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Synergistic Responses of Steroidal Estrogens *in Vitro* (Yeast) and *in Vivo* (Turtles)

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Many environmental agents exert estrogenic activity. Previous studies from our laboratories demonstrated that certain combinations of environmental estrogens (i) reverse the sex of male turtle embryos in a synergistic manner (Bergeron et al., (1994) *Environ. Hlth Perspect.* 102, 780–782), and (ii) synergistically transactivate the human estrogen receptor (hER) in yeast and mammalian cells (Arnold et al., (1996) *Science* 272, 1489–1492). Because our findings with synthetic estrogens suggested that combinations of naturally-occurring steroidal estrogens might also produce synergistic activity of the ER, we used the same model systems to measure the activity of combinations of steroidal estrogens. The activity of combinations of estrone, estradiol-17β or estradiol-17α in yeast strains expressing hER was synergistic at submaximal concentrations of both estrogenic compounds. However, synergy was not observed with mixtures of estrogens when the concentration of one of the estrogens alone was maximally active in yeast. Ligand-binding assays in yeast performed with various radiolabeled estrogens suggested that multiple estrogens may interact with the receptor. The estrogen-dependent process of sex-reversal of turtle embryos incubated at a male-producing temperature was used to determine whether steroidal estrogens also had synergistic activity *in vivo.* In this instance, a combination of estriol and estradiol-17β was effective in reversing the gonadal sex of turtle embryos from males to females in a synergistic manner. Our results suggest that the synergy of some combinations of estrogens, synthetic or steroidal, may play a role in the estrogen-dependent process of sexual development in certain species. © 1997 Academic Press

Ovarian estrogens regulate expression of secondary sexual characteristics and some reproductive functions in women. In some wildlife species, estrogens may actually participate in regulating the sex of the offspring (1). Compounds with estrogenic activity can be grouped into three classes; ovarian estrogens such as estradiol-17β; compounds produced by plants, such as coumestrol, called phytoestrogens; and synthetic chemicals found in the environment such as 1,1,1-trichloro-2,2-*bis*(*p*-chlorophenyl)ethane (DDT) and polychlorinated biphenyls (PCB). In general, these compounds share limited structural similarity but have been classified as estrogenic based on their capacity to produce classical estrogen-like effects in animals (2 - 5). For the most part, the *in vivo* effects of these compounds appear to correlate with the interaction of the chemicals through the estrogen receptor (ER) *in vitro* (3, 5). The affinity of most environmental estrogens for the ER is 1/50th to 1/10,000th that of estradiol-17β (6). The low affinity of most environmental estrogens for the ER raises the issue of whether the exposure of wildlife or humans to these chemicals produces any deleterious effects.

A well-characterized example of sexual development influenced by estrogen exposure is the red-eared slider turtle. The sex of many turtles is determined by temperature, a process known as temperature-dependent sex determination or TSD (1, 7); red-eared slider eggs

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Abbreviations: dichlorodiphenyltrichloroethane, DDT; human estrogen receptor, hER.

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incubated at 26 °C or 31 °C result in all male or all female hatchlings, respectively (8). Turtle embryos incubated at a male-producing temperature can be reversed by spotting the eggs with estradiol, estriol, estrone or high concentrations of hydroxylated PCBs (4, 7, 9). A combination of some hydroxylated PCBs was effective in sex-reversal at concentrations of the chemicals that had no activity alone (4). The slider turtles were used as an *in vivo* assay in the present study for measuring the activity of individual or combinations of estrogens that affect sexual development.

An *in vitro* cell model system using yeast expressing hER was developed to facilitate studies of the molecular mechanisms associated with the adverse effects of estrogens at critical developmental points (10). We have used yeast expressing hER to demonstrate that some combinations of synthetic chemicals produce estrogenic activity in a synergistic manner (6). A mixture of two hydroxylated PCBs produced estrogenic activity that was seven-fold greater than either chemical alone in yeast and Ishikawa cells, a human endometrial cancer cell line. These results suggested that the hER contains multiple estrogen-binding sites and that the interaction of hER with two different chemicals can produce synergistic transactivation.

In this paper, we determined whether combinations of steroidal estrogens have synergistic activity in both *in vitro* (yeast) and *in vivo* (turtle) systems. Some combinations of low doses of steroidal estrogens produced synergistic estrogenic activity in yeast expressing hER. Binding analysis suggested the interaction of two estrogens with the hER. Synergistic activity of a combination of steroidal estrogens was also observed to sex-reverse red-eared slider turtle embryos.

MATERIALS AND METHODS

Chemicals. Estradiol-17β, estrone, estriol and estradiol-17α were purchased from Sigma Chemical Co. (St. Louis, MO). 17β-3,4,6,7[^{3}H](N) estradiol (99 Ci/mmol) and 2, 4, 6, 7 [^{3}H](N) estrone (76 Ci/mmol) were purchased from DuPont/NEN (Wilmington, DE).

Yeast assays. Strain YPH499 (Mat a, ura 3-52, lys 2-801, ade 2-101, trp 1-Δ63, his 3-Δ200, leu 2-Δ1) (11) was transformed with the expression plasmid pRS303 containing wild-type hER under control of the glyceraldehyde-3-phosphate dehydrogenase promoter (12) and 2ERE-*lacZ* reporter (10). Strains were grown overnight at 30 °C in synthetic medium-uracil, tryptophan (for DY150) or histidine (for YPH499). The next day, 25 μl of the overnight culture was diluted into 975 μl of fresh medium and grown overnight with DMSO or estradiol-17β, estrone, estriol or estradiol-17α. All the estrogens were prepared in DMSO and the concentration of DMSO in the assay was less than 1.0%. The combinations of estrogens were pre-mixed and added so that the concentration of DMSO was equivalent to that used for the individual estrogens. None of the chemicals substantially inhibited the growth of the yeast at the concentrations tested.

β-galactosidase assays. These assays have been extensively described (6, 10). The absorbance of the reaction was measured at 420 nm (A_{420}). The growth of the yeast strains was monitored by measuring the absorbance at 600 nm (A_{600}). Miller units were determined using the following formula: [A_{420}/(A_{600} of 1/10 dilution of cells $\times$ volume of culture $\times$ length of incubation)] $\times$ 1000. Statistics were performed by one-way ANOVA least significant difference test (Microsoft Excel). Significant differences were defined when $p<0.05$. The data are the result of three independent experiments with three replicates each.

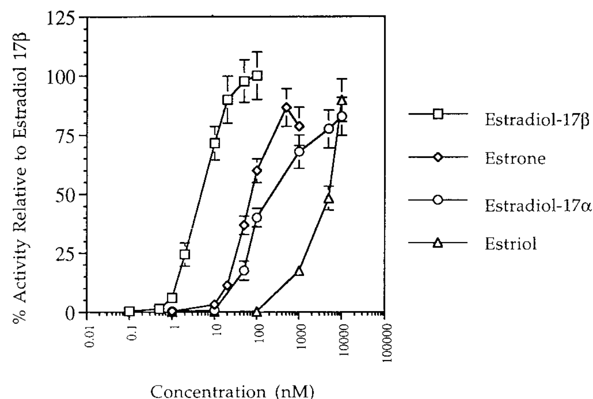

FIG. 1. Activity of various estrogens in yeast strain hER-ERE. Strain hER-ERE was grown overnight in the presence of the estrogens at increasing concentrations. β-galactosidase was measured after 14h, calculated as Miller Units as described in the Materials and Methods and presented as a percentage of estradiol-17β (100% at 10 nM; 6100 Miller Units). Each value is the mean of three independent experiments with three replicates.

Whole cell binding assays. Strain YPH499 was grown overnight at 30 °C in synthetic medium-tryptophan, uracil. The next day, 25 μl of the overnight culture was diluted into 975 μl of fresh medium and grown overnight with [^{3}H]estradiol-17β in the presence or absence of radioinert estrone. Alternatively, [^{3}H]estrone was added in the presence or absence of estradiol-17β. The combinations of estrogens were pre-mixed and added so that the concentration of DMSO was equivalent to that used for the individual estrogens. None of the chemicals inhibited the growth of the yeast at the concentrations tested. The cells were collected by centrifugation, washed three times with ice-cold PBS, suspended in PBS and the radioactivity measured in a liquid scintillation counter. Statistics were performed by one-way ANOVA least significant difference test (Microsoft Excel). Significant differences were defined when $p<0.05$. The data the result from three independent experiments with three replicates each.

Sex-reversal. Eggs were purchased from Robert Kliebert (Hammond, LA) and incubated at 26 °C on a layer of vermiculite: water (1:1) in temperature-controlled chambers (Precision). At the beginning of the period of gonadal differentiation (approximately five weeks from the date eggs are laid at this temperature), which coincides with the developmental stage at which the embryos are sensitive to the effects of exogenous estradiol-17β (9), eggs were randomly assigned to treatment groups and spotted with various concentrations of estriol, estradiol-17β, estradiol-17β plus 0.01 μg estriol, or with ethanol alone (5 μl) as a control. Incubation was continued at the experimental temperature until hatch (approximately seven weeks) and the hatchlings dissected to determine resulting sex ratios. We determined gonadal sex and status of genital ducts by visualization under a dissection microscope and verified sex histologically as described (8). The dosage effect of estradiol-17β and estriol alone and in combination was determined using polynomial logistic regression as described in Crews et al. (10). A one-tailed t-test was used to compare the regression coefficients under combined vs. single hormone treatment. Synergism between the paired estrogens would be indicated if a significantly larger dosage effect for the combined treat-

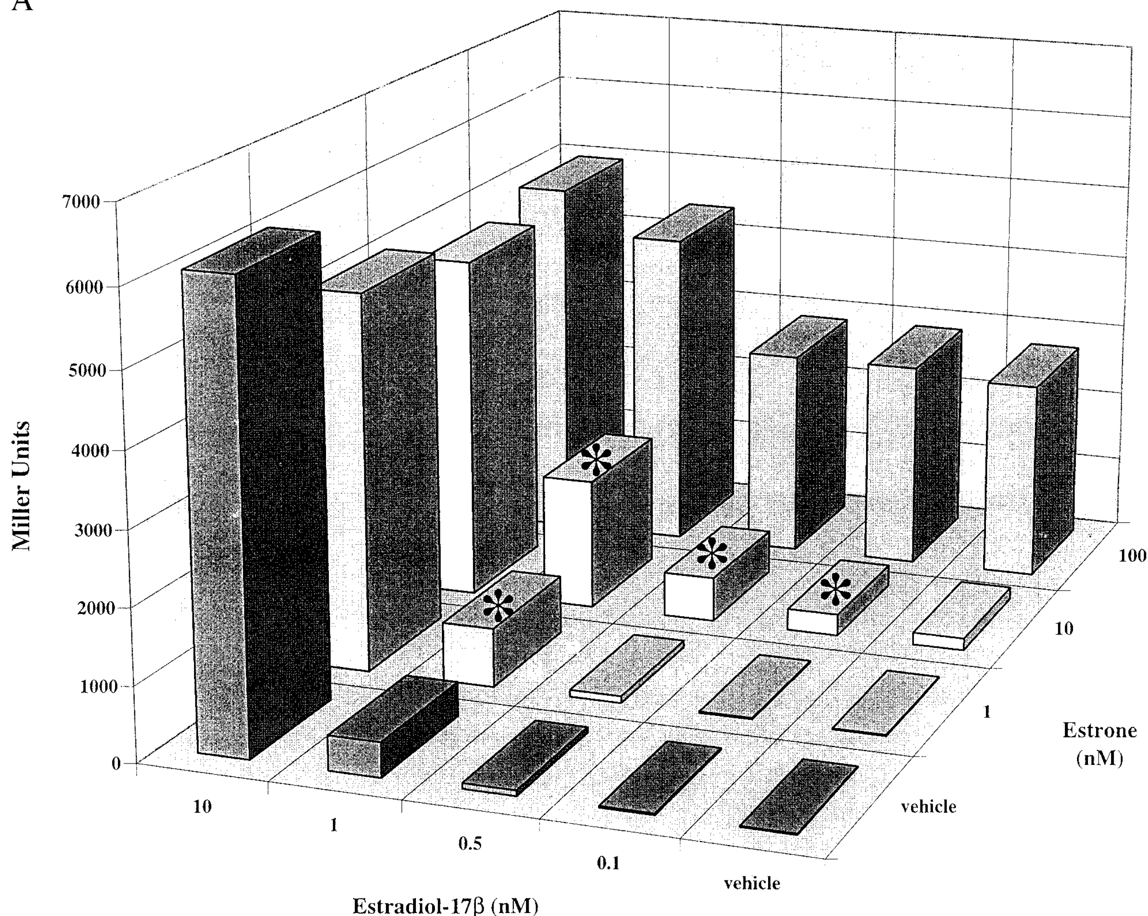

FIG. 2. Activity of combinations of various estrogens in strain hER-ERE. (A) Strain hER-ERE was grown overnight in the presence of estradiol-17β or estrone alone and in combinations at increasing concentrations. * represent combinations of estrogens that have significantly greater than additive activity ($p < 0.05$). (B) Strain hER-ERE was grown overnight in the presence of estradiol-17α and estrone. β-galactosidase was measured after 14h, calculated as Miller Units as described in the Materials and Methods. Each value is the mean of three independent experiments with three replicates.

ments of basal estriol and various concentrations of estradiol-17β compared to the dosage effect for estradiol-17β alone.

RESULTS AND DISCUSSION

Strain YPH499 containing hER and an estrogen-specific reporter was used to examine the activity of the steroidal estrogens estradiol-17β, estradiol-17α, estriol or estrone. Incubation of yeast with increasing concentrations of estradiol induced a dose-dependent increase in β-galactosidase activity (Fig. 1). This activity was maximum at 10 nM estradiol-17β. The activity of estrone, estradiol-17α and estriol was 1/20th, 1/40th and 1/1000th, respectively, that of estradiol-17β. The activity of the four steroidal estrogens in yeast strain hER-ERE is consistent with the activity of these estrogens in other yeast and mammalian cell systems (10, 19).

The activity of combinations of estrogenic compounds was investigated by generating dose-response relationships with one estrogen in the presence of a single dose of a second estrogen. Combinations of some submaximal concentrations of estradiol-17β and estrone produced a synergistic increase in reporter activity (Fig. 2A, Table 1). For example, 1 nM estradiol and 10 nM estrone produced 1808 Miller Units. A predicted value of 621 Miller units would have been observed if the two estrogens were additive. The combination of 1 nM estradiol and 10 nM estrone produced approximately three-fold synergy. However, a synergistic increase in reporter activity was not observed near or at the concentrations that produced maximal activity of estrone or estradiol-17β. This may indicate that the effect was saturable.

Synergy was also observed with combinations of estradiol-17α plus estrone (Fig. 2B) and estradiol-17β plus estradiol-17α (data not shown) in yeast. Consis-

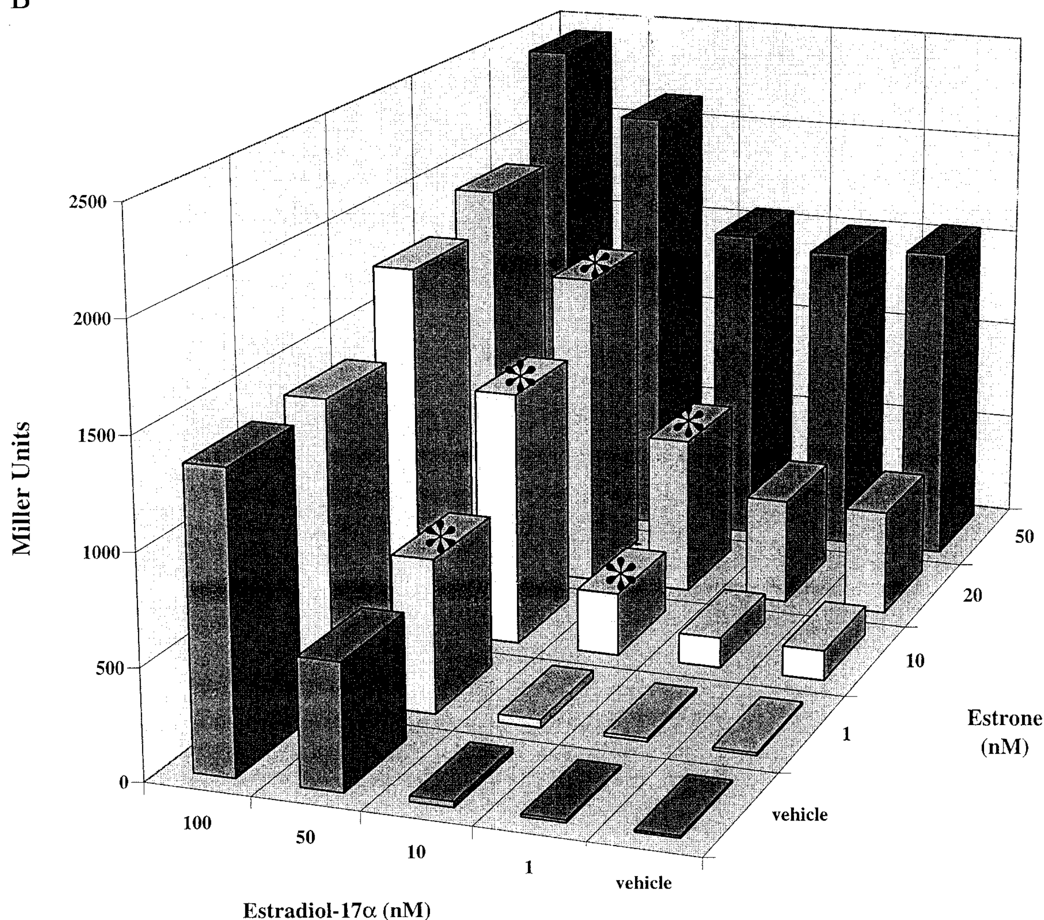

FIG. 2—*Continued*

tent with the results presented in Table 1, synergy was observed at submaximal, but not maximal, concentrations when combinations of the estrogens were examined. Furthermore, we also observed a greater than additive response with some combinations of steroidal estrogens using a similar hER-ERE unit in strain DY150 (data not shown). Synergistic responses were not observed with estradiol-17β and estriol with hER in strains DY150 and YPH499 (data not shown).

We have evaluated whether the synergistic transactivation of ER with a combination of steroidal estrogens resulted from two estrogens binding the ER.

TABLE 1

β-Galactosidase Activity of Estradiol-17β or Estrone Alone and in Combination in Yeast Strain YPH499 Expressing hER

	Vehicle	1.0 nM Estrone	10.0 nM Estrone	100.0 nM Estrone
Vehicle	14.6	16.4	163.8	2766.4
0.1 nM Estradiol-17β	21.8	20.0	291.2* (185)	2875.6
0.5 nM Estradiol-17β	72.8	91.0	609.08* (237)	2871.5
1.0 nM Estradiol-17β	327.6	564.2* (344)	1292.4* (492)	3130.4
10.0 nM Estradiol-17β	4182.0	3640.0	3367.0	3549.0

Note. The β-galactosidase activity is expressed as Miller units as described in the Materials and Methods. Values significantly greater than additive are noted with asterisk. Additive values in parentheses. Three independent experiments with three replicates were performed. The standard error was less than 10%.

TABLE 2

Radiolabeled Estrogen Binding in Yeast Strain YPH499 Expressing hER

	Binding Relative to [^{3}H]Estradiol (Estrone), percent
1.0 nM [^{3}H]Estradiol-17β + Vehicle	100.0
1.0 nM [^{3}H]Estradiol-17β + 200.0 nM Estradiol-17β	30.0*
1.0 nM [^{3}H]Estradiol-17β + 1.0 nM Estrone	111.0
1.0 nM [^{3}H]Estradiol-17β + 10.0 nM Estrone	116.0
1.0 nM [^{3}H]Estradiol-17β + 100.0 nM Estrone	70.0*
10.0 nM [^{3}H]Estrone + Vehicle	100.0
10.0 nM [^{3}H]Estrone + 100.0 nM Estradiol-17β	20.0*
10.0 nM [^{3}H]Estrone + 0.1 nM Estradiol-17β	125.0
10.0 nM [^{3}H]Estrone + 0.5 nM Estradiol-17β	176.0*
10.0 nM [^{3}H]Estrone + 1.0 nM Estradiol-17β	224.0*
10.0 nM [^{3}H]Estrone + 10.0 nM Estradiol-17β	76.0*

Note. Yeast were incubated with [^{3}H]estradiol-17β or [^{3}H]estrone in the presence of vehicle or increasing concentrations of the radioinert estradiol-17β or estrone. Values significantly greater or less than radiolabeled estrogen plus vehicle are indicated by asterisk.

Strain YPH499 expressing hER was incubated with 1 nM [^{3}H]estradiol in the presence or absence of various concentrations of estrone. A 200-fold excess of estradiol-17β significantly reduced [^{3}H]estradiol-17β binding to the hER (Table 2). Estrone did not affect binding of [^{3}H]estradiol-17β to the hER at 1 and 10 nM. These concentrations of estrone in combination with 1 nM estradiol-17β produced a synergistic increase in hER-mediated transactivation (Table 1). This indicated that some concentrations of estrone did not compete for [^{3}H]-estradiol-binding.

To evaluate the hypothesis that a second binding site on the ER may mediate the binding of some steroidal estrogens to the ER, 10 nM [^{3}H]estrone in the presence or absence of estradiol-17β was incubated in strain YPH499 expressing hER. Specific binding of [^{3}H]-estrone-binding to hER was demonstrated by incubation with a 10-fold excess of estradiol-17β (Table 2). Interestingly, the binding of [^{3}H]estrone to hER was increased with estradiol-17β. This suggested that estradiol-17β may induce an allosteric change in the ER promoting increased binding of [^{3}H]estrone.

In order to evaluate the biological significance of synergy in yeast, we investigated a mixture of steroidal estrogens on the sexual development of turtle embryos. Increasing concentrations of both estradiol-17β and estriol resulted in increasing numbers of female hatchlings (Table 3). The synergistic activity of a combination of steroidal estrogens was determined *in vivo* by measuring sex-reversal of hatchling turtles that had been treated during incubation at a male-producing temperature. Regression coefficients for the single hormone treatments were similar to those determined previously for the same estrogens at 26 °C (9); i.e., estriol produced a much larger dosage effect than estradiol-17β (Table 3; Fig. 3). The various concentrations of estradiol-17β in combination with a basal dosage of estriol was significantly greater than the comparable dosages of estradiol-17β alone (Fig. 3). Examination of regression coefficients indicate that this combination treatment produced two-fold synergy at the two lowest dosages of estradiol-17β. The combined treatment also showed a quadratic relationship, with greater effects at lower dosages and decreasing effect at higher dosage.

TABLE 3

Regression Results for the Effects of Increasing Dosages of Estradiol-17β or Estriol Applied Singly or in Combination on Hatchling Sex Ratio in the Red-Eared Slider Turtle.

Treatment	Regression coefficient
Single	
Estradiol-17β (E2)	8.95 ± 1.7 (Chi-square=29.2, $p<0.00001$)
Estriol (E3)	96.35 ± 16.0 (Chi-square=36.3, $p<0.00001$)
Combination	
E3 (0.01μg) + E2 [dose effect]	19.75 ± 5.9 (Chi-square=11.1, $p=0.0009$)*

Note. Regression coefficients ± 1 standard error with Chi-square and probability values in parentheses; there is 1 degree of freedom for each regression coefficient. Regression coefficient for combination treatment is for the second hormone treatment indicated as dosage effect in brackets. * Indicates synergism between combined estrogen treatment vs. single estrogen treatment.

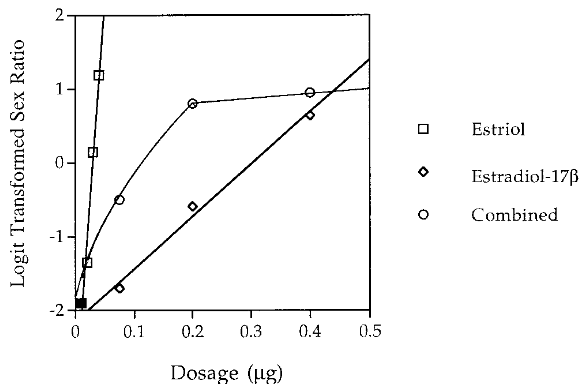

FIG. 3. Sex ratio of red-eared slider turtles at hatching. Depicted is the logit transformation of percentage data; zero indicates a 1:1 sex ratio, −2 indicates 100% males, and +2 100% females. Ethanol control (not shown) was 100% male hatchlings or −2. Filled square indicates the effect of 0.01 μg estriol, the lowest dosage used in the estriol dose-response (squares); this also was the basal dosage used in the combined treatment of estriol and increasing concentrations of estradiol-17β. Circles represent the effect of the combined treatment as compared to diamonds, which represent comparable dosages of estradiol-17β alone.

The results presented in this paper are similar to our previous results demonstrating synergistic hER-mediated transactivation in yeast treated with combinations of weakly estrogenic pesticides or hydroxylated PCBs (6) or turtle embryos with mixtures of hydroxylated PCBs (4). The synergistic activity in yeast with combinations of estrogenic chemicals correlated with the *in vitro* binding of two chemicals to the hER simultaneously. A recent technical report has indicated a failure to observe synergy of endosulfan and dieldrin using a variety of assays (14). We have suggested that the concentration of chemicals and ER are important determinants in modulating responses with mixtures of chemicals (15). Furthermore, our hypothesis may have important implications for estrogen-regulated responses that occur during development, when the concentration of ER has been reported to be low (16, 17). Further studies will be required to clarify why some types of developmental animal models, such as neonatal mouse (16) and turtle embryos (4), may be more sensitive to the effects of estrogenic chemicals alone and in mixtures than the corresponding adult.

In the red-eared slider turtle, endogenous estrogens are the physiological equivalent of a female-producing incubation temperature (8, 18, 19). Administration of exogenous estrogen to eggs incubating at a male-producing temperature will reverse sex (i.e., produce females) in an "all-or-none" manner; that is, hermaphrodites are not produced (7, 8). Interestingly, the steroidal estrogens estrone, estradiol-17β, and estriol are not equipotent at an incubation temperature that produces all males, but are physiologically equivalent at higher temperatures (9). Unlike estradiol-17β, ER binding with estrone and estriol changes with temperature in mammals (20). Female development can also be induced by synthetic compounds such as DES, R2858 (1, 21), and certain PCBs (4).

In this paper, we have shown that some combinations of steroidal estrogens have synergistic activity in yeast expressing hER and in sex-reversing male-determined turtle embryos. These results suggest that the presence of several endogenous estrogenic metabolites may play key roles during sexual development. The elucidation of the mechanism whereby combinations of steroidal estrogens influence sexual development will be important for understanding how some natural and synthetic estrogens in the environment may alter sexual development of sensitive organisms.

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