Studies on the Effect of 2-Methyl-3-ethyl-4-phenyl-Δ^4-cyclohexene carboxylic acid (ORF 3858) and Its *p*-Hydroxylated Metabolites on Uptake of ^{3}H-Estradiol-17β by the Uterus (35214)

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2-Methyl-3-ethyl-4-phenyl-Δ^4-cyclohexene carboxylic acid (ORF 3858) possesses a postcoital antifertility effect in rats when administered during the first 2 days of pregnancy (1, 2) and ORF 3588 causes lytic destruction of embryos *in vivo*. However, the compound is estrogenic. These facts open the question about the mechanism of action of ORF 3858. Does ORF 3858 interfere with gestation by causing changes in the reproductive tract or does it act directly on the embryo?

Grimes and Yard (3) have reported that ORF 3858 is metabolized to *p*-hydroxylated derivatives (ORF 5530 or ORF 5531). Further, when this biotransformation is inhibited, the antifertility activity of ORF 3858 is lost (4). Accordingly, to understand this compound's mode of action it becomes critical to ascertain whether the observed estrogenicity of ORF 3858 is dependent on its metabolism to the *p*-hydroxylated form.

The estrogen receptor concept (5, 6) provides a method for separating estrogens from proestrogens because it can be used as an *in vitro* assay for assessing target level responses to estrogens. This concept is based on the findings (i) that estrogen responsive tissues preferentially accumulate labeled estrogens *in vivo* and *in vitro*, and (ii) this uptake is competitively inhibited by nonlabeled estrogens. Receptor macromolecules from the uterus have been isolated by sucrose gradient centrifugation (7). We utilized *in vitro* parameters of estrogen binding for determining whether it is ORF 3858 or its *p*-hydroxylated metabolites which are estrogenic at the target level.

Materials and Methods. Tritium-labeled 2-methyl-3-ethyl-4-phenyl-Δ^4-cyclohexene carboxylic acid (ORF 3858-^{3}H) (sp act, 61 μCi/mg) was used. The radiopurity of this compound was determined by thin-layer chromatography on silica gel plates developed in toluene–glacial acetic acid (3:1). All radioactivity was found in a single peak at the point characteristic of the compound.

Unlabeled ORF 3858 as well as 2-methyl-3-ethyl-4-*p*-hydroxyphenyl-Δ^3-cyclohexene carboxylic acid (ORF 5530) and 2-methyl-3-ethyl-4-*p*-hydroxyphenyl-Δ^4-cyclohexene carboxylic acid (ORF 5531) were used.

^{3}H-Estradiol-17β (40 Ci/mmole) was purchased from New England Nuclear Corporation and found to be pure by thin-layer chromatographic analysis. All the unlabeled hormones cited in this report were purchased from Sigma.

CFN strain rats (20-25 days of age) were used in the first study. In the second (centrifugation) experiment, mature CFN female rats which had been ovariectomized 2 weeks previously were used. All animals were killed by cervical dislocation.

An Intertechnique model SL-30 liquid scintillation spectrophotometer was used for all the radioactivity determinations. Internal standards of tritiated toluene were used to determine counting efficiency.

In the tissue distribution studies, uterus and samples of large intestine were removed immediately after sacrifice of immature rats. Each tissue was placed in an individual vial and incubated with 5 ml of Kerbs–Ringer–phosphate buffer (pH 7.4) containing 1% serum, plus labeled hormone (1 μCi/100 ml; 5.5×10^{-10} *M*) and inhibitors as noted at a concentration of 10^{-6} *M*.

Tissues were incubated at 23° for 2 hr,

washed in buffer, blotted, and weighed wet. After homogenization in an all-glass Potter-Elvehjem grinder with 5 ml of chloroform:methanol (2:1), the homogenate was filtered through glass gauze and evaporated under nitrogen. Lipoidal material was taken up in 5 ml of methylene chloride, and radioactivity was determined. Data are expressed as dpm/mg of tissue wet weight.

Two separate experiments were performed. In the first, label uptake into tissues was determined for ^{3}H-estradiol-17β; the competitive effects of unlabeled estradiol-17β, progesterone, cholesterol, testosterone, cortisol, cholestane, ethinyl estradiol, diethylstilbesterol, pregnanediol, estriol, androstenedione, ORF 3858, ORF 5530, and ORF 5531 on uptake of ^{3}H-estradiol-17β into tissues was studied. In the second, label uptake into tissues was determined for ORF 3858-^{3}H, and the competitive effects of unlabeled ORF 3858 and estradiol-17β on this uptake were studied.

In each sucrose gradient isolation experiment, uteri of approximately 20 ovariectomized adult rats were removed and homogenized in 5 ml of phosphate buffer (0.2 *M* Na_2HPO_4, 0.2 *M* NaH_2PO_4, 0.001 *M* EDTA, pH 7.5). The homogenate was first centrifuged at 12,000*g* for 30 min and the resulting supernatant was then centrifuged at 273,000*g* for 1 hr. 0.5 ml of the final supernatant was incubated with 0.9 μl of either ^{3}H-estradiol-17β (100 μCi/ml) plus compounds to be tested where noted at a concentration of 2×10^{-6} *M* at 4° or incubated with ORF 3858-^{3}H (100 μCi/ml) for 1 hr. 200 μl of the incubation media was then layered on a 5–20% sucrose gradient and centrifuged at 273,000*g* for 16 hr. Fractions were collected in counting vials, scintillation fluid was added, and radioactivity in the fractions was determined. Quenching was corrected for by the use of an external standard.

Results. Intestine (control tissue) accumulated less label than did uterus (target tissue), and competition by unlabeled estradiol-17β depressed label uptake only in uterus (Table I). These findings are compatible with those reported by other workers.

The results obtained in our first set of tissue incubation experiments (Table I) show no evidence for competition by cholesterol, cholestane, progesterone, testosterone, cortisol, androstenedione, pregnanediol or ORF 3858. However, incubation with estradiol-17β, ethinyl estradiol, diethylstilbestrol, estriol, ORF 5530, and ORF 5531 all depressed label uptake in uterus but not in intestine.

TABLE I. Relative Ability of Compounds to Depress the Uptake of ^{3}H-Estradiol-17β into Uterus and Intestine.[a]

Potential competitors	Uterus	Intestine
None	904 ± 51	167 ± 17
Cholesterol	820 ± 93	152 ± 26
Cholestane	1038 ±170	112 ± 9
Progesterone	697 ± 54	158 ± 28
Testosterone	777 ± 41	116 ± 35
Cortisol	836 ± 29	127 ± 9
Androstenedione	709 ± 41	110 ± 2
Pregnanediol	994 ± 74	149 ± 2
ORF 3858	703 ± 66	222 ± 19
Estradiol-17β	317 ± 17	221 ± 19
Ethynyl estradiol	249 ± 22	109 ± 6
Diethylstilbestrol	225 ± 36	107 ± 4
Estriol	282 ± 20	160 ± 20
ORF 5530	351 ± 25	198 ± 26
ORF 5531	292 ± 58	159 ± 20

[a] Uptake of ^{3}H-estradiol-17β by uteri and samples of intestine after incubation in 5 ml of buffer containing 0.01 μCi/ml ^{3}H-estradiol-17β and 10^{-6} *M* potential competitors. Data are expressed as dpm/mg of wet tissue weight ± SE.

As opposed to results seen with ^{3}H-estradiol-17β (901 ± 51 dpm/mg of wet wt) very little incorporation of ORF 3858-^{3}H was seen in the uterus (58 ± 4 dpm/mg of wet wt). Uterine label content was no greater than that of intestine. Further, neither incubation with unlabeled ORF 3858 (62 ± 5 dpm/mg of wet wt) or incubation with unlabeled estradiol-17β (60 ± 7 dpm/mg wet wt) resulted in depressed incorporation of ORF 3858-^{3}H into uterus.

Sucrose gradient centrifugation of labeled uterine soluble fractions produced a peak of radioactivity as noted in Fig. 1a, and competition with estradiol-17β obliterated the peak. While neither cholesterol nor ORF 3858 competed for this peak (Fig. 1b), unlabeled ORF 5530 and ORF 5531 both obliterated the peak (Fig. 1c). No receptor peak

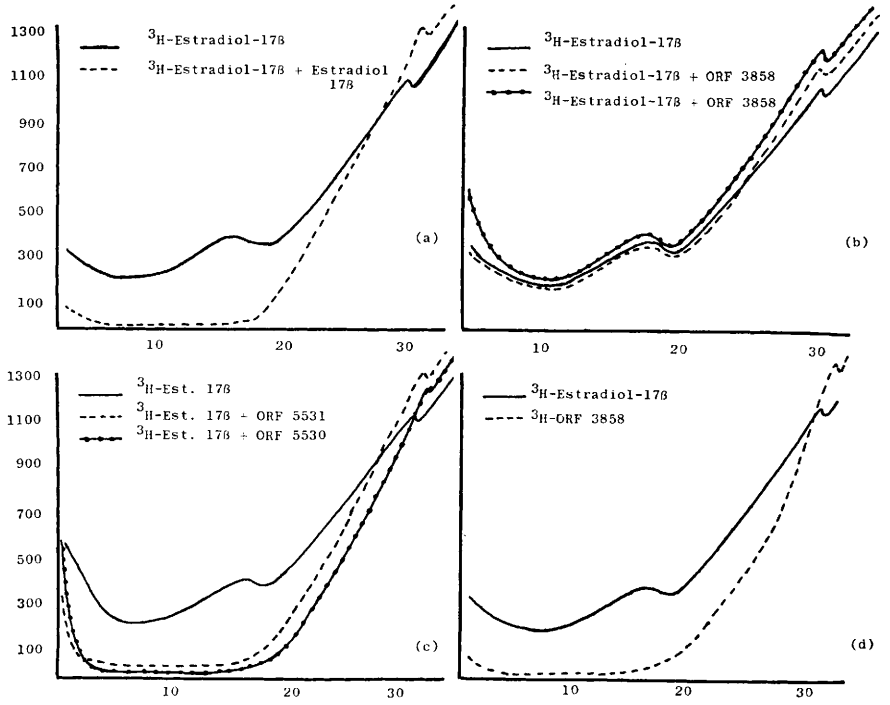

FIG. 1. Density gradient patterns of uterine cytosol fraction showing: (a) competion for the estrogen receptor region by estradiol-17β, (b) no competition by cholesterol and ORF 3858, and (c) competition by ORF 5530 and ORF 5531; (d) demonstrates the lack of a receptor region using ORF 3858-^{3}H. Two-tenths ml of soluble fraction containing isotope and potential competitors were layed on 5–20% sucrose gradients and centrifuged for 16 hr at 205,000g, 3°.

was seen after incubation of uterine soluble fractions with ORF 3858-^{3}H (Fig. 1d).

Discussion. This study is based on the observation that only estrogens appear to bind to estrogen receptors, and that nonlabeled estrogens will compete with labeled estrogens for existing estrogen receptors (8). While the majority of reported observations concern *in vivo* studies, *in vitro* techniques similar to those employed in this study have been reported in the past. In this experiment, uterus was chosen as the target issue and intestine was used as the control tissue.

Our findings indicate that ORF 3858 does not bind to estrogen receptors whereas its *para*-hydroxylated metabolites do bind. These findings lead one to conclude that ORF 3858 must be metabolized to its *p*-hydroxylated form to manifest its estrogenic effects. Estrogens have been demonstrated to interfere with pregnancy (10–12). Since metabolism of ORF 3858 to its *p*-hydroxylated form is required for its antifertility action (4), one might conclude that, at least in the rat, there is a relationship between the estrogenicity of the metabolites of ORF 3858 and the antifertility activity of ORF 3858.

Summary. The *para*-hydroxy metabolites of 2-methyl-3-ethyl-4-phenyl-Δ^4-cyclohexene carboxylic acid and known estrogens all depressed uptake of ^{3}H-estradiol-17β into uterus but not intestine. In contrast no evidence for competition was seen with ORF 3858 and nonestrogenic compounds. Little incorporation of ORF 3858-^{3}H was seen in target tissues, and neither estradiol-17β nor ORF 3858 competed for label uptake. Sucrose gradient centrifugation of uterine soluble fractions incubated with ^{3}H-estradiol-17β produced a peak of radioactivity believed to be the estrogen receptor complex. Neither cholesterol nor ORF 3858 depressed this peak; in contrast, incubation with estra-

diol-17β or the *para*-hydroxy metabolites of ORF 3858 did depress the peak. Incubation of uterine soluble fractions with ORF 3858-^{3}H did not result in any peak of activity which could be correlated with the estrogen receptor peak. These data indicate that ORF 3858 is not estrogenic at the target tissue level, but that the *para*-hydroxylated metabolities are estrogenic.

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