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Reversible and Time-Dependent Inhibition of the Hepatic Cytochrome P450 Steroidal Hydroxylases by the Proestrogenic Pesticide Methoxychlor in Rat and Human

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ABSTRACT: Methoxychlor, a currently used pesticide, is demethylated and hydroxylated by several hepatic microsomal cytochrome P450 enzymes. Also, methoxychlor undergoes metabolic activation, yielding a reactive intermediate (M*) that binds irreversibly and apparently covalently to microsomal proteins. The study investigated whether methoxychlor could inhibit or inactivate certain liver microsomal P450 enzymes.

The regioselective and stereoselective hydroxylation of testosterone and the 2-hydroxylation of estradiol (E_2) were utilized as markers of the P450 enzymes inhibited by methoxychlor. Both *reversible* and *time-dependent* inhibition were examined. Coincubation of methoxychlor and testosterone with liver microsomes from phenobarbital treated (PB-microsomes) male rats, yielded marked diminution of 2α- and 16α-testosterone hydroxylation, indicating strong inhibition of P4502C11 (P450h). Methoxychlor moderately inhibited 2β-, 7α-, 15α-, 15β-, and 16β-hydroxylation and androstenedione formation. There was only a weak inhibition of 6β-hydroxylation of testosterone. The methoxychlor-mediated inhibition of 6β-hydroxylation was competitive. By contrast, when methoxychlor was permitted to be metabolized by PB-microsomes or by liver microsomes from pregnenolone-16α-carbonitrile treated rats (PCN-microsomes) prior to addition of testosterone, a pronounced time-dependent inhibition of 6β-hydroxylation was observed, suggesting that methoxychlor inactivates the P450 3A isozyme(s). The di-demethylated methoxychlor (bis-OH-M) and the tris-hydroxy (catechol) methoxychlor metabolite (tris-OH-M) inhibited 6β-hydroxylation in PB-microsomes *competitively* and *noncompetitively*, respectively; however, these methoxychlor metabolites did not exhibit a time-dependent inhibition. Methoxychlor inhibited competitively the formation of 7α-hydroxytestosterone (7α-OH-T) and 16α-hydroxytestosterone (16α-OH-T) but exhibited little or no time-dependent inhibition of generation of these metabolites, indicating that P450s 2A1, 2B1/B2, and 2C11 were inhibited but not inactivated. Methoxychlor inhibited in a time-dependent fashion the 2-hydroxylation of E_2 in PB-microsomes. However, bis-OH-M exhibited solely reversible inhibition of the 2-hydroxylation, supporting our conclusion that the inactivation of P450s does not involve participation of the demethylated metabolites. Both competitive inhibition and time-dependent inactivation of human liver P450 3A (6β-hydroxylase) by methoxychlor, was observed. As with rat liver microsomes, the human 6β-hydroxylase was inhibited by bis-OH-M and tris-OH-M competitively and noncompetitively, respectively.

Testosterone and estradiol strongly inhibited the *irreversible* binding of methoxychlor to microsomal proteins. This might explain the "clean" competitive inhibition by methoxychlor of the 6β-OH-T formation when the compounds were coincubated. Glutathione (GSH) has been shown to interfere with the irreversible binding of methoxychlor to PB-microsomal proteins. The finding that the coincubation of GSH with methoxychlor partially diminishes the time-dependent inhibition of 6β-hydroxylation provides supportive evidence that the inactivation of P450 3A isozymes by methoxychlor is related to the formation of M*.

KEYWORDS: Cytochrome P450, P4503A, P4502B, P4502C11, P4502A1, P4503A4, CYP3A, CYP2B, CYP2C11, CYP2A1, CYP3A4, Liver Microsomes, Microsomal Steroid Hydroxylase, Pesticide, Methoxychlor, P450 Inhibitors, CYP3A Inactivation.

INTRODUCTION

Methoxychlor [1,1,1-trichloro-2,2-bis(4-methoxyphenyl)ethane] is currently used as a substi-

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tute for certain pesticidal activities of DDT, which has been banned in the industrially developed countries. The current popularity of methoxychlor stems from its relatively low toxicity, apparently associated with its rapid metabolism (1). Methoxychlor is demethylated by mouse, rat and human liver microsomal monooxygenases into mono- and bis-phenolic (bis-OH-M)[1] products (1–5). Additionally, liver microsomes from phenobarbital (*PB*)-treated rats (PB-microsomes) catalyze the aromatic hydroxylation of methoxychlor, yielding a tris-hydroxy (catechol) metabolite (tris-OH-M) (5) (Figure 1). However, the rapid metabolism of methoxychlor may be of some concern, because the mono- and bis-demethylated metabolites exhibit considerable estrogenic activity in mammals, indicating that methoxychlor is a proestrogen (4). Recently, we observed that the mono and bis-demethylation of methoxychlor in rat liver are catalyzed by constitutive cytochrome P450 enzymes and that ring hydroxylation is primarily catalyzed by the PB-induced 2B1/2B2 isozymes (S. Dehal and D. Kupfer, unpublished).

Additionally, methoxychlor undergoes metabolic activation by liver microsomes, yielding a reactive intermediate (M*) that binds irreversibly to proteins (6,7). PB treatment markedly elevates the irreversible binding of methoxychlor, apparently catalyzed by P450 2B1/2B2 [(7), and unpublished results)]. The major radiolabeled protein adduct generated during the incubation of PB microsomes with [^{14}C]-methoxychlor exhibited on SDS-PAGE a band with an Mr value in the P450 region. However, metabolism of methoxychlor did not diminish the spectrally assayed microsomal P450 (H-C. Li and D. Kupfer, unpublished), indicating that there was no loss of heme or of CO-binding (8). Nevertheless, our study considered the possibility that inhibition/inactivation of hepatic microsomal P450 enzymes may occur during the metabolism of methoxychlor.

[1]*Abbreviations and common names*: Methoxychlor, [1,1,1-trichloro-2,2-bis(4-methoxyphenyl)ethane]; mono-OH-M, [1,1,1-trichloro-2-(4-hydroxyphenyl)-2-(4-methoxyphenyl)ethane]; bis-OH-M, [1,1,1-trichloro-2,2-bis(4-hydroxyphenyl)ethane]; tris-OH-M, [1,1,1-trichloro-2-(4-hydroxyphenyl)-2-(3,4-dihydroxyphenyl)ethane]; DDT, [1,1,1-trichloro-2,2-bis(p-chlorophenyl)-ethane].

MATERIALS AND METHODS

[^{14}C]-testosterone (55.4 or 54.5 mCi/mmol) and 2-[^{3}H]-estradiol (22.0 Ci/mmol) were obtained from Du Pont NEN Research Products (Boston, MA). [^{14}C]-methoxychlor (9.2 mCi/mmol) was purchased from Sigma Chemical Co. (St. Louis, MO). The authentic samples of the various hydroxylated testosterone derivatives were provided gratis by Prof. D. N. Kirk (Univ. of London, UK) or purchased from Steraloids (Wilton, NH). Methoxychlor was purchased from Chem Service (West Chester, PA). Bis-OH-M was provided gratis by T. Fujita (Kyoto Univ., Japan) and J. Sanborn (Illinois Natur. History Survey, Urbana, IL). Tris-OH-M was obtained through custom synthesis from Chemsyn Science Labs (Lenexa, KS). NADPH, glucose 6-phosphate, glucose-6-phosphate dehydrogenase, EDTA disodium salt, glutathione were purchased from Sigma Chemical Co. (St. Louis, MO). Phenobarbital sodium salt and reagent grade solvents were purchased from Mallinckrodt (St. Louis, MO). Corn oil (USP grade) was from Matheson, Coleman, and Bell (Cincinnati, OH). Pregnenolone-16α-carbonitrile was a gift from G. D. Searle (Skokie, IL). N,N-diethyl-4-methyl-3-oxo-4-aza-5α-androstane-17β-carboxamide (4-MA) is a gift from Merck Sharp & Dohme Research Labs (Rahway, NJ). Ultima-Gold, biodegradable scintillation fluid, was from Packard (Downers Grove, IL). Human

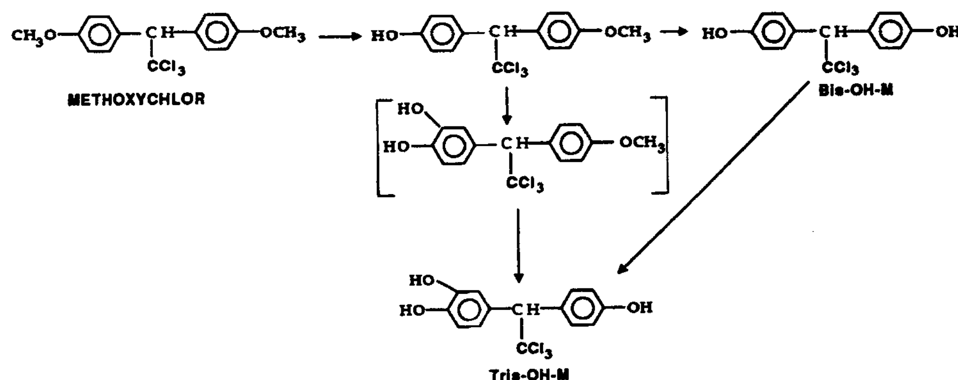

FIGURE 1. Proposed route of metabolism of methoxychlor by liver microsomes. The alternate pathway involving the initial ring hydroxylation of methoxychlor followed by sequential demethylation to tris-OH-M, is under investigation.

P450 3A4 constitutively expressed in h3A4v2 cell line (derived from human lymphoblastoid cell line) was purchased as a microsomal suspension (15 pmol P450 3A4/mg protein) from Gentest Co (Woburn, MA). All other chemicals were of reagent grade quality and were used without further purification.

Animals and Treatment

Male and female Sprague-Dawley CD rats (90–100 g) were purchased from Charles River Breeding Laboratories (Wilmington, MA) and were kept in a room with controlled temperature (22°C) and light (12-hr light/dark cycle; lights off at 7:00 p.m. EDT). Phenobarbital (PB) treatment (37.5 mg/kg, i.p. in 0.2 ml H_2O twice daily) was for 4 days. Liver microsomes were prepared 12 hr after the last PB injection. Pregnenolone-16α-carbonitrile (PCN) was injected (50 mg/kg, i.p. as a suspension in 0.4 ml corn oil daily for 3 days) and microsomes were prepared 24 hr after the last dose. Control animals from each treatment group received injections of the vehicle only. Segments of human livers were obtained from National Disease Research Interchange (Philadelphia, PA) and kept frozen at −70°C until use. Microsomes from rat or human livers were prepared as previously described (9). Rat microsomes usually represented a pool of 4–8 livers. Human microsomes were from individual livers. The microsomal pellet was suspended in 1.15% aqueous KCl solution, followed by centrifugation at 105,000 g for 1 hr. The supernatant was discarded and the microsomal pellet was covered with about 2 ml of 1.15% KCl and stored at −70°C until use.

Incubations

Microsomes were thawed, the KCl solution was discarded and the microsomes were resuspended in fresh 1.15% KCl. A modified Lowry procedure was used for determination of protein concentration (10,11). Incubations were performed in 20 ml glass scintillation vials containing the following constituents: 0.6 ml sodium phosphate buffer (pH 7.4, 60 μmol) containing EDTA (1 μmol), 0.1 ml $MgCl_2$ solution (10 μmol), microsomes (0.5 mg protein in 0.1 ml of 1.15% aqueous KCl), methoxychlor (40 or 120 nmol in 10 μl ethanol), [^{14}C]-testosterone (400,000 dpm, 120 nmol in 10 μl ethanol, unless otherwise stated), 4-MA (1 nmol in 5 μl ethanol, to inhibit 5α-steroid reductase (31)), NADPH regenerating system (glucose 6-phosphate, 10 μmol; NADPH, 0.5 μmol; glucose-6-phosphate dehydrogenase, 2 IU) added in 0.1 ml sodium phosphate buffer (pH 7.4, 10 μmol) and H_2O for a final volume of 1.0 ml.

Kinetic Analysis of Reversible Inhibition (Competitive and Noncompetitive)

After 2 min warm up at 37°C, the reaction was initiated with the NADPH regenerating system and the samples were incubated for 3 min (female rat) or 5 min (male rat). Reactions were terminated with 7.5 ml methylene chloride. The incubation mixtures were extracted with methylene chloride and subjected to thin layer chromatography TLC analysis and radioscanning (see below) and the testosterone metabolites were quantitated. The nature of the inhibition of testosterone hydroxylation by methoxychlor, was determined by Lineweaver-Burk, Eadie-Hofstee, and Dixon plots.

Time-Dependent Inhibition Studies

After a 2 min warm-up at 37°C of the incubation mixture lacking testosterone (with or without methoxychlor), NADPH regenerating system was either added or omitted and the preincubation was conducted for various time intervals. Subsequently, [^{14}C]-testosterone (120 μM, 250,000 dpm) was added in 10 μl ethanol, supplemented when needed with NADPH regenerating system, and the incubation continued for 10 min at 37° and terminated with 7.5 ml methylene chloride. Extraction and quantitation of testosterone metabolites is described next.

Analysis of Steroid Hydroxylation

The incubation mixtures were extracted twice with 7.5 ml methylene chloride and the organic phase was placed in 20 ml extraction tubes and evaporated to dryness at room temperature under a stream of nitrogen. The extract was dissolved in methylene chloride, transferred into 1 dram vials and evaporated to dryness, and the resulting residue was dissolved in 75 μl ethanol. An aliquot (10 μl) was taken for quantitation of radioactivity by scintillation counting and an aliquot containing approximately 120,000 dpm was subjected to TLC. To separate 15α-, 16α-, 7α-, 15β-, 6β-, and 2α-hydroxylated testosterone metabolites, a solvent system of methylene chloride:acetone (9:2.5) was used.

Chromatography was conducted for approximately 3 hr under a temperature gradient achieved by placing the chromatography tank in ice-cold

water with a water level being approximately at two-thirds of the height of the tank. This procedure maintained a higher temperature at the top of the tank, facilitating solvent evaporation at the top and permitting several solvent passes during the chromatography. Plates were thoroughly dried and scanned for radioactivity with a Bioscan 200 imaging scanner (Bioscan, Washington DC) and the various testosterone metabolites were quantitated. In the data presented in Table 1 and Figure 2, the chromatographic system was modified. TLC plates were first chromatographed in methylene chloride:acetone (6:1) in a closed tank at room temperature, to approximately three-fourths of the height of the plates. The tanks containing the plates were then placed in ice-cold water for about 8 min. Subsequently, the tanks were taken out of the cold water and the plates were chromatographed again to the same height as before. The plates were then allowed to dry for 30 min at room temperature and chromatographed in methylene chloride:acetone (3:1) at room temperature, allowing the solvent to reach about 1 cm below the top of the plate.

When the Bioscan imaging scanner autointegration mode was unable to integrate precisely the minor metabolites of proximate chromatographic mobilities,[2] the plates were subjected to autoradiography with Kodak imaging film X-Omat AR, for 15 days at 4°C, followed by densitometric analysis with LKB Ultra Scan XL Laser densitometer. Alternatively, the radioactive zones were quantitated by *manual* integration of the TLC plates with the Bioscan System 200 imaging scanner. There was linearity and direct proportionality of product formation with time (correlation coefficient (r) > 0.99). However, there was linearity (r > 0.99) but not direct proportionality of product formation with respect to protein concentration. Therefore, the data is presented as nmol product/min at the specified protein concentration, which was usually 0.5 mg.

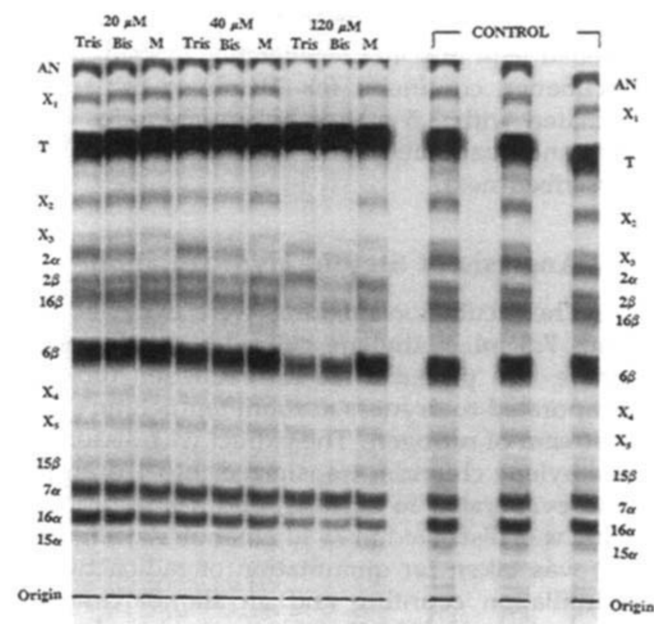

FIGURE 2. Autoradiogram of a TLC plate from incubations of [4-^{14}C]-testosterone (120 μM, 400,000 dpm) with live microsomes from *PB*-treated male rats (0.5 mg protein) for 10 min; in the absence or presence of methoxychlor, bis-OH-M or tris-OH-M at 20, 40, and 120 μM. As described for coincubation of methoxychlor with testosterone in Materials and Methods. AN = androstenedione, T = testosterone, X_1–X_5 = unidentified metabolites (X_1 appears to be 5α-dihydrotestosterone); the rest are hydroxylated metabolites at the indicated carbon sites.

Assay of Estradiol-2-Hydroxylase

Incubations were essentially as previously described, except that the substrate was 2-[^{3}H]-estradiol and 4-MA was deleted. The mode of termination of the reaction and the determination of the rate of hydroxylation of E_2 from the ^3H_2O generated during the hydroxylation were as previously described (12).

Assay of Inhibition of the Irreversible Binding of Methoxychlor to Microsomal Proteins

[^{14}C]-methoxychlor was incubated with PB-microsomes in the presence of NADPH and in the presence of radioinert testosterone or estradiol. Controls lacked testosterone and estradiol. The binding of methoxychlor equivalents was determined from the radioactivity irreversibly (apparently covalently) bound to the microsomal proteins. Briefly, the labeled microsomal proteins were precipitated with ethanol and collected on a glass fiber filter, the filter exhaustively washed with various organic solvents, and the protein-bound radioactivity was determined as previously described (6,7).

RESULTS

Testosterone Hydroxylation by Rat Liver Microsomes

Reversible Inhibition

Methoxychlor and [^{14}C]-testosterone (T) were coincubated with liver microsomes from PB-treated

[2]Because of low levels and occasional difficulties in the TLC separation of the 15α-, 15β-, 2β-, and 16β-hydroxytestosterone derivatives, only approximate values were obtained for these compounds with the Bioscan monitor. However, after autoradiography and densitometry, we were able to accurately assay these compounds.

TABLE 1. Inhibition of Testosterone Hydroxylation by Methoxychlor (M), Bis-OH-Methoxychlor (Bis-OH) and Tris-OH-Methoxychlor (Tris-OH) in PB-Male Rat

Addition		Testosterone Hydroxylation (nmol/min)[a]								
(μM)		15α	16α	7α	15β	6β	16β	2β	2α	AN
None (control)		0.07 ± 0.00	0.60 ± 0.02	0.26 ± 0.01	0.11 ± 0.01	1.84 ± 0.08	0.33 ± 0.02	0.20 ± 0.01	0.27 ± 0.02	0.59 ± 0.06
M	(1)	0.06 ± 0.01(86)	0.54 ± 0.02(90)[b]	0.25 ± 0.01(96)	0.10 ± 0.01(91)	1.84 ± 0.07(100)	0.29 ± 0.03(88)	0.19 ± 0.02(95)	0.21 ± 0.03(78)[b]	0.59 ± 0.04(100)
	(5)	0.05 ± 0.01(71)[b]	0.39 ± 0.02(65)[d]	0.23 ± 0.01(88)[b]	0.09 ± 0.01(82)	1.69 ± 0.03(92)[b]	0.25 ± 0.02(76)[c]	0.18 ± 0.01(90)	0.13 ± 0.02(48)[c]	0.61 ± 0.03(103)
	(10)	0.05 ± 0.01(71)[b]	0.31 ± 0.01(52)[d]	0.23 ± 0.01(88)[b]	0.07 ± 0.01(64)[c]	1.64 ± 0.06(89)[b]	0.23 ± 0.02(70)[c]	0.18 ± 0.01(90)	0.10 ± 0.00(37)[d]	0.57 ± 0.04(97)
None (control)		0.09 ± 0.01	0.72 ± 0.03	0.42 ± 0.01	0.11 ± 0.01	1.70 ± 0.02	0.32 ± 0.01	0.19 ± 0.01	0.30 ± 0.05	0.51 ± 0.01
M	(20)	0.05 ± 0.00(56)[c]	0.32 ± 0.01(44)[d]	0.33 ± 0.02(79)[c]	0.06 ± 0.01(55)[c]	1.47 ± 0.04(86)[d]	0.23 ± 0.01(72)[d]	0.14 ± 0.00(74)[c]	0.05 ± 0.00(17)[d]	0.33 ± 0.00(65)[d]
	(40)	0.05 ± 0.01(56)[c]	0.24 ± 0.01(33)[d]	0.33 ± 0.01(79)[d]	0.06 ± 0.01(55)[c]	1.45 ± 0.03(85)[d]	0.21 ± 0.01(66)[d]	0.14 ± 0.01(74)[c]	0.05 ± 0.01(17)[c]	0.28 ± 0.01(55)[d]
	(120)	0.04 ± 0.00(44)[c]	0.16 ± 0.02(22)[d]	0.31 ± 0.02(74)[c]	0.05 ± 0.00(45)[d]	1.31 ± 0.05(77)[d]	0.15 ± 0.03(47)[d]	0.13 ± 0.01(68)[c]	0.03 ± 0.01(10)[d]	0.21 ± 0.02(41)[d]
Bis-OH	(20)	0.06 ± 0.01(67)[b]	0.43 ± 0.01(60)[d]	0.38 ± 0.01(90)[c]	0.05 ± 0.00(45)[d]	1.29 ± 0.02(76)[d]	0.26 ± 0.02(81)[c]	0.15 ± 0.01(79)[c]	0.11 ± 0.01(37)[c]	0.38 ± 0.00(75)[d]
	(40)	0.04 ± 0.01(44)[c]	0.27 ± 0.01(38)[d]	0.35 ± 0.01(83)[c]	0.04 ± 0.00(36)[d]	0.94 ± 0.03(55)[d]	0.16 ± 0.01(50)[d]	0.11 ± 0.01(58)[d]	0.09 ± 0.01(30)[c]	0.31 ± 0.01(61)[d]
	(120)	0.02 ± 0.00(22)[d]	0.08 ± 0.00(11)[d]	0.29 ± 0.00(69)[d]	0.02 ± 0.00(18)[d]	0.42 ± 0.02(25)[d]	0.09 ± 0.01(28)[d]	0.05 ± 0.00(26)[d]	0.03 ± 0.01(10)[d]	0.11 ± 0.00(22)[d]
Tris-OH	(20)	0.06 ± 0.01(67)[b]	0.51 ± 0.02(71)[d]	0.38 ± 0.02(90)[b]	0.04 ± 0.00(36)[d]	1.21 ± 0.05(71)[d]	0.26 ± 0.01(81)[c]	0.14 ± 0.02(74)[b]	0.20 ± 0.02(67)[b]	0.41 ± 0.01(80)[d]
	(40)	0.04 ± 0.00(44)[c]	0.38 ± 0.01(53)[d]	0.34 ± 0.01(81)[d]	0.04 ± 0.01(36)[c]	0.93 ± 0.03(55)[d]	0.21 ± 0.01(66)[d]	0.12 ± 0.02(63)[c]	0.18 ± 0.01(60)[b]	0.34 ± 0.01(67)[d]
	(120)	0.02 ± 0.00(22)[d]	0.11 ± 0.01(15)[d]	0.28 ± 0.02(67)[d]	0.02 ± 0.00(18)[d]	0.34 ± 0.03(20)[d]	0.10 ± 0.02(31)[d]	0.12 ± 0.01(63)[c]	0.06 ± 0.00(20)[c]	0.12 ± 0.01(24)[d]

Incubations containing methoxychlor, Bis-OH or Tris-OH and ^{14}C-testosterone (120 μM) were conducted for 10 min with 0.5 mg PB-microsomes.
[a]Average of triplicate incubations ± S.D. Values in parentheses are percent of control.
[b]$P < 0.05$.
[c]$p < 0.01$.
[d]$p < 0.001$.
AN = Δ^4-androstene-3,17-dione.

male rat (male PB-microsomes) and NADPH at 37° for 10 min, and the inhibition of hydroxylation at various sites of the testosterone molecule was determined (Figure 2, Table 1). Inhibition was most pronounced toward the 2α-, 16α-, and 15α-hydroxylations, indicating that methoxychlor strongly inhibited the constitutive male-specific P450 2C11 and 2A2.[3] Less pronounced inhibition of the 16β- and 7α-hydroxylation, apparently catalyzed by 2B1/2B2 and 2A1, respectively, was also observed. The relatively weak inhibition of 2β-, 6β-, and 15β-hydroxylations by methoxychlor is attributed to low inhibition of P450 3A1/A2. The pronounced differences in the potency of inhibition at the various sites of hydroxylation indicate a great variability in sensitivity of P450 enzymes to methoxychlor. Inhibition of testosterone hydroxylation by the major methoxychlor metabolites (bis-OH-M and tris-OH-M) was also observed (Figure 2, Table 1). Similar to methoxychlor, bis-OH-M exhibited strong inhibition of 2α- and 16α-hydroxylation. Formation of androstenedione was moderately inhibited by methoxychlor and by its metabolites. Several unidentified compounds (X_1, X_2, and X_3) were observed and their accumulation was strongly inhibited by bis-OH-M and tris-OH-M (Figure 2). Based on chromatographic mobility and inhibition by 4-MA, X_1 appears to be 5α-dihydrotestosterone. X_1 represents approximately 3% of the amount usually formed in incubations conducted in the absence of 4-MA.

Because 6β- and 16α-hydroxylations represent major pathways of testosterone hydroxylation in the PB-microsomes, the characteristics of inhibition of these reactions were examined by the Lineweaver-Burk, Eadie-Hofstee, and Dixon plots. Results indicate the following (1) Methoxychlor competitively inhibited 6β-hydroxylation in male and female PB-microsomes (Figure 3A, 3B, Table 2), exhibiting Ki = 89 μM (PB-male) and 66 μM (PB-female). Similarly, bis-OH-M competitively inhibited the 6β-hydroxylation; Ki = 21 μM (PB-male) and 19 μM (PB-female). However, the tris-OH-M inhibition was *noncompetitive* (Figure 3B, Table 2), Ki = 29 μM (PB-male) and 32 μM (PB-female). (2) Methoxychlor *inhibited* 16α-hydroxylation competitively (Figure 4, Table 2); Ki = 9 and 17 μM, in PB-male (Figure, not shown) and female, respectively. The inhibition of 16α-hydroxylations by

[3]P450 enzymes in rat liver microsomes catalyze the following testosterone hydroxylations. 2C11(P450h): 2α, 16α; 3A1/2: 2β, 6β, 15β, and Δ^6-T; 2A1(P450a): 7α and Δ^6-T; 2B1/B2(P450 b/e): 16α, 16β, androstenedione; 2A2(RLM2): 15α, 12α (15–21); in parentheses are P450s according to Levin's nomenclature (15), except for RLM2 (18).

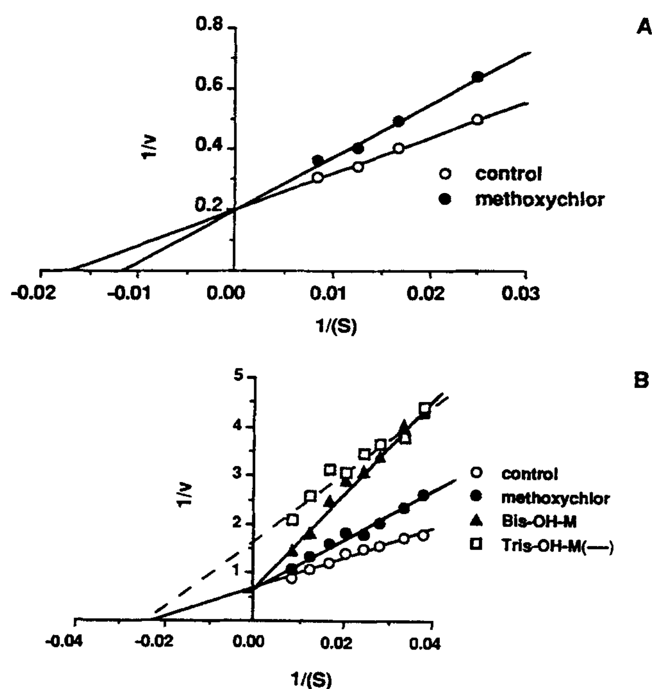

FIGURE 3A. Lineweaver-Burk plot of the inhibition of 6β-hydroxylation of testosterone by 40 μM methoxychlor in liver microsomes from PB-treated male rats. (S) = substrate concentration (μM), v = nmol product/min. Incubations were conducted with 0.5 mg microsomal protein for 5 min. **3B.** Lineweaver-Burk plot of the inhibition of 6β-hydroxylation of testosterone by 40 μM methoxychlor, bis-OH-M, and tris-OH-M in liver microsomes from *PB*-treated *female* rats. (S) = μM, v = nmol product/min. Incubations were conducted with 0.5 mg microsomal protein for 3 min.

bis-OH-M, in PB-male and PB-female, was competitive (Ki = 11 and 13 μM, respectively) and by tris-OH-M was noncompetitive (Figure 4) with Ki = 18 and 21 μM, respectively.

Time-Dependent Inhibition

When methoxychlor was permitted to be metabolized by male PB-microsomes prior to the addition of testosterone, we observed a pronounced time-dependent inhibition of 6β-hydroxylation of testosterone (Figure 5A, B).[4] The inhibition was not due to depletion of NADPH during the preincubation period, because the supplemental addition of NADPH prior to testosterone did not obviate the inhibition (not shown). We examined the possibility that the time-dependent inhibition was

[4]Preincubation of PB-microsomes with NADPH, prior to addition of testosterone, consistently diminished 6β-hydroxylation and elevated 7α-hydroxylation, indicating an NADPH-mediated inactivation of P450 3A and activation of 2A1, respectively.

TABLE 2. Characteristics of Inhibition of Testosterone 6β- and 16α-Hydroxylation by Methoxychlor (M) and by Its Demethylated Metabolites

Source of liver Microsomes	Inhibitor	*Ki (μM)*[a] *Hydroxylation* 6β	16α
PB-male rat	Methoxychlor	88.8(c)[b]	9.2(c)
	Bis-OH-M	21.0(c)	10.5(c)
	Tris-OH-M	28.7(n)	17.8(n)
PB-female rat	Methoxychlor	65.7(c)	16.6(c)
	Bis-OH-M	18.5(c)	13.2(c)
	Tris-OH-M	31.7(n)	20.7(n)
Human-male	Methoxychlor	60.0(c)	
	Bis-OH-M	30.6(c)	
	Tris-OH-M	28.2(n)	
Cell line h3A4v2	Methoxychlor	74.8(c)	

[a]Km(μM) for 6β-OH = 59 (male PB rat), 44 (female PB rat), 119 (human) and 752 (cell line); Km(μM) for 16α = 124 (male PB rat), 102 (female PB rat).
[b]*c* = competitive inhibition; *n* = noncompetitive inhibition.

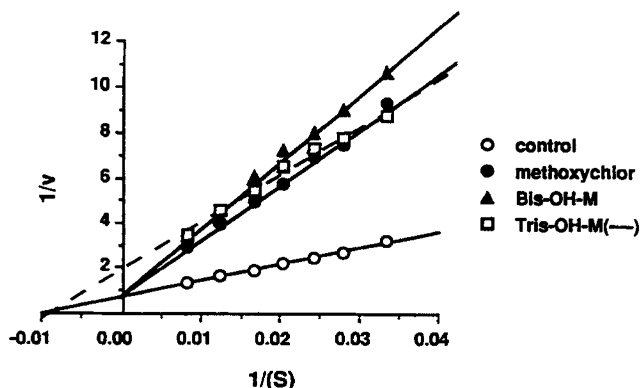

FIGURE 4. Lineweaver-Burk plot of the inhibition of 16α-hydroxylation of testosterone by 40 μM methoxychlor, bis-OH-M, and tris-OH-M in liver microsomes from *PB*-treated *female* rats. (S) = μM, v = nmol product/min. Incubations were conducted with 0.5 mg microsomal protein for 3 min.

elicited by methoxychlor metabolites formed during preincubation. A mixture of methoxychlor (12.6 μM), bis-OH-M (10.5 μM), and tris-OH-M (7.7 μM) formed in incubations of 40 μM methoxychlor with PB-microsomes inhibited 6β-hydroxylation merely by 14% more than methoxychlor alone. By contrast, the time-dependent inhibition of 6β-OH-T by methoxychlor after 30 min was pronounced, being approximately 39% of control values versus 91% in incubations of methoxychlor (minus NADPH) (Figure 5A, B). Additionally, 40μM bis-OH-M or tris-OH-M, after 30 or 60 min, did not exhibit a significant time-dependent inhibition of 6β-hydroxylation (not shown). These findings indicate that though methoxychlor is only a weak reversible inhibitor of 6β-hydroxylation, its metabolism produces a time-dependent inhibition of the P4503A1/3A2 isozymes and the inhibition is largely not caused by the formation of bis-OH-M and tris-OH-M.

Methoxychlor did not elicit time-dependent inhibition of the 16α- and 7α-hydroxylations (not shown), indicating that methoxychlor does not inactivate P450 2B1/2, 2C11 and 2A1.

Young female rats (*ca.* 40 days old) exhibited 0.04 nmol 6β-OH-T/min/0.5 mg protein, indicating the presence of low level of P450 3A2 (13,14). PCN treatment increased that enzymatic activity by approximately thirtyfold (not depicted), apparently caused by induction of 3A1 (17). In these PCN-microsomes, methoxychlor inhibited in a time-dependent manner the 6β-hydroxylation of testosterone, indicating inhibition of P450 3A1 (Figure 5C, D). By contrast, bis-OH-M and tris-OH-M did not elicit a time-dependent inhibition of 6β-hydroxylation (not shown), though both compounds are reversible inhibitors of P450 3A1 in female rats. In the PCN-female rat, as in the PB-male, there was no time-dependent inhibition of the 7α- and 16α-hydroxylases (not shown). Because PCN-microsomes produce only minimal amounts of tris-OH-M (S. Dehal and D. Kupfer, unpubl.), this finding indicates that the time-dependent inhibition of P450 3A does not proceed via the formation of tris-OH-M but occurs through another pathway, possibly involving the generation of a reactive intermediate.

Testosterone effectively inhibits the irreversible binding of metabolically activated methoxychlor to PB-microsomal proteins (Figure 6).[5] The

[5]Irreversible binding (most probably covalent) of metabolically activated [^{14}C]-methoxychlor refers to the radioactiv-

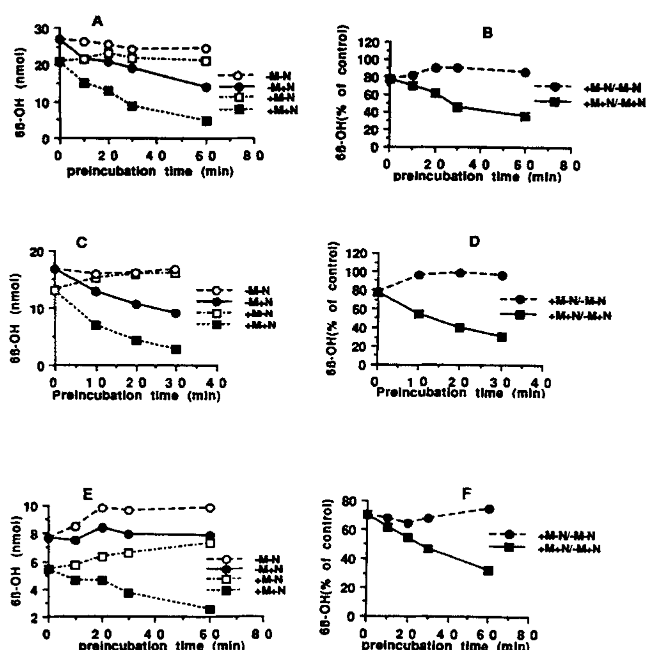

FIGURE 5. Time-dependent inhibition by methoxychlor of 6β-hydroxylation of testosterone. Preincubations were conducted with methoxychlor (40 μM), in the presence or absence of NADPH with 0.5 mg liver microsomes from PB-treated male rats (A, B), PCN-treated female rats (C, D), Human (E, F) for various time intervals. Subsequently, [^{14}-C]-testosterone (120 μM; 250,000 dpm) was added, supplemented with NADPH, and incubated for an additional 10 min. *Notations* (preincubation): −M−N = minus methoxychlor and minus NADPH, −M+N = minus methoxychlor plus NADPH, +M−N = plus methoxychlor minus NADPH, +M+N = plus methoxychlor and NADPH. A, C, E. = nmol 6β-OH-T formed; B, D, F (6β-OH-T expressed as % of control) obtained from the ratios of +M−N/−M−N and of +M+N/−M+N.

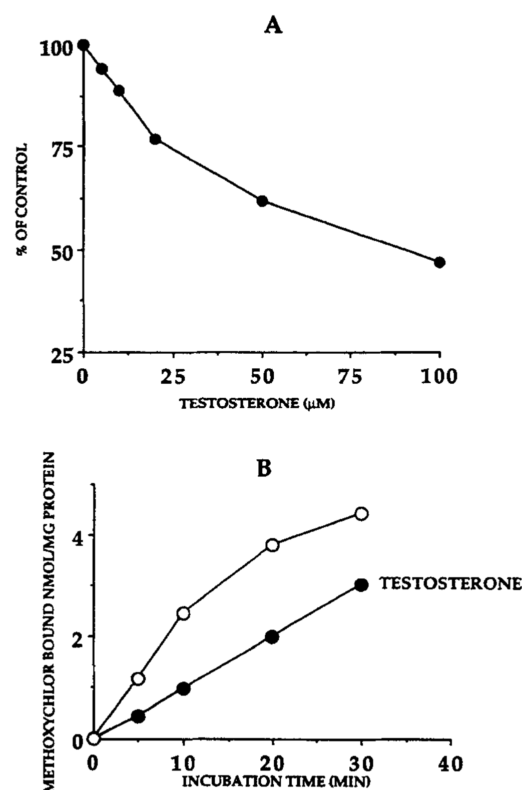

FIGURE 6. Inhibition by testosterone of the irreversible binding of methoxychlor to liver microsomal proteins from PB-treated *male* rats. Effect of various concentrations of testosterone (A). Time course of inhibition: testosterone (120 μM) and ^{14}C-methoxychlor (20 μM) were coincubated with 0.8 mg liver microsomes. Filled dots represent incubations with testosterone (B).

inhibition of the formation of the reactive intermediate of methoxychlor (M*), may explain the reason for the relatively "clean" competitive inhibition of testosterone hydroxylation by methoxychlor when co-incubated with testosterone for 5 minutes.

Glutathione (GSH), at 5 mM, effectively interferes with the irreversible binding of methoxychlor to PB-microsomal proteins (6). The observation that the coincubation of 5 mM GSH with methoxychlor, partially overcomes the time-dependent inhibition of the 6β-hydroxylation of testosterone (Figure 7) supports our presumption that the inactivation of P450 3A1/2 is related to the formation of M*.

ity associated with the microsomal proteins after numerous washings with organic solvents and which remain bound to proteins on SDS-PAGE.

Estradiol 2-Hydroxylation in Rat Liver

Reversible Inhibition

Coincubation of methoxychlor or of bis-OH-M with E_2, markedly inhibited its 2-hydroxylation by PB-male microsomes (Figure 8). Bis-OH-M was less inhibitory than methoxychlor. However, whereas the inhibition by methoxychlor levelled off at 10 μM methoxychlor, the inhibition by bis-OH-M continued to increase up to 100 μM, possibly caused by the higher solubility of bis-OH-M.

Time-Dependent Inhibition

Methoxychlor inhibits the 2-hydroxylation of E_2 in a time-dependent manner (Figure 9). The possibility that the time-dependent inhibition reflected the depletion of NADPH during preincubation with methoxychlor was ruled out, because the addition of supplementary amounts of NADPH after the preincubation period did not diminish the

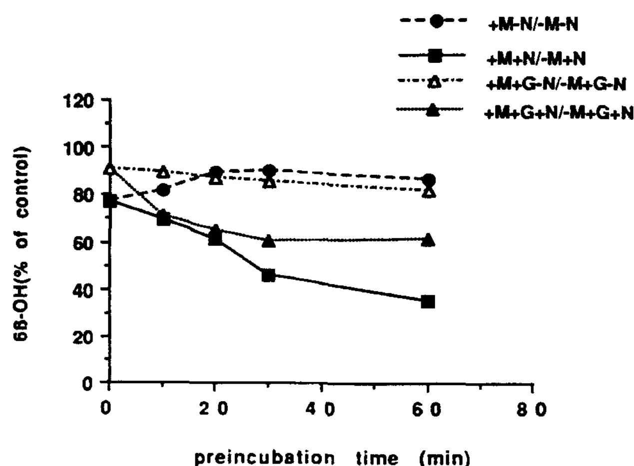

FIGURE 7. Diminution by GSH of the time-dependent inhibition of 6β-hydroxylase by methoxychlor. Preincubation was conducted for various time intervals with 0.5 mg PB-male rat liver microsomes and 40 μM methoxychlor, in the presence or absence of GSH (5 mM), and plus or minus NADPH, at 37°. Subsequently, [^{14}C]-testosterone (120 μM) and a supplement of NADPH was added and the incubation was continued for 10 min. The formation of 6β-OH-T was monitored as described in Materials and Methods. *Notations* (preincubation): +M = contained methoxychlor, −M = no methoxychlor, +N = contained NADPH, −N = no NADPH, +G = contained GSH, −G = no GSH. On ordinate: 6β-OH-T (expressed as % of corresponding control) obtained from the ratios of +M−N/−M−N, +M+N/−M+N, +M+G−N/−M+G−N and +M+G+N/−M+G+N.

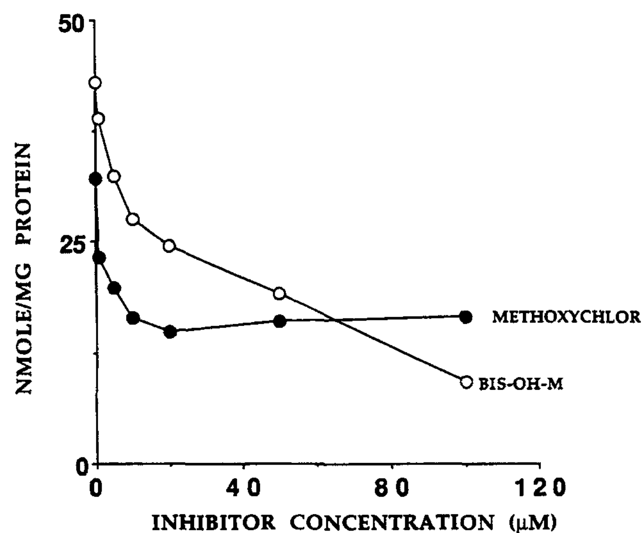

FIGURE 8. Inhibition of 2-hydroxylation of estradiol by various concentrations of methoxychlor and bis-OH-M. Incubations were conducted with E_2 (100 μM; 200,000 dpm) with various concentrations of methoxychlor or bis-OH-M and *male* PB-microsomes (0.2 mg protein) for 20 min.

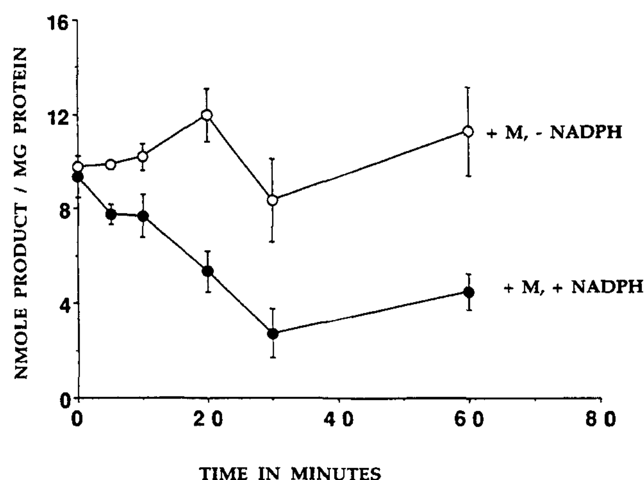

FIGURE 9. Time-dependent inhibition by methoxychlor of 2-hydroxylation of estradiol. Preincubations were conducted with methoxychlor (120 μM), in the presence or absence of NADPH with 0.2 mg liver microsomes from PB-treated male rats, for various time intervals. Subsequently, 2-[^{3}H]-E_2 (100 μM, 200,000 dpm) was added, supplemented with NADPH, and incubated for an additional 20 min. Product (2-OH-E_2) was quantitated as described in Materials and Methods.

inhibition (not shown). The inhibition was not due to the formation of the major methoxychlor metabolite, because bis-OH-M exhibits a lower inhibition of 2-hydroxylation than methoxychlor. Furthermore, a 5:1 or 20:1 dilution of the microsomal preparation after the preincubation with methoxychlor in the presence of NADPH, followed by the addition of [^{3}H]-estradiol, did not obviate the inhibition (not shown), indicating that the inhibition was not caused by the time-dependent formation of methoxychlor metabolites with stronger inhibitory potency than methoxychlor. By contrast, a similar dilution experiment with bis-OH-M (100 μM) did not exhibit a time-dependent inhibition, providing additional evidence that bis-OH-M is not involved in the inactivation of the E_2-hydroxylase by methoxychlor. These findings indicated that methoxychlor inactivates the enzyme catalyzing the 2-hydroxylation of estradiol.

Testosterone 6β-Hydroxylation in Human Liver

We addressed the question of whether methoxychlor inhibits reversibly or irreversibly testosterone hydroxylation in the human. To identify an active source of testosterone 6β-hydroxylase, we examined liver microsomes from several human donors. Liver microsomes from a single subject (28-

year-old male) were selected and used in the subsequent studies.

Reversible Inhibition

Methoxychlor or bis-OH-M, co-incubated with testosterone, inhibited competitively the formation of 6β-OH-T (Figure 10). By contrast, tris-OH-M, exhibited noncompetitive inhibition. To obtain support that the methoxychlor-mediated inhibition of the 6β-hydroxylase in human liver was caused by inhibition of the P450 3A4 isozyme, we carried out a similar experiment with microsomes from the h3A4v2 cell line, containing solely human P450 3A4. This preparation exhibited active, albeit low, testosterone 6β-hydroxylation (87 pmol/min/mg protein) as compared with rat PB microsomes (3,400 pmol/min/mg protein). Indeed, in microsomes from these cells, methoxychlor inhibited the 3A4 isozyme and the inhibition was strictly competitive, evidenced from both Lineweaver-Burk and Eadie-Hofstee plots (Figure 11).

Time-Dependent Inhibition

When methoxychlor was metabolized by human liver microsomes similarly to the process for the rat, prior to the addition of testosterone, a time-dependent inhibition of the 6β-hydroxylase was observed (Figure 5E, F).

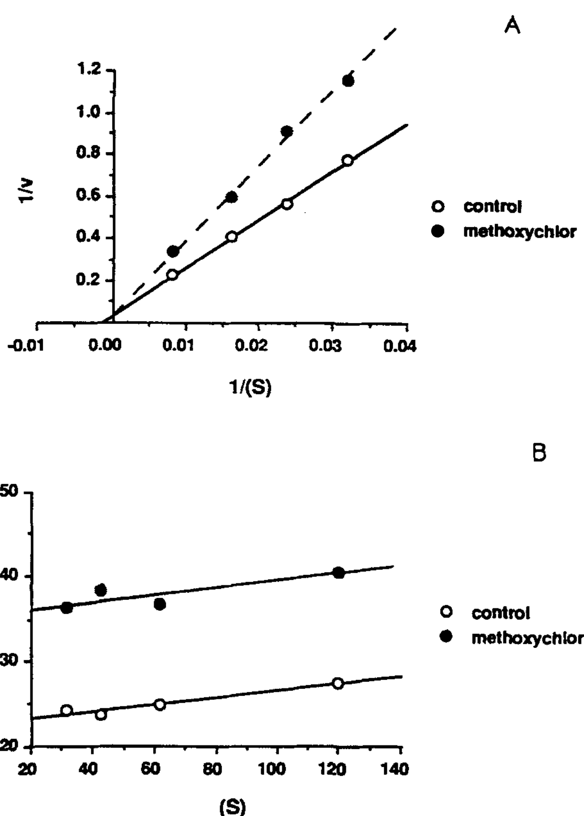

FIGURE 11. Inhibition of 6β-hydroxylation of testosterone by 40 μM methoxychlor in microsomes from cell line h3A4v2, containing human P450 3A4. Incubations were with 0.5 mg microsomal protein and conducted for 20 min. Lineweaver-Burk plot, (S) = μM, v = nmol product/min (A). Eadie-Hofstee plot; (S) = μM, v = nmol product/min (B).

DISCUSSION

To determine which P450 enzymes are inhibited by methoxychlor and its major metabolites, we used the regioselectivity and stereospecificity of hydroxylation of testosterone by liver microsomes from rats and human as markers for the P450 enzymes catalyzing those reactions. Additionally, we examined the inhibition of 2-hydroxylation of estradiol (E_2) by methoxychlor. Methoxychlor inhibited reversibly essentially all of the P450s catalyzing testosterone hydroxylation. This was not surprising, because methoxychlor is a good substrate for constitutive and PB induced P450s and probably serves as an alternate substrate (S. Dehal and D. Kupfer, unpublished). However, a pronounced time-dependent inhibition of P450 3A isozymes in rat and human liver by methoxychlor was also observed.

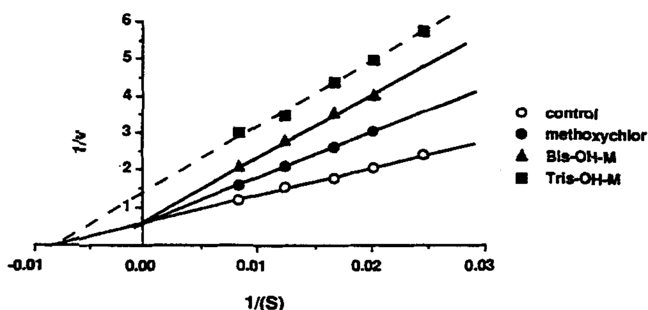

FIGURE 10. Lineweaver-Burk plot of the inhibition of 6β-hydroxylation of testosterone by 40 μM of methoxychlor, bis-OH-M or tris-OH-M in microsomes from *human* liver. (S) = μM, v = nmol product/min. Incubations were with 0.5 mg microsomal protein and were conducted for 6 min.

The reversible inhibition of testosterone 6β-hydroxylation was demonstrated to be strictly competitive with methoxychlor and its prime demethylated metabolite, the bis-OH-M; however, a noncompetitive inhibition was elicited by the catechol metabolite, tris-OH-M. A portion of the irreversible binding of tris-OH-M to liver microsomal protein was found to be NADPH-independent (S. Dehal and D. Kupfer, unpublished), suggest-

ing the possibility that tris-OH-M may be causing some inactivation of the P450 3A isozymes. The time-dependent inhibition appears to be elicited through the generation of the reactive intermediate of methoxychlor (M*). This presumption is supported by our finding that methoxychlor forms an irreversible adduct with protein(s), which on SDS-PAGE exhibit an Mr in the P450 region. Furthermore, GSH inhibits the formation of the M*-protein adducts (6–8) and GSH partially alleviated the methoxychlor-mediated time-dependent inhibition of the 6β-hydroxylase. The finding that testosterone strongly inhibits the formation of M*-protein adducts, may provide a rational for the dilemma of why coincubation of methoxychlor with testosterone exhibits strict competitive inhibition.

The reversible inhibition of 2-hydroxylation of E_2 by methoxychlor and bis-OH-M is of particular interest. Methoxychlor is a proestrogen with bis-OH-M being its most potent estrogenic metabolite (3,4). In turn, the hydroxylations of either bis-OH-M or E_2 result in their respective catechol metabolites. However, whereas formation of the catechol methoxychlor (tris-OH-M) is catalyzed almost solely by PB-induced P4502B1/B2 (S. Dehal and D. Kupfer, unpublished), catechol estradiol formation is catalyzed by P450 2C11, 1A2 and 3A (19,22). Indeed, support for the possible involvement of P450s other than 1A2 in E_2-2-hydroxylation by PB-microsomes, stems from our observation that titration of the microsomes with antibodies against 1A2 inhibited the 2-hydroxylation by only 30% (not shown). Of additional interest is our observation that similarly to testosterone, E_2 strongly inhibits the irreversible binding of methoxychlor to microsomal proteins (data not presented). This action of E_2 may be the cause for the protection of the E_2-hydroxylase during the coincubation of methoxychlor and E_2 with liver microsomes.

We also observed time-dependent inhibition of estradiol 2-hydroxylation by methoxychlor in rat liver. Hence the inhibition of estradiol hydroxylation by methoxychlor in rat liver may have involved the inactivation of P450 1A2 and/or 3A1/3A2. The time-dependent inactivation appears to be irreversible, because the dilution of the incubation mixture, from the preincubation of methoxychlor with PB-microsomes and NADPH, did not obviate the inhibition. Nevertheless, we did not rule out the possibility that the inhibition of the 2-hydroxylation of E_2 was caused by the formation of a hitherto uncharacterized methoxychlor metabolite possessing unusually high lipid solubility, because such a compound would not be expected to diffuse readily from the microsomal membrane. Of interest is our observation that though the irreversible binding of methoxychlor to proteins is catalyzed primarily by P450 2B1, there was no time-dependent inhibition of that isozyme but instead an apparent inactivation of the P450 3A isozyme(s). This suggests that the reactive intermediate of methoxychlor is stable long enough to be able to diffuse from its site of formation and react with another protein. Whether the protein adduct—previously observed by us in PAGE analysis—is indeed composed of M* and P450 requires further study.

In humans and guinea pigs, cortisol is the major glucocorticoid. Both species form 6β-hydrocortisol, which in the human is apparently formed by the P450 3A catalyzed 6β-hydroxylation of cortisol (23,24). Catechol estrogen metabolites are formed in humans via the 2- and 4-hydroxylation of estrogens (25,26). It has been suggested that catechol estrogens are involved in the induction of hormonal carcinogenesis (27,28). However, an opposing view has also been presented: that the formation of catechol estrogens renders estrogens innocuous, but 16α-hydroxylation of estradiol/estrone generates hormonal carcinogens (29,30) that are possibly involved in mammary and endometrial cancer. Hence, the inhibitory effects by methoxychlor of the steroidal 6β-hydroxylase and of the estradiol 2-hydroxylase may have physiological and pharmacological ramifications. Future studies will explore the effects of methoxychlor on the *in vivo* pathways of cortisol and estradiol metabolism.

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