Biochemical Pharmacology, Vol. 33, No. 4, pp. 559–564, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00

HEPATIC MICROSOMAL *p*-NITROANISOLE *O*-DEMETHYLASE

EFFECTS OF CHLORDECONE OR MIREX INDUCTION IN MALE AND FEMALE RATS*

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(*Received* 3 *March* 1983; *accepted* 28 *July* 1983)

Abstract—The effects of chlordecone (CD) or mirex treatment on the hepatic microsomal monooxygenase system of male and female rats were investigated, and kinetic parameters (apparent V_{max} and apparent K_m) for *p*-nitroanisole *O*-demethylase were studied in detail. Both pesticides elevated the levels of cytochrome P-450 in a time- and dose-dependent manner. The maximum rate of *p*-nitroanisole metabolism in males was increased about 100 and 50% and the apparent K_m was elevated about 40- and 18-fold by CD and mirex respectively. *p*-Nitroanisole metabolism in females was reduced slightly by treatment with either agent, and the apparent K_m was increased about 14-fold by CD but was relatively unaffected by mirex treatment.

Chlordecone† (Kepone, CD‡) and its fully chlorinated structural analog, mirex§, are insecticides and flame retardants [1, 2]. While CD is no longer used, mirex is used as a flame retardant and requests have been made to allow its use as an insecticide to combat the fire ant. Exposure to CD results in toxicity involving the liver and the neuromuscular, endocrine and reproductive systems in several mammalian species including man [3]. In the liver, these effects include hepatocarcinoma [4, 5], morphological changes [6–8], induction of the microsomal monooxygenase system [9–13], hepatobiliary dysfunction [14, 15], and the potentiation of the hepatotoxicity of chloromethanes [16–20]. In contrast, exposure to mirex results in induction of the hepatic microsomal monooxygenase system [9, 21–23] without the toxicity observed for CD.

Previous studies of the effects of CD on the hepatic monooxygenase system have reported alterations in the rate of metabolism of various substrates of this enzyme system [9–13, 21–23]. In a recent study [13], the rate of metabolism of a number of substrates was investigated in the male rat as a function of CD treatment. While the metabolism of these substrates was affected by exposure of the animal to CD, a larger effect was observed in K_m than V_{max}. The hypothesis was advanced [13] that these effects were primarily the result of inhibition of metabolism by residual CD [24].

The experiments described below were designed to further test this hypothesis with mirex as well as CD by using both male and female rats. *p*-Nitroanisole was selected for these studies since the effect on K_m is most pronounced with this substrate [13].

MATERIALS AND METHODS

NADPH, NADH, $NADP^+$, cytochrome *c*, sodium DL-isocitrate, isocitrate dehydrogenase Type IV, sodium cholate, Lubrol PX, phenobarbital (PB), 3-methylcholanthrene (3-MC) (Sigma); *p*-nitroanisole (Eastman); chlordecone 99% (Chem Service); mirex 98+% (Supelco); and DEAE-cellulose, DE52 (Whatman) were obtained from the indicated sources. All other chemicals used were of the highest quality commercially available and were used without further purification.

The microsomal fraction was prepared, as previously described [25], from the livers of 200–250 g male or female Sprague–Dawley rats (Harlan Industries, Madison, WI). The rats received CD or mirex dissolved in corn oil or corn oil alone by intraperitoneal injection (0.2 ml corn oil/100 g body wt). The animals were fasted for about 24 hr after the last injection prior to being decapitated. When severe tremors developed (CD treatment), the animals continued to eat if food pellets were placed in the bottom of the cages. These rats apparently lacked the coordination necessary to feed from hanging containers.

Cytochrome P-450 and b_5 were measured by the method of Omura and Sato [26] using a Perkin–Elmer 557 spectrophotometer. NADPH–cytochrome P-450 reductase and NADH–cytochrome b_5

* This study was supported by USPHS Grant ES-02045 and Hatch Project VA-0612304.

† Decachlorooctahydro-1,3,4-metheno-2*H*-cyclobuta-[*cd*]-pentalene-2-one.

‡ Abbreviations: CD, chlordecone; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PB, phenobarbital; and 3-MC, 3-methylcholanthrene.

§ Dodecachlorooctahydro-1,3,4-metheno-2*H*-cyclobuta-[*cd*]pentalene.

reductase activities were assayed as cytochrome *c* reductase activities at 25° following the reduction of cytochrome *c* at 550 nm ($\varepsilon = 21\ mM^{-1}\ cm^{-1}$) using a Cary 219 spectrophotometer. The assay system contained 50 mM potassium phosphate buffer, pH 7.7, 0.05 mM cytochrome *c*, and 0.1 mM NADPH or NADH. Protein was determined by the method of Lowry *et al.* [27] by using bovine serum albumin as the standard.

The production of *p*-nitrophenolate [28] was used to measure *p*-nitroanisole *O*-demethylase. This activity was monitored continuously with a Perkin–Elmer spectrophotometer equipped with a cuvette stirring accessory. The assay system maintained at 37° was composed of 50 mM Hepes buffer, pH 8.0, 150 mM KCl, 10 mM $MgCl_2$, 10 mM sodium DL-isocitrate, 0.4 units/ml isocitrate dehydrogenase, 1 mg microsomal protein/ml and 0.2mM NADPH. Estimations of V_{max} and K_m were made by linear least squares analysis of Lineweaver–Burk reciprocal plots. The correlation coefficients were at least 0.998. At least seven different concentrations of *p*-nitroanisole were used. A minimum range of substrate concentrations (highest to lowest) was 40-fold. These experiments were performed at least twice, and the initial velocity measurements were determined in duplicate at each substrate concentration. The V_{max} varied within ± 10%, while K_m values varied within ± 15%. The actual concentrations of *p*-nitroanisole were estimated from the absorbance at 317 nm. An extinction coefficient of $10\ mM^{-1}\ cm^{-1}$ was determined (unpublished).

The CD content of microsomal fractions was analysed by electron capture gas–liquid chromatography in the laboratory of R. W. Young of this department using a technique developed by Consolidated Laboratories (Richmond, VA). Since small amounts of CD (and perhaps mirex) adhere to glass and plastic, glass and plasticware exposed to these compounds were washed with ethanol prior to use in subsequent experiments.

DEAE-chromatography of detergent-solubilized microsomal samples was performed using a modification of the procedure of Warner *et al.* [29]. The microsomal fraction (200 mg protein) was stirred for 30 min in 5 mM potassium phosphate buffer, pH 7.4, contained 20% glycerol, 0.1 mM EDTA, 0.5% sodium cholate and 0.2% Lubrol PX. The final microsomal protein concentration was 2 mg/ml. Following centrifugation (100,000 *g* for 60 min), the supernatant fraction containing 85–95% of the cytochrome P-450 was applied to a DE52 column (2.5 × 45 cm) equilibrated with 10 mM potassium phosphate buffer, pH 7.7, containing 20% glycerol, 0.1 mM EDTA, 0.2% sodium cholate and 0.1% Lubrol PX (buffer A). The column was washed with 100 ml buffer A and then eluted with a salt gradient formed with 500 ml buffer A and 500 ml buffer A containing 0.25 M NaCl. Cytochrome b_5 was not eluted under these conditions. The flow rate was 40 ml/hr, and 4-ml fractions were collected. The entire procedure was performed at room temperature. The use of Lubrol PX in place of Emulgen 911 allowed the use of $A_{280\,nm}$ as a protein monitor of column fractions.

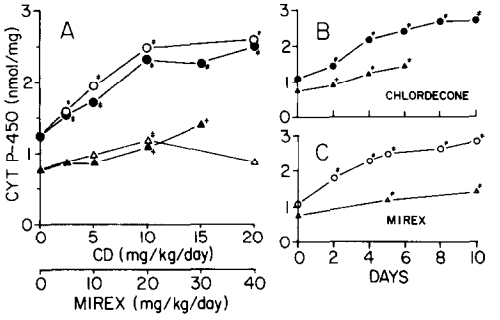

Fig. 1. Cytochrome P-450 induction as a function of CD or mirex treatment. (A) Dose level — i.p. injections once per day for 5 days. Key: closed symbols (CD), open symbols (mirex), circles (males), and triangles (females). (B and C) Days of treatment. CD, 10 mg/kg/day; mirex, 20 mg/kg/day. Symbols as in A. Statistically different than controls at $P < 0.05$ (+) or $P < 0.005$ (≠).

RESULTS

Induction. Intraperitoneal injection of male and female rats with CD resulted in a dose- and time-dependent increase in hepatic microsomal cytochrome P-450 (Fig. 1, A and B) and a decrease in NADH–cytochrome b_5 (*c*) reductase activity (Table 1). Cytochrome b_5 and NADPH–cytochrome P-450 (*c*) reductase were elevated significantly in male but

Table 1. Changes in microsomal enzyme content following CD or mirex treatment*

	Cytochrome P-450 (nmoles/mg)	Cytochrome b_5 (nmoles/mg)	NADPH–cytochrome P-450 reductase (nmoles/min/mg)	NADH–cytochrome b_5 reductase (nmoles/min/mg)
Control				
Male	1.23 ± 0.07	0.46 ± 0.03	105 ± 10	507 ± 71
Female	0.76 ± 0.05	0.43 ± 0.05	132 ± 20	1273 ± 188
CD				
Male	2.32 ± 0.14†	0.57 ± 0.05†	217 ± 24†	347 ± 11†
Female	1.08 ± 0.01†	0.41 ± 0.03	120 ± 18	687 ± 11†
Mirex				
Male	1.93 ± 0.13†	0.52 ± 0.05†	124 ± 6†	407 ± 11†
Female	0.94 ± 0.04	0.43 ± 0.02	186 ± 15	1120 ± 85

* Five daily i.p. injections: controls, corn oil; CD or mirex, 10 mg/kg/day.
† Statistically different from control, $P < 0.05$.

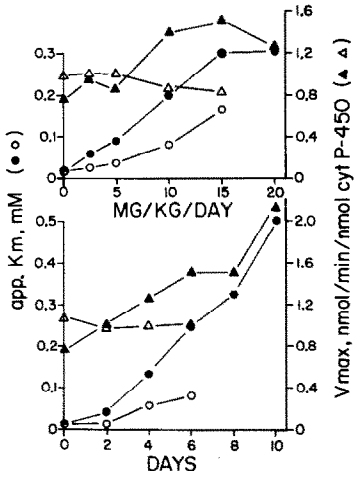

Fig. 2. Influence of CD treatment on *p*-nitroanisole *O*-demethylase activity of microsomes isolated from male (closed symbols) and female (open symbols) rats. Top: variable dose, five daily doses. Bottom: variable treatment period, 10 mg/kg/day.

not female rats (Table 1). All of the female rats receiving CD for 5 days at a dosage of 20 mg/kg/day or 8 days of CD at 10 mg/kg/day died.

Similarly, treatment with mirex resulted in increases in cytochrome P-450, cytochrome b_5, and NADPH–cytochrome P-450 (*c*) reductase and a decrease in NADH–cyctochrome b_5 (*c*) reductase in male rats (Fig. 1, A and C, Table 1). Female rats were less responsive to mirex than to chlordecone.

p-*Nitroanisole O-demethylase*. Treatment of male rats with CD caused about a 2-fold increase in the rate of *p*-nitroanisole metabolism (nmoles/min/nmole cytochrome P-450) while similar treatment of female rats caused no apparent change (Fig. 2). The apparent K_m for *p*-nitroanisole was increased dramatically in both males and females following treatment with CD although this effect was much more pronounced in the male (Fig. 2). The maximum

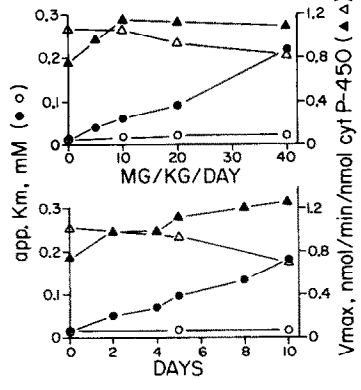

Fig. 3. Influence of mirex treatment on *p*-nitroanisole *O*-demethylase activity of microsomes isolated from male (closed symbols) and female (open symbols) rats. Top: variable dose, five daily doses. Bottom: variable treatment period, 20 mg/kg/day.

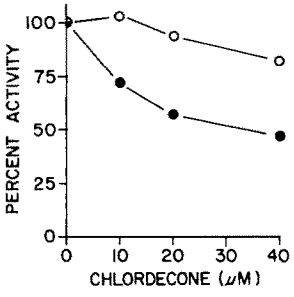

Fig. 4. Influence of CD on *p*-nitroanisole *O*-demethylase activity of microsomes isolated from untreated male (closed symbols) and female (open symbols) rats. The concentration of *p*-nitroanisole was 100 μM. Activity is presented as a percentage of an ethanol control.

increase was about 40-fold for male rats and 14-fold for females.

Treatment with mirex resulted in about a 50% increase in *p*-nitroanisole O-demethylation in male rats and a slight decrease in turnover in females (Fig. 3). The apparent K_m for *p*-nitroanisole was not altered significantly as a result of mirex treatment of female rats. In males, apparent K_m was increased about 18-fold following treatment with mirex (40 mg/kg/day) for 5 days (Fig. 3).

In vitro addition of CD to microsomes isolated from untreated male or female rats inhibited *p*-nitroanisole *O*-demethylase activity. The inhibition was more pronounced with microsomes isolated from male rats (Fig. 4). The extent of inhibition in males at the level of substrate and inhibitor used is consistent with the apparent spectral binding constant of CD to cytochrome P-450 and the inhibition constant [24].

Chlordecone and mirex content of liver and hepatic microsomes. Following treatment of rats with CD or mirex, a larger fraction of the administered dose of CD was sequestered in the liver than with mirex (Table 2). Comparable results were obtained for the pesticide content of the hepatic microsomal fraction isolated from treated animals. No dramatic differences were observed between males and females (Table 2). The microsomal content of CD was directly dependent upon the dose and time of administration (data not shown).

DEAE-chromatography of detergent-solubilized microsomes. Using a modification of the procedure of Warner *et al.* [29], various forms of hepatic microsomal cytochrome P-450 were separated using anion exchange chromatography (Fig. 5). Different $A_{417\,nm}$ elution profiles, representing cytochrome P-450, were noted dependent upon animal treatment. Following treatment with PB, 3-MC or mirex, dramatic increases, compared to controls, were observed in the cytochrome P-450 content around fraction 160. In the case of CD treatment, the major change in elution profile was an increase around fraction 80 which was also the major peak eluted using microsomes isolated from control animals. The profile resulting from CD treatment was distinctly different from that of mirex treatment although these compounds are structurally related.

Table 2. CD and mirex content of liver and hepatic microsomes following treatment of male and female rats*

	Pesticide content			
	Male		Female	
Treatment	Liver (μg/g)	Microsomes (μg/mg)	Liver (μg/g)	Microsomes (μg/mg)
CD	75	1.03	73	1.05
Mirex	1.4	0.036	1.4	0.027

* Rats were treated with CD or mirex, 10 mg/kg/day, for 5 days. All values are averages of two determinations.

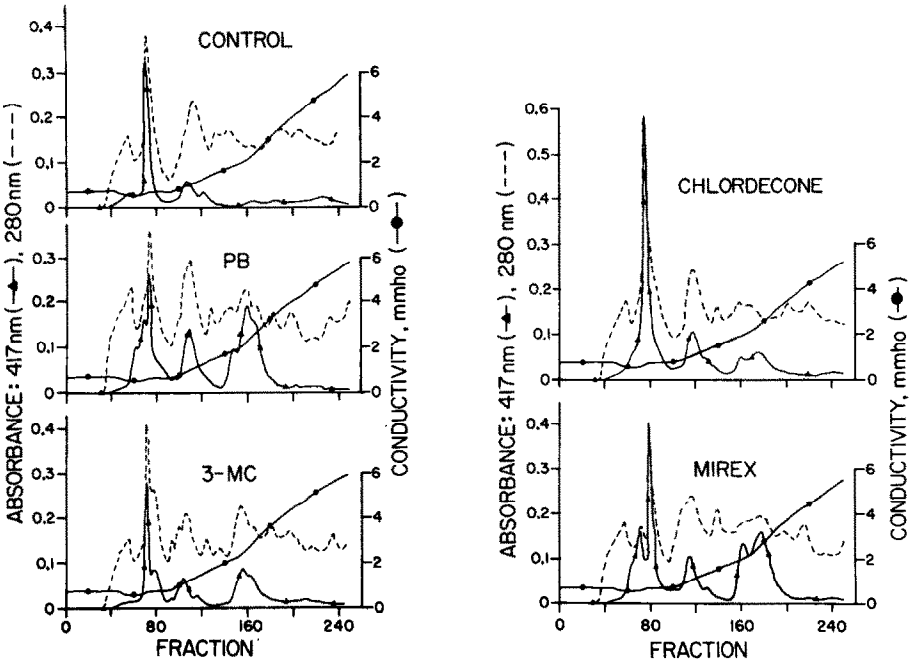

Fig. 5. DEAE-chromatography of hepatic microsomes isolated from male rats either untreated or treated with PB (80 mg/kg/day for 4 days), 3-MC (20 mg/kg/day for 4 days), CD (10 mg/kg/day for 5 days), or mirex (20 mg/kg/day for 5 days). The details of this procedure are described in Materials and Methods.

DISCUSSION

The observed induction of the hepatic microsomal monooxygenase in rats by the insecticides CD and mirex (Fig. 1, Table 1) confirmed earlier reports [9–13, 21–23]. In contrast to earlier studies which reported similar values of LD_{50} for male and female rats [30, 31], female rats used in these studies died when treated with CD (20 mg/kg/day for 5 days or 10 mg/kg/day for 8 days). The difference between the current study and earlier reports may involve the route of administration of CD, intraperitoneal versus oral.

As reported previously [13], treatment of male rats with CD results in about a 2-fold increase in the maximum rate of *p*-nitroanisole metabolism (nmoles/min/nmole cytochrome P-450) and a dramatic increase in apparent K_m. The increase in apparent K_m for *p*-nitroanisole was progressive with the dose and the time period during which CD was administered (Fig. 2). With females, the apparent K_m increased but less dramatically than with males while metabolism was not affected.

Mirex treatment of male rats produced qualitatively similar results (Fig. 3). The maximum rate of metabolism (nmoles/min/nmole cytochrome P-450) was increased by about 50%, while apparent K_m increased progressively with dose and time of treatment. In contrast, the metabolism of *p*-nitroanisole in females was reduced slightly as a function of mirex treatment, while the apparent K_m was relatively constant.

Changes in the kinetic parameters of xenobiotic metabolism as a function of monooxygenase system

induction can reflect an alteration in the makeup of the cytochrome P-450 pool. The existence of multiple forms of cytochrome P-450 in the rat liver microsomal fraction and changes in the pool composition as a function of induction have been documented [32–34]. The DEAE-chromatography microsomal cytochrome P-450 elution profile (Fig. 5) and SDS-PAGE microsomal protein patterns [13] from CD-treated rats more closely resembled those of untreated than of PB- or 3-MC-treated animals, suggestive of an increase in constitutive cytochromes as a result of CD treatment. Mirex treatment resulted in a cytochrome profile different from that of untreated rats as well as that of PB, 3-MC or CD treatment.

Kinetic parameters, particularly apparent K_m, can be altered by the presence of a competitive inhibitor. CD and mirex bind to the active site of cytochrome P-450 [21, 24], and CD has been shown to competitively inhibit xenobiotic metabolism [24]. CD and, to a lesser extent, mirex accumulate in the liver ([19, 35–37], Table 2). Progressive increases in liver content of these pesticides as a function of dose and time of treatment would be consistent with the increases in apparent K_m. Mirex, stored in higher concentration in fat than in liver [19, 35–37], would be expected to produce a lesser effect on apparent K_m than CD (Figs. 2 and 3). The disparity between males and females with regard to apparent K_m can most readily be attributed to sex differences in the inhibitory power of the pesticides (Fig. 4).

The experimental results are consistent with the concept that CD may be an indirect or permissive inducer of cytochrome P-450. In this model the high levels of CD in the microsomal membrane (Table 2) interact with cytochrome P-450, resulting in inhibition of the metabolism of monooxygenase substrates, e.g. lipophilic dietary components, to which the animal is normally exposed. This inhibition results in elevated levels of these substrates which, in turn, induce formation of cytochrome P-450 to reestablish normal clearance of these compounds. That CD interacts with cytochrome P-450 *in vitro* causing inhibition of drug turnover has been established ([21, 24], Fig. 4). Increases in high-spin cytochrome P-450 [13] and apparent K_m values for monooxygenase substrates as a function of CD exposure are consistent with similar phenomenon *in vivo*. An interesting consequence of this model is that it would predict that the pool of cytochrome P-450 following CD exposure should be qualitatively similar to that of controls. This prediction is based on the assumption that the cytochrome pool in control animals is that required to metabolize endogenous compounds and xenobiotics to which the animal is normally exposed (e.g. from the diet). DEAE-chromatography (Fig. 5) and SDS-PAGE [13] patterns are consistent with this qualitative similarity between controls and CD-exposed animals. Obviously, this comparison cannot be definitively made until the identity of each cytochrome is established.

With respect to the hepatic microsomal monooxygenase system, the ultimate consequences of exposure to xenobiotics such as CD and mirex will be determined not only by the alterations in the level and makeup of the cytochrome P-450 pool but also by the amount of residual compound present in the liver. This latter will depend upon the total body burden of the material as well as its particular tissue distribution.

Acknowledgements—The valuable technical assistance of W. Lon Fanning and Joy Burroughs was greatly appreciated.

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