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Effect of Estradiol on the Induction of Porphyria by Hexachlorobenzene in the Rat

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ABSTRACT. Hexachlorobenzene (HCB) is porphyrinogenic in adult female but not in male rats. This study aimed to assess the role of 17β-estradiol in the induction of porphyria by HCB in both sexes by adding or removing the hormone. Groups of intact females, ovariectomized females (Ova), castrated males (Cas), and Cas receiving 17β-estradiol (4 mg/kg, i.m., once a week beginning 2 weeks prior to HCB) were given five consecutive daily doses of HCB (100 mg/kg in corn oil, p.o.). Porphyria was assessed by urinary uroporphyrin excretion measured at days 16, 31, 38, 45, 52, 59, and 87. The percentage of porphyric rats in intact females increased from day 31 (58%) to day 87 (75%), whereas none of the Ova or Cas rats responded. However, administration of estradiol (days 120–169) and another sequence of HCB doses (days 134–138) to the same Ova rats caused porphyria (50% at day 186). Cas rats given estradiol also developed porphyria (43 and 86% on days 31 and 87, respectively). HCB-treated Ova rats given two doses of estradiol at either days 1 and 8 or days 22 and 29 developed a porphyria of similar magnitude (day 52). The role of estradiol cannot be explained by a reduction of pentachlorothiophenol formation, a putative detoxication pathway. Overall, results show that both sexes have the ability to respond to HCB when 17β-estradiol is present and suggest that the sexual dimorphism in HCB-induced porphyria in the rat is related to the hormonal status. BIOCHEM PHARMACOL **54**;1:19–25, 1997.

KEY WORDS. estradiol; hexachlorobenzene; liver; porphyria; rat; toxicology

HCB§, a polyhalogenated aromatic hydrocarbon, is an environmental contaminant found in fatty tissues and maternal milk of virtually all Canadians [1, 2] and citizens of other industrialized countries. HCB induces hepatic porphyria in humans and laboratory animals [3]. The hepatic porphyria induced by HCB is characterized by an increased hepatic accumulation as well as a greater urinary excretion of highly carboxylated porphyrins, particularly uroporphyrin. In female rats, but not in male rats, chronic administration of HCB induces hepatic porphyria similar to human porphyria cutanea tarda. Female rats have been found to be more susceptible than male rats to the porphyrinogenic effects of HCB [4–8]. This last observation suggests a role for the sexual hormones. Although it is theoretically possible that androgens may play an inhibiting role in the male rat, it seems more likely that estrogens exert a positive action in the induction of HCB-induced porphyria. Grant *et al.* [4] observed that removal of the ovaries in females receiving a diet containing HCB decreased the level of porphyrins accumulated in the liver. Smith and Francis [9] observed small changes in hepatic porphyrin concentrations and uroporphyrinogen decarboxylase activity, a critical heme synthesis enzyme, in male rats given HCB in the diet for more than 100 days. However, when the same animals received multiple doses of the estrogenic drug diethylstilbestrol, as the dipropionate or chlorotrianisene, during the later period of HCB feeding, there was a large increase in porphyrin levels accompanied with a reduction in uroporphyrinogen decarboxylase activity.

The sexual dimorphism observed in the rat could, in part, result from differences in biotransformation of HCB. It is postulated that hepatic porphyria is related to oxidation of HCB to pentachlorophenol and tetrachlorohydroquinone [10–12], whereas conjugation of HCB to GSH has been proposed to represent a detoxication pathway [13]. The latter pathway leads to the formation of PCTP. The biliary excretion of PCTP is much higher in male rats than in female rats [14].

The present study takes advantage of a standardized reproducible experimental protocol of HCB administration previously developed in our laboratory [14]. This protocol offers the advantage of giving a minimal amount of HCB to the rat to induce a fully developed porphyria in a well-defined and predictable time frame. This experimental

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§ *Abbreviations:* Cas, castrated male rats; GSH, glutathione; HCB, hexachlorobenzene; PCTP, pentachlorothiophenol; and Ova, ovariectomized female rats.

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model consists of five consecutive daily doses of 100 mg HCB/kg body weight followed by a latent period of 30 days (delay phase) without treatment before the onset of porphyria (marked liver and urinary uroporphyrin accumulation). Male rats are totally resistant to the induction of porphyria using such a protocol.

The present study aimed to demonstrate that the presence of 17β-estradiol during the delay phase is at the basis of the sexual dimorphism in the induction of porphyria by HCB. This was achieved by using an approach testing the ability of HCB to induce porphyria in both sexes upon addition or removal of 17β-estradiol through surgical (ovariectomy, castration) and/or hormonal manipulations (17β-estradiol injections). Temporal progression of porphyria was assessed by measuring urinary uroporphyrin excretion. The effect of the presence of 17β-estradiol at specific times during the delay phase was also studied. A mechanistic explanation of the effect of estradiol was sought in HCB detoxication activity through PCTP formation.

MATERIALS AND METHODS

Chemicals

HCB (100 ± 0.5%) was purchased from the Aldrich Chemical Co., Inc. (Milwaukee, WI). Estradiol valerate (Delestrogen®) was obtained from Squibb Canada (Division of Bristol–Myers Squibb Canada Inc.) (Montreal, Québec). An estradiol-17-β ^{125}I RIA Kit was purchased from ICN Biochemicals Inc., Diagnostics Division (Costa Mesa, CA). Uroporphyrin III was obtained from Porphyrin Products Inc. (Logan, UT). Corn oil was purchased from Best Foods Canada Inc. (Etobicoke, Ontario). Pentachlorothiophenol (90–95%) was obtained from the Aldrich Chemical Co. (Milwaukee, WI). Ketamine and xylazine were purchased from CDMV Inc. (St-Hyacinthe, Québec). Finally, all other reagents were bought commercially at the highest purity available.

Animals and Treatments

REPEATED ADMINISTRATION OF ESTRADIOL VALERATE. Intact female (150–175 g), ovariectomized female (150–175 g), and castrated male (175–200 g) Sprague–Dawley rats (Charles River Canada Inc., St-Constant, Québec) were used after a 7-day acclimation period. The gonadectomies were carried out by veterinarians at Charles River Canada Inc. The animals were housed in stainless steel open-bottom cages. Room temperature was kept at 21° with an artificial 12-hr light/dark cycle (6:00 a.m. to 6:00 p.m.). Rats had access to food (Purina Rat Chow 5012, Chow Mills, St. Louis, MO) and water *ad lib.*

Two series of rats were used. The first series of animals consisted of four groups receiving the HCB treatment: normal female rats (N = 12), Ova rats (N = 13), Cas rats (N = 10), and Cas rats receiving 17β-estradiol valerate (N = 10). The 17β-estradiol treatment given to Cas rats consisted of i.m. injections in the rear legs of 4 mg/kg 17β-estradiol valerate dissolved in sesame oil (10 mg/mL), once a week starting 2 weeks prior to HCB administrations. During this time, the other groups were kept without treatment. Then all groups of rats received a total dose of 500 mg HCB/kg of body weight in the form of five consecutive daily administrations of 100 mg/kg dissolved in corn oil (10 mL/kg, p.o.). A group of normal control female rats received corn oil only (10 mL/kg, p.o.). Urinary uroporphyrin excretion was measured on days 16, 31, 38, 45, 52, 59, and 87. The animals were placed individually in metabolism cages, and 24-hr urine samples were collected in plastic containers that were kept protected from light inside a box filled with ice. Urine samples were centrifuged quickly and analyzed for porphyrin levels; if not analyzed immediately, they were stored at −20° for later analysis.

The second series of treated animals consisted of the Cas and Ova rats kept from the experiment described above. The Cas rats which had been given HCB from days 1 to 5 received seven weekly injections of 17β-estradiol valerate beginning on day 60. Urinary uroporphyrin excretion was subsequently measured on days 76 and 112. The Ova rats were given ten weekly 17β-estradiol injections beginning on day 120 plus another sequence of five consecutive daily doses of HCB beginning on day 134. Urinary uroporphyrin excretion was measured subsequently on day 186.

SEQUENTIAL ACUTE ADMINISTRATION OF 17β-ESTRADIOL. This series of experiments aimed to identify the temporal relationship between the administrations of 17β-estradiol and HCB. Cas rats (175–200 g) were divided into five groups of 10 rats each given HCB according to the protocol described above: [Cas:(1); Cas:(1, 8); Cas:(15); Cas:(22, 29); Cas:(29)]; the numbers in parentheses indicate the day(s) on which the rats received 17β-estradiol (4 mg/kg, i.m.). Urinary uroporphyrin excretion was measured on days 38, 45, 52, and 59. Blood was collected from the tail vein for the groups of Cas rats given 17β-estradiol either on day 1 or on days 1 and 8. Plasma was obtained by centrifugation at 1000 *g* for 5 min of the heparinized samples. Plasma estradiol concentration was measured daily from days 2 to 8 [Cas:(1)] or days 2 to 15 [Cas:(1, 8)] using the ICN [^{125}I]Estradiol-17β ImmuChem™ Coated Tube RIA Kit. A part of the same protocol was repeated using Ova rats (150–175 g) distributed into two groups of 8 rats, i.e. Ova:(1, 8) and Ova:(22, 29). Urinary uroporphyrin excretion was then measured on days 37 and 44. Plasma estradiol concentration was measured daily from day 9 to day 15 [Ova:(1, 8)] and every other day from day 30 to day 36 [Ova:(22, 29)].

Finally, in Ova rats given the HCB (days 1–5) treatment and the two early doses of 17β-estradiol (days 1 and 8), urinary uroporphyrin excretion was measured on day 16.

Measurement of Biliary PCTP

Groups of intact female (N = 5), Ova (N = 4), male (N = 4), and Cas rats (N = 4) were anesthetized with ketamine

(100 mg/kg body wt, i.p.) and xylazine (12 mg/kg body wt, i.p.) immediately after they received their fifth daily dose of HCB. The bile duct was cannulated using PE-10 polyethylene tubing (Becton–Dickinson, Parsippany, NJ). The tubing was inserted under the skin toward the back of the animal to exit in the neck area. Finally, the cannula was tied to the abdominal muscle before closing. Rats were placed individually in a 6 in. × 6 in. stainless steel metabolism cage. The tubing was inserted in a spring attached at the top of the cage to allow the rat to move freely in the cage while preventing damage to the tubing. Bile was collected for 24 hr in polycarbonate containers wrapped with aluminum foil. Rats had free access to food and water. After collection, bile samples were kept frozen until analyzed.

PCTP was measured by gas chromatography after alkaline hydrolysis, using a modification of the method described by D'Amour and Charbonneau [14]. Bile samples (0.5 mL), 2 N NaOH (0.6 mL), and 0.7% aqueous ascorbic acid solution (1 mL) were heated for 1 hr at 70°. Hydrolysates were cooled, and then acidified to pH 1 with concentrated HCl. Samples were extracted twice with 2 mL of ethyl acetate. Each time, the mixture was vortexed and centrifuged at 1000 *g* for 10 min, and then the ethyl acetate layers were separated. The combined extract was analyzed directly by gas chromatography, i.e. without concentrating it.

PCTP quantitation was performed using a Hewlett–Packard (HP model 5730 A) gas chromatograph equipped with an automatic injector (HP model 7673) and an electron capture detector. A DB-5 (5% phenyl–95% methyl) capillary column (30 m × 0.25 mm, i.d., 0.25 μm coating thickness) was used with helium as the carrier gas at a flow rate of 1 mL/min. The argon:methane (95:5) make-up flow rate was 30 mL/min. The injector and detector port temperatures were 280° and 300°, respectively. The initial temperature of the column was at 60°; it was increased to 200° at a rate of 10°/min, then to 280° at a rate of 5°/min and held for 5 min. The split/splitless capillary injector was operated in the splitless mode, and the injection volume was 1 μL. The retention time of PCTP under these chromatographic conditions was 17.2 min. Peak areas were recorded with an integrator (HP model 3396, Series II). The calibration curve of PCTP was linear from 250 to 2000 pg/μL. The identity of the PCTP peak was confirmed by gas chromatography–mass spectrometry.

Urinary Uroporphyrin Analysis

Urinary uroporphyrin excretion was determined as described by Krishnan *et al.* [8] using a Perkin–Elmer Luminescence Spectrometer LS 50B operated with a 486 IBM computer. The band width for emission was 20 nm and for excitation was 10 nm. The excitation wavelength (370–420 nm) giving maximal fluorescence intensity was 404.3 and 407.7 nm for solutions containing 0 and 100% uroporphyrin. The emission wavelength was 596 nm. Rats were considered porphyric when the percentage of uroporphyrin III in urine was higher than 10%.

Statistical Analyses

Data on urinary uroporphyrin excretion were analyzed using a multifactor ANOVA (sex, time, treatment) and the differences among means assessed by Duncan's multiple range test. The ANOVA procedure was also used to evaluate differences in PCTP levels between intact female, Ova, male, and Cas plus estradiol rats. In all cases, $P < 0.05$ was used as the level of significance.

RESULTS

Figure 1 shows the importance of 17β-estradiol during the delay phase. In intact female rats, the HCB treatment caused a marked porphyria detectable only after day 31 (Fig. 1, top panel), with approximately 75% of the animals in the group developing porphyria (Fig. 1, bottom panel). The removal of the 17β-estradiol component through ovariectomy protected all the rats from being porphyric (Fig. 1). Male rats were non-responsive to the HCB treatment (data not shown), and castration was without effect on their susceptibility to develop porphyria (Fig. 1). However, when Cas rats were given 17β-estradiol, they resembled female rats in their response, as 75% of animals in the group became porphyric (Fig. 1, bottom panel). The urinary uroporphyrin excretion on day 87 of Cas rats given 17β-estradiol was higher but not statistically different (large inter-individual variations) compared with that of intact female rats (Fig. 1, top panel).

The same rats of the two non-responsive groups, i.e. Ova and Cas, were used to further evaluate the importance of the 17β-estradiol component. Urinary uroporphyrin levels for the Ova rats, which then received 17β-estradiol (weekly beginning on day 120) concomitantly with a second treatment of HCB (days 134–138) were comparable to those for normal females measured at the same time, i.e. at 52 days after the first HCB dose; porphyria developed in 55% of the animals in the ovariectomized plus 17β-estradiol group (data not shown). Knowing the effect exerted by 17β-estradiol in HCB-treated Cas rats and considering that liver HCB levels are much lower at day 60 (17 μg/g compared with 70 μg/g at day 6), the Cas rats given HCB from days 1 to 5 were injected from day 60 with 17β-estradiol for 7 weeks. None of the animals in this group was porphyric 16 or 52 days later (data not shown).

Since GSH conjugation leading to PCTP formation appears to be a detoxication pathway with higher activity in male than in female rats, we have sought differences in biliary PCTP excretion upon removal and addition of the 17β-estradiol component. As expected, biliary excretion of PCTP was 2.4 times higher ($P < 0.05$) for males than females (Fig. 2). The administration of 17β-estradiol to Cas rats reduced ($P < 0.05$) the PCTP level to that of intact

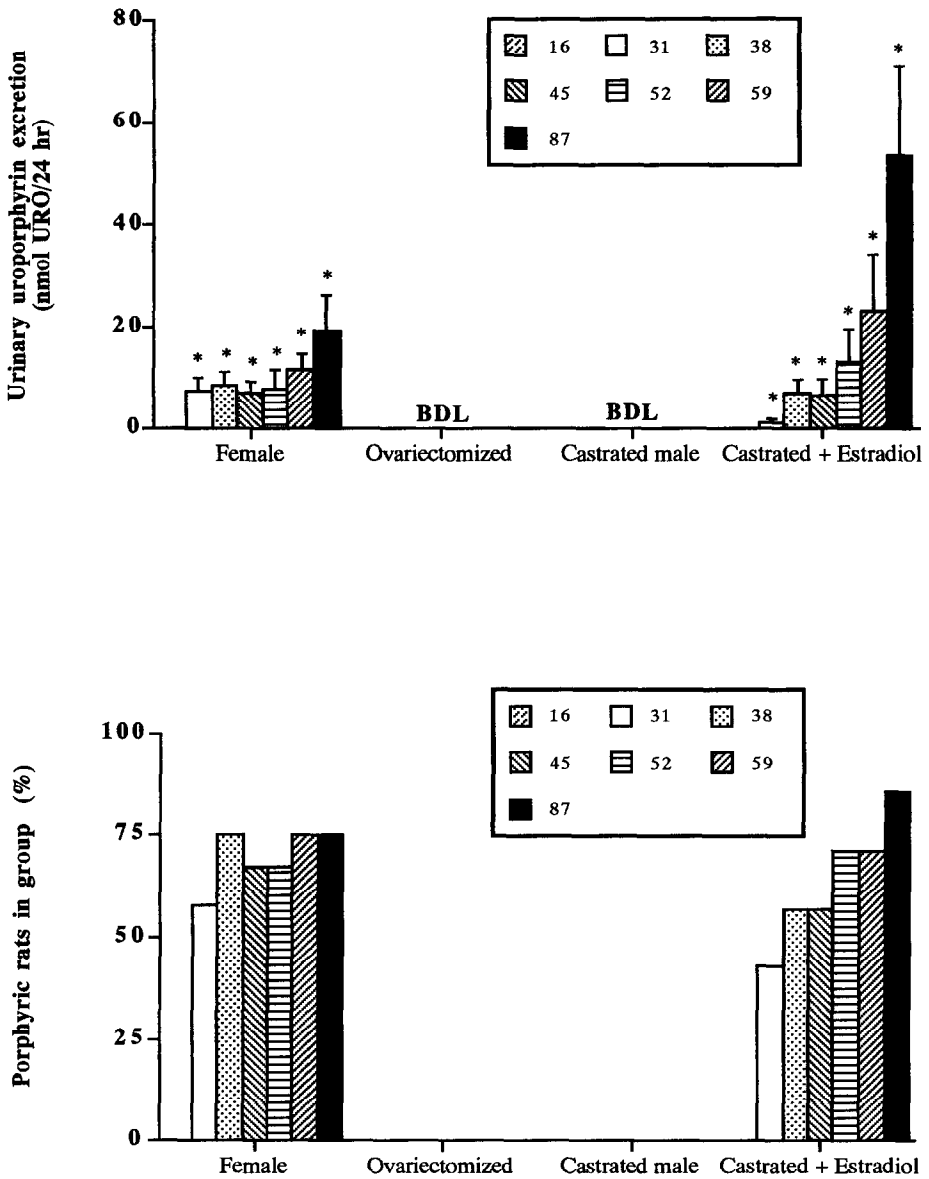

FIG. 1. Temporal profile for (top panel) urinary uroporphyrin excretion and (bottom panel) percentage of porphyric rats in each group. Rats received a total dose of 500 mg HCB/kg body weight (five consecutive administrations of 100 mg HCB/kg over 5 days, p.o., 10 mL/kg). Results in the top panel are expressed as means ± SEM and in the bottom panel as the mean percentage (12, 13, 10, and 10 animals per group for the female, ovariectomized, castrated, and castrated + estradiol rats, respectively). Asterisks indicate that the mean is significantly different ($P < 0.05$) from the group at day 16 for the same sex. BDL = below the detection limit. Note that values at day 16 for all groups were below the detection limit.

females (Fig. 2). However, the ovariectomy was without effect in modifying the biliary PCTP excretion of intact female rats (Fig. 2).

The dominant role observed for repeated administrations of 17β-estradiol given prior to the HCB treatment and maintained throughout the delay phase prompted us to assess, in Ova and Cas rats, the efficiency of one or two injections of 17β-estradiol given at specific time points during the delay phase. In HCB-treated Ova rats, the administration of two 17β-estradiol doses at a 1-week interval caused porphyria on day 52 for both the early (days 1 and 8) and late (days 22 and 29) administrations (Table 1). The two 17β-estradiol injections to HCB-treated Cas rats resulted in a lower proportion of rats with porphyria and lower levels of urinary uroporphyrin excretion compared with Ova rats (Table 1). In HCB-treated Cas rats receiving a single injection of 17β-estradiol at the beginning (day 1), middle (day 15), or end (day 29) of the delay phase, none of the animals was porphyric on day 52.

The plasma 17β-estradiol level varies according to the estrus cycle. In the non-synchronized control females used, the mean plasma 17β-estradiol concentration was approximately 50 pg/mL. We have assessed plasma 17β-estradiol concentrations resulting from the exogenous injections. The level in Ova rats given a dose of 4 mg/kg peaked at 27,000 pg/mL and decreased to 2,200 pg/mL over 6 days

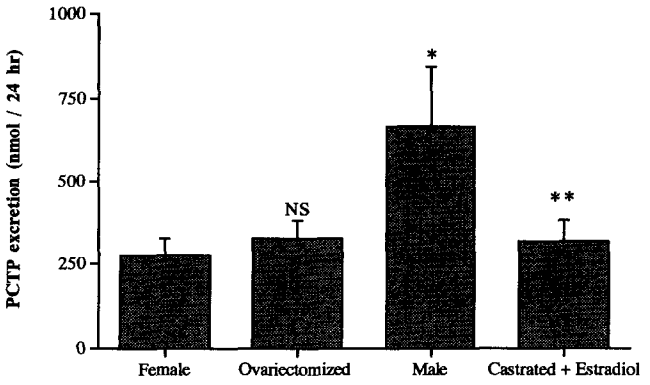

FIG. 2. Biliary excretion of PCTP in HCB-treated rats with or without the estradiol component. Bile was collected for 24 hr after the fifth dose of 100 mg HCB/kg body weight. Results are expressed as means ± SEM (5, 4, 4, and 4 animals per group for the female, ovariectomized, male, and castrated + estradiol rats, respectively). NS indicates that the mean was not significantly ($P > 0.05$) different from intact female rats. A single asterisk (*) indicates that the mean was significantly ($P < 0.05$) different from HCB-treated female rats, whereas double asterisks (**) indicate a significant difference ($P < 0.05$) from HCB-treated male rats.

(Fig. 3), with an estimated half-life of 36.8 hr. Plasma 17β-estradiol concentration in Cas rats given the same dose was approximately three times lower (Fig. 3). The administration of 17β-estradiol during the early (days 1 and 8) or late (days 22 and 29) part of the delay phase resulted in similar plasma levels (Fig. 3). The profile observed in Cas rats for the 7-day period after the second injection (day 8) was identical to that for the first injection (day 1), indicating no accumulation phenomenon when two doses were given. Pharmacokinetic analysis assuming a first-order rate of elimination [log concn (ng/mL) = 3.124 − 0.196x, where x is the time in days] indicated that for Ova rats injected at days 1 and 8 the normal control female level was reached at day 23, indicating that the plasma estradiol level remained above the supra-physiologic level throughout the delay phase.

DISCUSSION

The results showed that the responsiveness of female rats, but not male rats, to the porphyrinogenic effect of HCB depends on the presence of estrogens rather than on the absence of androgens; theoretically, the latter could have exerted an inhibitory role in the male. This is clearly exemplified by the fact that in male rats castration alone was without effect on the porphyrinogenic activity of HCB, whereas castration and 17β-estradiol administration yielded to porphyria. Moreover, removal of the estrogen component through ovariectomy caused female rats to become insensitive to the porphyrinogenic effect of HCB. The same Ova animals were, however, subsequently made responsive to a second round of five daily doses of HCB when 17β-estradiol was given concomitantly. This clearly demonstrates the important role of 17β-estradiol in the induction of porphyria by HCB in the rat. This is in accord with observations of two previous studies that implied a role of estrogens in HCB-induced porphyria: HCB-treated male rats became strongly porphyric when injected with DES, an estrogenic compound [9], and liver porphyrin accumulation in HCB-treated female rats was reduced following ovariectomy [4]. The present study offers the advantage of being focussed on the effect of the biologically relevant estrogenic component, 17β-estradiol, using a study design addressing the issue of sexual dimorphism.

TABLE 1. Effects of two weekly doses of 17β-estradiol on the induction of porphyria in HCB-treated ovariectomized female or castrated male rats

Injections of estradiol on days:	% of Porphyric rats in group		Urinary uroporphyrin excretion (nmol/24 hr)	
	Ova	Cas	Ova*	Cas
1 and 8	100	22	60 ± 49	2 and 8†
22 and 29	88	10	22 ± 13	35†

* Values are means ± SEM for 10 rats.
† All other values in the group (N = 10) were equal to zero.

In female rats, the protocol of five consecutive daily HCB doses caused porphyria from approximately day 30–40, indicating the presence of a delay phase before porphyria fully developed (marked increase in liver uroporphyrin accumulation and urinary uroporphyrin excretion). This protocol has allowed us to assess the role of 17β-estradiol in a time-dependent manner. In the first series of experiments, 17β-estradiol was administered weekly to Ova or Cas rats from 2 weeks prior to HCB treatment to the end of the delay phase. The temporal patterns for the percentage of porphyric rats in each of these two groups, as well as in the group of HCB-treated normal female rats, were identical; however, differences in the level of urinary uroporphyrin excretion from days 31 to 87 were observed. Subsequently, sequential administrations at the beginning or at the end of the delay phase were studied. Two weekly doses of 17β-estradiol at the beginning (days 1 and 8) or at the end (days 22 and 29) were shown to be equally effective in causing porphyria in HCB-treated Ova and Cas rats. The early administrations did not accelerate the onset of porphyria, as porphyria developed in both groups at the usual time point (day 38). Plasma 17β-estradiol levels were markedly higher for both groups compared with the control normal female level: the maximal level was three times higher in female Ova rats compared with Cas rats, but the level 6 days later was similar for both groups. Pharmacokinetic analysis assuming a first-order rate of elimination indicated that the normal control female level was reached at day 23 for Ova rats injected at days 1 and 8. The normal plasma level measured (50 pg/mL) in non-synchronized control females was in agreement with the observations of Davis *et al.* [15]. It is to be noted that the estrus cycle in female rats lasts 5 days, such that during the 5-day HCB treatment each female completed one cycle.

Biotransformation of HCB has been postulated to play an

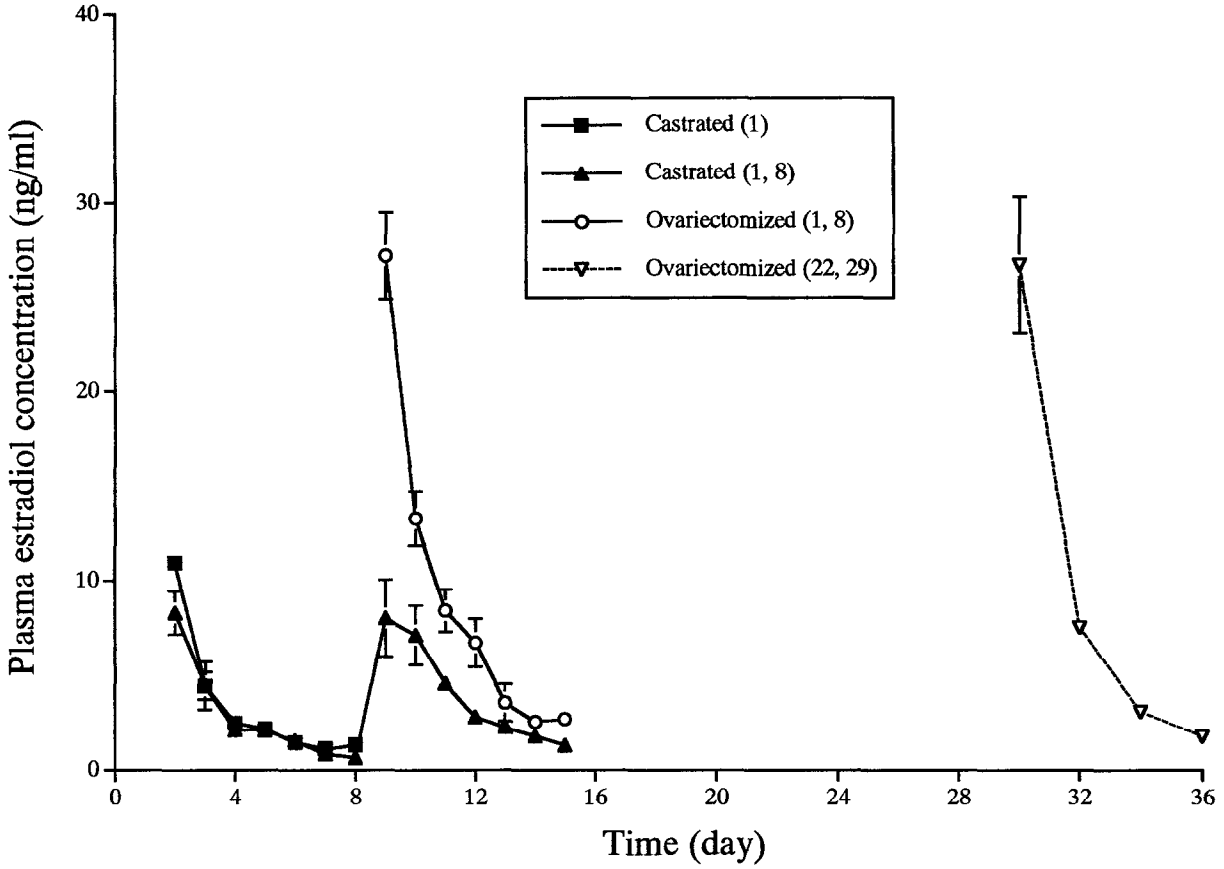

FIG. 3. Temporal profile for plasma estradiol concentration in castrated male and ovariectomized female rats. Rats received a total dose of 500 mg HCB/kg body weight (five consecutive administrations of 100 mg HCB/kg over 5 days, p.o., 10 mL/kg). Values are means ± SEM for 10 rats. Numbers in parentheses indicate days on which the rats received estradiol (4 mg/kg).

essential role in the development of porphyria [10, 12, 13]. D'Amour and Charbonneau [14] suggested that the sex-related difference in the induction of hepatic porphyria by HCB may partly arise from differences in GSH conjugation since biliary excretion of PCTP, the major metabolite arising from this pathway, was higher for male rats than for female rats. Results in this study showed that 17β-estradiol administration to Cas rats reduced the biliary PCTP excretion to the level of female rats; however, ovariectomy of female rats was without effect. This suggests that GSH *S*-transferases, such as those likely to be involved in the conjugation of HCB to GSH, are under the control of testosterone. In support of this hypothesis, Hatayama *et al.* [16] reported that upon castration the level of the 7-7 form of hepatic GSH *S*-transferase in male mice decreases to that in females, while that in females increases to the adult male level upon administration of testosterone. Thus, the role of 17β-estradiol in the porphyrinogenic effect of HCB cannot be explained by a modulation of the level of PCTP formation since, despite its protective effect, the ovariectomy does not change biliary PCTP excretion compared with normal females.

Two interesting observations may help to further understand the mechanism of HCB-induced porphyria: (i) in Ova rats, the supra-physiologic plasma levels of 17β-estradiol did not accelerate the development of porphyria [Ova:(1, 8) rats were porphyric at day 38 but not at day 16]; however, uroporphyrin accumulation occurred shortly after injection of the hormone at a very late stage of the delay phase [day 38 for Ova:(22, 29)]; (ii) in this five daily dose model, liver HCB concentrations decreased exponentially from day 6 [8], such that levels for the "days 22–38" period were much lower than those for the "days 1–16" period. These observations indicated that the delay phase was not related to a delay in obtaining a certain liver HCB level or to a slow action of the 17β-estradiol. Nevertheless, a threshold liver HCB level was required for the development of porphyria, as exemplified by the fact that HCB-treated Cas rats given 17β-estradiol during the "days 60–102" period did not develop porphyria, contrary to those receiving it throughout the "days 0–38" period; liver HCB concentration decreased from 69.4 to 16.5 μg/g tissue during the "days 6–60" period, and then the reduction progressed to 0.8 μg/g at day 102 [8]. Using the five-dose model, we have shown recently that uroporphyrinogen decarboxylase (UROD) is inhibited in porphyric rats only when porphyria occurs (end of delay phase), but not in non-porphyric females or males [17]. We also observed that

a heat stable inhibitor of UROD is present in the cytosol from female rat livers, and to a lesser extent in the cytosol from male rat livers (unpublished experiments). One may speculate that the hormone acts by exacerbating the UROD inhibition caused by HCB through a stimulation of the heme biosynthesis cycle leading to accumulation of uroporphyrin.

In summary, results showed that the sexual dimorphism in HCB-induced porphyria in the rat is related to the presence of 17β-estradiol and that both sexes have the ability to respond when proper hormonal status is present. These observations will permit further mechanistic studies to understand the porphyrinogenic action of HCB.

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