterine weight and to induce peroxidase in the estrogen-primed rat uterus. We have also tested a weak estrogenic analogue of hexestrol for anti-estrogenic activity and shown that 2-hydroxyestradiol-17$\beta$ antagonized the uterine peroxidase-inducing action of hexestrol without affecting the weight increase in this organ.

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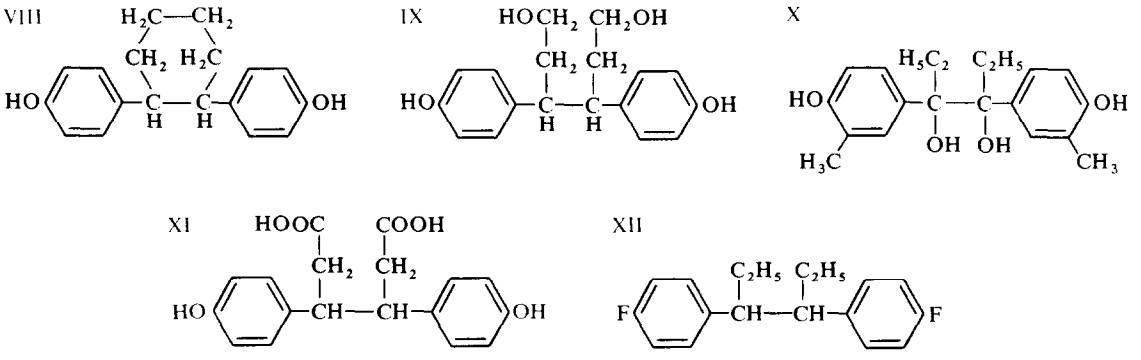
Table 2. Hexestrol derivatives without uterotrophic or peroxidase-inducing activity*




* The compounds were tested for uterotrophic and peroxidase-inducing activities as in Table 1.

Table 3. Effects of dihydroxyhexestrol, 3,3′-difluorobutestrol and 2-hydroxyestradiol on the induction of uterine peroxidase by hexestrol*

| Compound given in combination with hexestrol | Dose ($\mu$g) | Uterine wt (mg) | No. of rats | Percent of added [4-$^{14}$C]estradiol converted to water-soluble products | Rate of oxidation of guaiacol [$\Delta E_{470} \cdot \text{min}^{-1}$ (g tissue)$^{-1}$] |
|---|---|---|---|---|---|
| | | 87.8 ± 1.7 | 90 | 37.7 ± 2.7 | 8.2 ± 1.2 |
| 3,3′-Difluorobutestrol (compound VI) | 5 | 87.3 ± 1.9 | 30 | 39.0 ± 3.6 | 8.9 ± 2.1 |
| | 10 | 91.0 ± 1.7 | 45 | 31.9 ± 4.7 | 8.0 ± 2.1 |
| | 25 | 96.2 ± 2.7 | 10 | 39.9 (38.5–41.2) | 9.8 (8.8–10.8) |
| 2-Hydroxyestradiol-17$\beta$ | 100 | 91.7 ± 2.9 | 30 | 24.2 ± 1.2† | 4.1 ± 0.3† |

* Estrogen-primed immature rats were given 1 $\mu$g hexestrol (I) alone or in combination with 3,3′-difluorobutestrol or 2-hydroxyestradiol in oil by subcutaneous injection. Values for peroxidase are the means ± S.E.M., using uteri pooled from groups of five rats. The range is given for experiments with only two groups (ten rats).

† $P < 0.001$ ($t$-test), compared with rats treated with hexestrol only.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