Can Increased Hepatic Estrogen Metabolism Interfere with Ovulation in the Rat? Effects of Chronic Phenobarbital or Chlordane Treatment[1] (37685)

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(Introduced by A. R. Midgley, Jr.)

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In the rat, LH release and ovulation are dependent upon an increase in serum concentrations of 17β-estradiol (E_2) on the last day of diestrous and the morning of functional proestrous. An injection of antiserum to E_2 blocks the preovulatory release of LH and prevents subsequent ovulation in both adult rats and in immature rats treated with pregnant mare's serum gonadotrophin (PMS) (1, 2). Properly timed injections of anesthetic doses of barbiturates will also prevent the preovulatory release of LH (3–6). It has been suggested that either short-term or chronic treatment with phenobarbital or chlordane might block ovulation by increasing the metabolism of E_2, thus producing a decrease in serum concentration of E_2 below that required for triggering the ovulatory release of LH in the rat (7–12).

Short-term treatment of rats with phenobarbital or chlordane has been shown to enhance the activities of drug metabolizing enzymes in hepatic microsomes (13, 14). The many similarities between the oxidative drug-metabolizing enzymes and the steroid hydroxylases of hepatic microsomes has led to the suggestion that the oxidative drug-metabolizing system may also be primarily responsible for steroid metabolism (15). The ovarian sex steroid E_2 is a natural body substrate for these hepatic enzymes, as evidenced by its ability to serve as the competitive inhibitor of drug-metabolizing systems in liver microsomes (16, 17). Thus, treatment of rats with phenobarbital or chlordane increases hepatic microsomal steroid hydroxylases which accelerate the metabolism of E_2. This effect is paralleled by a decreased gain in uterine weight and a decreased incorporation of E_2 in the uterus following an injection of physiological amounts of this steroid (18–21). These observations prompted us to determine if the induction of hepatic microsomal enzymes could prevent or influence LH release and ovulation in the rat.

Methods. Immature (22-day-old, 45–55 g) and mature (240–260 g) female rats of the Holtzman strain were given Rockland rat chow and water *ad libitum*. They were housed in air-conditioned quarters (70 ± 5°F) where the lights went on at 5 AM and off at 7 PM automatically.

Experiment I. Immature PMS-treated. Four groups of immature rats (20/group) were given an ip injection of phenobarbital sodium (37 mg/kg in saline), Chlordane[4] (12.5 mg/kg, dissolved in corn oil), or an injection of corn oil (0.1 ml) at 8 AM and 5 PM for 4 consecutive days beginning on the 22nd day of age. All groups except 1 of the 2 phenobarbital-treated groups were given 15 IU of PMS[5] sc at 10:30 AM on the 25th day of age. Five rats from each group were anesthetized with ether 24, 48, 54, and 72 hr

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[4] Reference grade chlordane (1,2,4,5,6,7,8,8-octachloro-3a,4,5,5a-tetrahydro-4,7-methanoindane), City Chemical Company, Chicago, Ill.

[5] Equinex, Ayerst Laboratories, Little Falls, N. J.

after the PMS injections and exsanguinated via the abdominal aorta. The livers (2.5 g/rat) were used for microsomal enzyme studies. The blood was centrifuged and the serum was collected and frozen. Aliquots of 20–40 μl of serum were analyzed for LH using a double antibody radioimmunoassay (22). Serum LH concentrations were expressed in terms of B160, a partially purified rat anterior pituitary gland extract, 1 mg of which is equivalent to 0.17 mg of NIH-LH-S_1, as determined by ovarian ascorbic acid depletion bioassay. Oviducts from the 72–hr groups were examined visually for the presence of ova as indicated by a transparent dilated portion of the oviduct.

Experiment II. Cycling adults. Mature female rats were exposed to a regular lighting schedule (lights on 5 AM to 7 PM) for 2 weeks. Vaginal smears were taken at 9–10 AM daily for 18 days prior to treatment and were continued throughout the experiment. Rats with either 4- or 5-day estrous cycles were used and the animals were weighed on the first, seventh, and last days of treatment. Three groups of animals were given ip injections of phenobarbital (37 mg/kg), chlordane (25 mg/kg), or an appropriate volume of corn oil (0.15 ml) at 5 PM daily for 14 consecutive days except on days of functional proestrous. On the 14th day of treatment, 10 proestrous rats were selected from each group and heart punctures without anesthesia were performed at 4:30 PM. The blood samples were left in the syringes and allowed to clot for 24 hr at 4°. Serum was collected and 25–μl aliquots were eventually assayed for LH. The following morning at 9 AM the same animals were anesthetized with ether and exsanguinated via the abdominal aorta. The oviducts were carefully examined for the presence of ova and approximately 5 g of liver from each rat was rapidly collected for microsomal enzyme studies.

Experiment III. Hypophysectomized castrates. Three groups of mature female rats (5/group) which had been ovariectomized 1 mo previously and consequently had elevated serum LH and FSH levels (23, 24) were given ip injections of either phenobarbital (37 mg/kg), chlordane (12.5 mg/kg) or comparable injections of corn oil (0.18 ml) at 9 AM and 5 PM for 4 consecutive days. Forty hours after the last injection they were hypophysectomized using a stereotaxic transauricular method (25). Blood was obtained by cardiac puncture without anesthesia at 1, 30, and 120 min after hypophysectomy. Serum was collected and 10- or 50-μl aliquots were analyzed for LH and FSH (26). The serum FSH concentrations are expressed in terms of B310, a partially purified rat anterior pituitary gland extract, 1 mg of which is equivalent to 0.33 mg of NIH-FSH-S_1 as determined by a modified Steelman-Pohley bioassay (27). Immediatelly after the last blood sample was taken the animals were anesthetized with ether and exsanguinated via the abdominal aorta. Approximately 5 g of liver from each animal was prepared for individual microsomal enzyme activity determinations. The pituitary fossa of each animal was examined for pituitary fragments with a dissecting microscope.

Microsomal enzyme studies. Approximately 2.5 or 5 g of liver from each animal was weighed to the nearest 0.01 g and homogenized individually in 10 ml of buffer (0.15 *M* KCl containing 0.05 *M* tris base, pH 7.4) at 4° using a drill press technique according to the method of Hiddeman (28). The homogenates were centrifuged (13,000*g*) at 4° for 20 min and the supernatant which contained the microsomes and the soluble fraction was used for the enzyme studies (29). The incubation mixture used for the hydroxylation studies contained 2 ml of 0.1 *M* phosphate buffer (pH 7.4), 1 ml of liver homogenate[6], and 2 ml of cofactors and substrate[7] (glucose-6-phosphate, 25 μmoles; triphosphopyridine nucleotide, 2.25 μmoles; diphosphopyridine nucleotide, 2.5 μmoles; nicotinamide, 20 μmoles; magnesium sulfate, 25 μmoles; and 2.4 mg of acetanilide as substrate). Blanks were incubated at the same time. The incubation was performed in open 25-ml Erlenmeyer flasks at 37° for 20 min in a Dubnoff metabolic shaker (100 rpm) under 100%

[6] The μg of *p*-aminophenol formed/g liver/20 min for the 166 mg/ml liver homogenate was computed by multiplying OD × slope factor × 6 which gives a quantity of product formed comparable to using a 333 mg/ml liver homogenate and multiplying OD × slope factor × 3.

[7] Sigma Chemical Company, St. Louis, MO.

TABLE I. Effect of Phenobarbital or Chlordane Treatment on Hepatic Hydroxylating Enzyme Activity of PMS-Treated Immature Rats.

Treatment[a]	Hr after PMS[b]		
	24	48	72
Oil control + PMS	70 ± 5.5[c]	72 ± 8.8	76 ± 4.2
Phenobarbital	596 ± 14.4	489 ± 31.0	364 ± 6.6
Phenobarbital + PMS	613 ± 26.9	471 ± 20.1	338 ± 11.0
Chlordane + PMS	491 ± 24.0	461 ± 9.7	459 ± 12.6

[a] The immature rats were given ip injections of phenobarbital (37 mg/kg), chlordane (12.5 mg/kg dissolved in corn oil), or a comparable dose of corn oil (0.1 ml) at 8 AM and 5 PM for 4 consecutive days beginning on the 22nd day of age.

[b] 15 IU of PMS was injected sc at 10:30 AM on the 25th day of age.

[c] Hydroxylation of acetanilide; reaction carried out for 20 min at 37° under 100% oxygen; μg of *p*-aminophenol formed/g liver/20 min; each value represents the mean ± SE from 5 rats.

oxygen. At the end of the incubation period the reaction was stopped by the addition of 1 ml of 6 *N* HCl to each flask. The determination for total *p*-aminophenol was performed according to the method of Brodie and Axelrod (30).

Results. Experiment I. An 8-fold increase in hepatic hydroxylating enzyme activity in the PMS-treated immature rats following phenobarbital or chlordane treatment is shown in Table I. The enzyme activity of the phenobarbital-treated controls (without PMS) was not significantly different from the phenobarbital + PMS group, indicating that PMS did not influence hepatic enzyme induction.

Table II indicates LH concentrations in sera collected at various times after the PMS injections and the incidence of ovulation as determined 72 hr after the PMS injections. Fifty-four hours after the PMS injections a significant increase in serum LH occurred, indicating a preovulatory release of LH. Neither phenobarbital nor chlordane blocked ovulation in these rats.

Experiment II. The increase in hepatic enzyme activity resulting from phenobarbital or chlordane treatment is presented in Table III. During the 14-day treatment period none of the rats lost weight or showed irregularities in their estrous cycles as determined by vaginal smears. The LH concentrations for the serum samples obtained by heart puncture at 4:30 PM on the day of proestrous are also indicated in Table III.

TABLE II. Failure of Phenobarbital or Chlordane Treatment to Prevent LH Release and Ovulation in PMS-Treated Immature Rats.

Treatment[a]	Serum LH + PMS (ng B160/ml)				Ovulating total
	Hr after PMS				
	24	48	54	72	
Oil control	226 ± 17.8[b]	94 ± 0.7	194 ± 23.3[c]	43 ± 4.3	5/5
Phenobarbital + PMS	208 ± 10.0	89 ± 3.6	203 ± 32.0[c]	44 ± 1.6	5/5
Chlordane + PMS	188 ± 15	91 ± 5.1	173 ± 25.9[c]	43 ± 4.9	4/5

[a] The immature rats were given ip injections of phenobarbital (37 mg/kg), or chlordane (12.5 mg/kg dissolved in corn oil), or a comparable injection of corn oil (0.1 ml) at 8 AM and 5 PM for 4 consecutive days beginning on the 22nd day of age.

[b] The assay system used for the determinations of serum LH concentrations also measures PMS; each value represents the mean ± SE from 5 rats.

[c] This increase in serum LH activity represents a preovulatory release of endogenous LH.

TABLE III. Failure of Phenobarbital or Chlordane Treatment to Prevent LH Release and Ovulation in the Adult Rat.

Treatment[a]	Hepatic microsomal hydroxylating enzyme activity	Ovulating total	Serum LH[b]	With elevated serum LH Total
Oil control	81 ± 3.2[c]	10/10	337.5 (149.0–526.9)	7/10
Phenobarbital	283 ± 10.2	10/10	209.8 (133.5–489.7)	8/10
Chlordane	288 ± 10.7	7/10	170.8 (36.7–286.4)	5/10

[a] Rats were given ip injections of either phenobarbital (37 mg/kg), chlordane (25 mg/kg dissolved in corn oil), or a comparable injection of corn oil (0.15 ml) at 5 PM daily for 14 consecutive days except on the days of functional proestrous. The rats were sacrificed 18 hr after the last injection.

[b] LH concentrations (ng B160/ml) in serum obtained at 4:30 PM on the day of functional proestrous (14th day of treatment); each value represents the mean and the 95% confidence limits of the mean from 10 rats, not all of which had elevated serum LH at the time of sampling. Serum levels were considered to be elevated if they were greater than 20 ng/ml.

[c] Hydroxylation of acetanilide; μg of *p*-aminophenol formed/g liver/20 min; reaction carried out for 20 min at 37° under 100% oxygen; each value represents the mean ± SE from 10 rats.

The failure to find elevated serum LH concentrations in some rats which subsequently ovulated was anticipated, since LH release could have occurred a few hours before or after the sample was taken. Only 7 of 10 rats in the chlordane-treated group ovulated; whereas all of the control and phenobarbital-treated rats ovulated. This suggests that ovulation may be partially inhibited by chlordane treatment, but the mechanism of such inhibition is not clear.

Experiment III. Half-life of LH and FSH in hypophysectomized castrates. The hepatic microsomal hydroxylation enzyme activities of the phenobarbital- (300 ± 31.5) or chlordane- (237 ± 17.6) treated ovariectomized rats was increased significantly over the controls (60 ± 2.3).[8] Forty-eight hours after the last injection the treated rats were hypophysectomized. The LH concentrations in serum collected at 1 and 30 min after hypophysectomy (the early phase of serum LH decline) indicated a half life of approximately 30 min (Table IV). No significant differences were calculated among the disappearance rates for serum LH or FSH between the 1-, 30-, and 120-min samples with respect to the chlordane-treated, phenobarbital-treated, or control groups.

Discussion. It is well established that properly timed phenobarbital treatment inhibits ovulation in the rat (3–6). The ability of such treatment to alter the metabolism and biological activity of exogenous estrogen is also well established (14, 19–21), but the data presented here indicate these effects in the intact animal are not sufficient to prevent ovulation. It appears that the animal may compensate for any increased rate of steroid metabolism either by increasing estrogen production or by responding to existing estrogen levels with an adequate release of LH. The estrogen requirements for a normal preovulatory LH peak in the rat have not been established, but it has been shown that a quantity of LH in excess of that required for ovulation is normally released during proestrous (31).

Chronic or short-term treatment of adult or immature rats with phenobarbital or chlordane increases the activity of hepatic microsomal enzymes which metabolize estrogens, inhibits uterine weight increases, and decreases incorporation of E_2 into the uterus

[8] The hepatic microsomal hydroxylation activity (expressed as μg of *p*-aminophenol formed/g liver/20 min at 37° under 100% oxygen) is with 5 rats/group (mean ± SE).

TABLE IV. Disappearance of LH and FSH in Phenobarbital- or Chlordane-Treated One-Month Ovariectomized Adult Rats.[a]

	Minutes after hypophysectomy					
	0		30		120	
Treatment	LH[b]	FSH[c]	LH	FSH	LH	FSH
Oil control	153.5 ± 68.5[d]	11.5 ± 0.5	76.5 ± 19.5	9.1 ± 0.5	56.0 ± 2.0	6.1 ± 0.1
Phenobarbital	129.8 ± 22.3	11.4 ± 0.9	45.8 ± 5.7	8.1 ± 0.6	23.4 ± 8.0	5.2 ± 0.3
Chlordane	150.5 ± 21.1	9.8 ± 1.3	61.7 ± 7.7	8.4 ± 0.4	32.0 ± 7.3	5.4 ± 0.3

[a] The animals were given ip injections of phenobarbital (37 mg/kg), chlordane (12.5 mg/kg), or corn oil (0.18 ml) at 9 AM and 5 PM for 4 consecutive days. Forty hours after the last injection they were hypophysectomized. Blood samples taken via heart puncture at the indicated times were analyzed for LH and FSH by radioimmunoassay.

[b] Expressed as ng B160/ml serum.

[c] Expressed as μg B310/ml serum.

[d] Each value represents the mean ± SE from 5 rats.

following injections of physiologic amounts of tritiated E_2 (14, 19–21).

The ability of these compounds to stimulate the hepatic metabolism of E_2 and to alter the effects of E_2 on the uterus suggests a potential to alter reproductive function. However, short-term treatment of PMS-injected immature rats with phenobarbital or chlordane did *not* prevent the preovulatory increase in serum LH concentrations or ovulation. Ying and Meyer (12) reported that phenobarbital treatment reduced the incidence of ovulation in PMS-treated immature rats given 3 IU of PMS. Our use of 15 IU of PMS in such rats may have resulted in abnormally large follicular development and excess production of E_2. Such an explanation of overstimulated ovaries cannot, however, be applied to the mature cyclic females in these studies, which also functioned normally in the presence of enhanced hepatic function. Induction of hepatic microsomes with phenobarbital or chlordane did not significantly effect the disappearance rate of the glycoproteins, LH or FSH, in serum. The half-life of FSH approximated 120 min and the initial decline in serum LH approximated a half-life of 30 min. These rates of disappearance are consistent with previously reported values for normal rats (32, 33).

Treatment of the adult female rats with chlordane for two weeks produced a slight inhibition of ovulation. Ambrose *et al.* (7) reported that chlordane treatment decreased fertility in female rats and Welch *et al.* (14) found that this insecticide decreased fertility in female mice. The suggestion of an inhibition of ovulation in the present study does not appear to be related to the increased activity of the steroid hydroxylating enzymes since the level of enzyme activity was not significantly different between the chlordane- and phenobarbital-treated animals. Fertility encompasses many stages of reproductive function and a multitude of processes including tubal transport, fertilization, and implementation may be influenced by chlordane. Unpublished observations in our laboratory indicate that a single 25 mg/kg ip injection of chlordane in adult female rats at 10 AM on the morning of functional proestrous does not prevent the preovulatory increase in serum LH or ovulation. This suggests that a single dose of chlordane does not function to directly block the ovulatory process. However, multiple doses leading to accumulation appear to adversely effect fertility and ovulation.

It appears that the effects of phenobarbital cannot be attributed to alterations in hepatic function, but are dependent on its anesthetic action, since neither phenobarbital nor other compounds which stimulate hepatic enzymes are capable of preventing ovulation by altering the metabolism of circulating steroids.

Summary. Female Holtzman rats were treated for 3–14 days with daily doses of

phenobarbital (37 mg/kg, ip) or chlordane (25 mg/kg, ip) which caused a 10-fold increase in the hydroxylation of acetanilide by hepatic microsomes. Mature females treated with phenobarbital continued to cycle, exhibited preovulatory increases in serum luteinizing hormone (LH), and ovulated normally during a 2-week treatment period. In similar rats, ovariectomized 1 month earlier, the rate of disappearance of LH and follicle stimulating hormone (FSH) from the serum following hypophysectomy was unaltered by 2 weeks of phenobarbital or chlordane treatment. In immature females in which ovulation was induced with pregnant mare's serum, the preovulatory increase in serum LH concentration and subsequent ovulation were not altered by activation of hepatic microsomal enzymes. These results suggest that treatment of rats with phenobarbital does not block ovulation by mechanisms involving hepatic enzymes and/or estrogen metabolism.

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