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CHARACTERISTICS OF THE ACTIVE OXYGEN IN COVALENT BINDING OF THE PESTICIDE METHOXYCHLOR TO HEPATIC MICROSOMAL PROTEINS

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Abstract—This study examined the characteristics of the active oxygen species involved in generation of the reactive intermediate of methoxychlor which covalently binds to liver microsomal proteins. The possibility that the active oxygen participating in the above reaction is the superoxide anion (O_2^-) or a species generated from O_2^- was examined with the help of superoxide dismutase (SOD) and with an SOD-mimetic agent, CuDIPS [Cu^{2+}(3,5-diisopropylsalicyclic acid)$_2$]. It was observed that, whereas CuDIPS inhibited covalent binding of methoxychlor metabolite(s), SOD did not. However, ZnDIPS [Zn^{2+}(3,5-diisopropylsalicyclic acid)$_2$], which exhibits *no* SOD-mimetic activity, did not inhibit covalent binding. Furthermore, both CuDIPS and ZnDIPS had little or no effect on the formation of demethylated (polar) metabolites of methoxychlor, demonstrating that the inhibition of covalent binding by CuDIPS was not merely due to a general inhibition of the hepatic monooxygenase system. These findings suggested that O_2^- was involved in covalent binding, but was not accessible to SOD. Additional support for O_2^- involvement stems from the observation that α-tocopheryl acid succinate markedly inhibited covalent binding of methoxychlor. The possibility that hydrogen peroxide (H_2O_2) was involved in covalent binding of methoxychlor appears unlikely. Catalase had no effect on covalent binding when NADPH was the cofactor, and the use of H_2O_2 in place of NADPH did not yield covalent binding. Certain scavengers of hydroxyl radical (ethanol, *t*-butanol and benzoate) inhibited, and other known scavengers (DMSO and mannitol) did not inhibit, covalent binding. EDTA stimulated binding, desferal (desferrioxamine) exhibited no effect on binding, and diethylenetriaminepentaacetic acid (DETAPAC) inhibited binding. A possible explanation for this observation is that the Fe^{2+} needed for generation of ·OH is much more easily obtained from Fe^{3+}-EDTA than from Fe^{3+}-desferal, which resists reduction. The inhibitory effect by DETAPAC may be due to chelation of another metal which is needed for the reaction. Lastly, certain scavengers of singlet oxygen inhibited covalent binding with little effect on the formation of polar metabolites of methoxychlor. In conclusion, these studies support the involvement of ·OH and singlet oxygen, possibly derived from O_2^-, in the formation of the reactive methoxychlor intermediate. However, the less likely possibility that these compounds, used to probe for active oxygen, merely inhibit the activity of a cytochrome P-450 which specifically catalyzes the formation of the reactive methoxychlor intermediate but not of the demethylation of methoxychlor cannot be ruled out.

Methoxychlor† is used widely as an insecticide to substitute for DDT, whose use was banned primarily because of its extensive persistence in the environment. The rapid biodegradability of methoxychlor and its low overt toxicity (high LD_{50}) in mammals [1–3] have contributed significantly to its increased usage. However, these apparently beneficial characteristics of methoxychlor resulting from its rapid metabolism may also yield certain long-range toxic manifestations. For instance, methoxychlor is metabolized by the hepatic cytochrome P-450 monooxygenase system into relatively potent estrogens, which could be toxic particularly at the neonate stage of development [4–6]. Also, potential toxicity of methoxychlor may arise from the metabolic "activation" of methoxychlor and its covalent binding to macromolecules [7]. With respect to the latter, our studies demonstrated that the metabolism of [^{14}C]methoxychlor by rat liver microsomes, fortified by NADPH, yields a reactive intermediate which covalently binds to microsomal proteins [7].

Based on indirect evidence, the covalent binding of methoxychlor to liver microsomal proteins appears to be catalyzed by the microsomal cytochrome P-450 monooxygenases [7]. This binding was found to be dramatically stimulated by phenobarbital treatment of rats and to require an oxygen atmosphere and NADPH. Incidentally, there was no significant binding to microsomal RNA (W. H. Bulger and D. Kupfer, unpublished observations). The lack of stimulatory effect on the covalent binding of methoxychlor to proteins by 1,1,1-trichloropropane-2,3-oxide, an epoxide hydrase inhibitor [8], and the marked inhibition of the covalent binding by low concentrations of various antioxidants [7], suggested that the methoxychlor reactive intermediate is not an epoxide but is probably a free radical or is derived from a free radical intermediate.

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† Abbreviations and common names: Methoxychlor, 1, 1, 1 - trichloro - 2, 2 - bis(*p* - methoxyphenyl) - ethane; CuDIPS, Cu^{2+}(3,5-diisopropylsalicylic acid)$_2$; ZnDIPS, Zn^{2+}(3,5-diisopropylsalicylic acid)$_2$; DMSO, dimethyl sulfoxide; desferal, desferrioxamine; DETAPAC, diethylenetriaminepentaacetic acid; and HPLC, high performance liquid chromatography.

The question concerning the nature of the active oxygen species in enzyme-mediated generation of reactive intermediates from a variety of compounds has been the subject of numerous studies. In those studies, the involvement of various active oxygen species, such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) and hydroxyl radical ($\cdot OH$), has been proposed [9–11].

The finding that O_2^-, generated in aprotic medium, metabolizes *p,p'*-DDT and methoxychlor by dechlorination [12, 13] suggested the possibility that the cytochrome P-450-mediated covalent binding of methoxychlor also involves the participation of O_2^-. Also, since the microsomal cytochrome P-450 monooxygenase system generates H_2O_2 [14, 15] and O_2^- [16, 17] and the resulting H_2O_2 could react with O_2^- to yield $\cdot OH$ radicals via the Haber–Weiss reaction [18], the possibility that $\cdot OH$ was involved in covalent binding of methoxychlor was considered in the current study. Lastly, the possibility that $^1O_2$, possibly generated by the reaction of H_2O_2 with O_2^- or by dismutation of O_2^- [19, 20], or as a product of the radical decay of lipid hydroperoxides [21], is involved in the enzymatic metabolism of methoxychlor into reactive species was explored.

METHODS

Materials. EDTA disodium salt, NADPH, glucose - 6 - phosphate, glucose - 6 - phosphate dehydrogenase, superoxide dismutase (2800 units/mg), catalase (25,000 units/mg), xanthine, xanthine oxidase (0.42 units/mg), mannitol, DETAPAC, β-carotene, L-tryptophan, and sodium azide were from the Sigma Chemical Co. (St. Louis, MO). Phenobarbital sodium was from Mallinckrodt (St. Louis, MO). Chelex-100 was from Bio-Rad Laboratories (Richmond, CA). H_2O_2 and *t*-butanol were from the Fisher Scientific Co. (Pittsburgh, PA). DMSO and HPLC solvents were from Burdick & Jackson (Muskegon, MI). Benzoic acid (converted by us to the sodium salt) was from Matheson Coleman Bell (Norwood, OH). Desferal was a gift from Ciba-Geigy (Summit, NJ). L-Histidine was from Mann Research Laboratories (New York, NY). Insta-Gel was from Packard (Downers Grove, IL). Liquifluor was from New England Nuclear (Boston, MA). Methoxychlor was from Chem. Service (West Chester, PA). Uniformly ring-labeled [^{14}C]methoxychlor (1.8 mCi/mmole) was obtained from the California Bionuclear Corp. (Sun Valley, CA). Routinely, the [^{14}C]methoxychlor was purified by a previously described extraction method [4, 22]. The radiochemical purity of 99+% was established by TLC [7]. Occasionally, the radioactive methoxychlor was purified by HPLC as previously described [6]. CuDIPS and ZnDIPS were provided by Dr. Thomas Kensler. α-Tocopheryl acid succinate (converted by us to the sodium salt) was provided gratis by the Henkel Co., Fine Chem. Div. (Minneapolis, MN).

Animals. Male Sprague–Dawley CD rats (110–120 g) were obtained from the Charles River Breeding Laboratories and were injected i.p. with phenobarbital (37.5 mg/kg in 0.2 ml H_2O twice daily) for 4 days, and liver microsomes were prepared 12 hr after the last injection. Control animals for each treatment group were injected with the vehicle only. The preparation of liver microsomes was as previously described [23].

Incubations. Incubations were carried out in 20-ml glass scintillation vials containing the following constituents: 0.6 ml (60 μmoles) of sodium phosphate buffer (pH 7.4); 0.1 ml (10 μmoles) $MgCl_2$; microsomal suspension (0.6 to 0.8 mg protein in 0.1 ml of 1.15% aqueous KCl); EDTA (1 μmole); [^{14}C]methoxychlor (100,000 or 70,000 dpm in 5 μl ethanol); NADPH-regenerating system (glucose-6-phosphate, 10 μmoles; NADPH, 0.5 μmole; glucose-6-phosphate dehydrogenase, 2 I.U.) in 0.1 ml (10 μmoles) phosphate buffer; and H_2O to render a final volume of 1 ml. The reaction was initiated by adding the NADPH-regenerating system and incubating at 37° in a water-bath shaker. Incubations were conducted under an atmosphere of air. The reaction was terminated after 10 min by adding 5 ml ethanol. After termination of the incubation, the precipitate was trapped on a 2.4 cm Whatman GF/C glass microfiber filter adapted to a vacuum filter flask with a Schleicher & Schuell filter holder. The trapped precipitate was washed by passing the following solvents through the filter: ethanol, 10 ml; hexane, 40 ml; methanol–ether (3:1), 60 ml; and methanol–ether (1:3), 40 ml. At this point there was little or no radioactivity in the final wash. In initial experiments, the filter was placed at the bottom of a 20-ml glass scintillation vial containing 4 ml Liquifluor, and the radioactive content was determined. Because of occasional lack of congruency among radioactive values in duplicate or triplicate incubations, presumably due to differences in amount of protein trapped on the filters, a modified procedure was adopted. In this procedure, the filters were extracted with 2 ml SDS (2%) for 1 hr at 37° and washed with an additional 1 ml SDS. The volume was adjusted to 3 ml, and radioactivity in a 1-ml aliquot was determined in 10 ml Insta-Gel. Two 0.3-ml aliquots of the SDS extract were used for protein determination by the method of Lowry *et al.* [24] with the modification of Stauffer [25].

Polar metabolites. Polar metabolites of [^{14}C]methoxychlor, which constitute primarily the mono- and bis-hydroxy derivatives [4, 6], were determined in the following manner. The ethanol fraction (15 ml) from the above filtration procedure was saved and evaporated to dryness with a stream of nitrogen. The resulting residue was dissolved by adding 3 ml of 0.1 N NaOH. Unmetabolized [^{14}C]methoxychlor was removed by extraction with hexane (four times with 6 ml each), and the resulting aqueous phase was neutralized (pH 6 to 7) or made acidic with HCl to pH *ca.* 3. The radioactive content of 1 ml of the aqueous fraction was determined by liquid scintillation counting employing 10 ml Insta-Gel. Occasionally, the polar metabolites were extracted from the acidified aqueous phase (pH *ca.* 3) with ether, and the radioactivity in both phases was determined by liquid scintillation counting.

RESULTS AND DISCUSSION

To determine the possibility that O_2^- generated by the liver microsomes was involved in covalent bind-

Table 1. Effect of superoxide dismutase (SOD), catalase and H_2O_2 on covalent binding of [^{14}C]methoxychlor metabolite(s) to hepatic microsomal proteins

Addition(+)/Deletion(−)	Covalent binding of [^{14}C]methoxychlor equivalents (% of control)
Expt. 1	
None (control)	100*
+ SOD (250 units)	102.7
+ SOD (500 units)	90.3
+ Catalase (250 units)	101.7
+ Catalase (500 units)	103.5†
+ SOD (500 units) + catalase (250 units)	88.9
+ SOD (500 units) + catalase (500 units)	94.2†
Expt. 2	
None (control)	100‡
− NADPH	1.4
− NADPH + H_2O_2 (25 mM)	3.5
− NADPH + H_2O_2 (50 mM)	5.6
− NADPH + H_2O_2 (100 mM)	7.1
− NADPH + H_2O_2 (200 mM)	4.9
− NADPH + H_2O_2 (100 mM) + sodium azide (1 mM)	5.3
− NADPH + H_2O_2 (100 mM) + sodium azide (10 mM)	3.3

Incubations of [^{14}C]methoxychlor (100,000 dpm, 25 nmoles) were carried out in 1 ml volume for 10 or 30 min with liver microsomes, from phenobarbital-treated rats, fortified with NADPH or, when so specified, H_2O_2 was used instead. Conditions were as described in Materials and Methods.

* Control (100%) yielded 144.4 pmoles bound/min/mg protein (30-min incubation).

† Control (100%) yielded 162 pmoles bound/min/mg protein (10-min incubation).

‡ Control (100%) yielded 96.4 pmoles bound/min/mg protein; [^{14}C]methoxychlor was added in 10 μl acetone (10-min incubation).

ing of methoxychlor to microsomal proteins, the effect of superoxide dismutase on the covalent binding of [^{14}C]methoxychlor was examined (Table 1). Results demonstrated that superoxide dismutase (SOD) had no effect on covalent binding. The possibility that the SOD preparation used in this study was inactive was ruled out. We observed that this SOD preparation inhibited the rate of reduction of cytochrome *c* by O_2^- generated during a reaction of xanthine and xanthine oxidase [26] (not shown). Also, under those conditions, methoxychlor did not inhibit either O_2^- generation or SOD activity (not shown), and hence it appears unlikely that methoxychlor could have prevented SOD action on the microsomal O_2^--mediated covalent binding of methoxychlor. These findings suggested that O_2^- was probably not involved in generating the reactive methoxychlor intermediate. However, CuDIPS, a SOD mimetic agent [27, 28], inhibited the covalent binding of methoxychlor, while ZnDIPS, which has no SOD activity, did not inhibit covalent binding (Table 2). Since CuDIPS could have diminished covalent binding by merely inhibiting the NADPH–P-450 reductase, as observed with $CuSO_4$ and Cu–

Table 2. Effect of CuDIPS and ZnDIPS on covalent binding of [^{14}C]methoxychlor metabolite(s) to hepatic microsomal proteins and on formation of polar methoxychlor metabolites

	Covalent binding (% of control)*			Polar metabolites (% of control)*	
Addition (μM)	Expt. 1†	Expt. 2†	Expt. 3†	Expt. 2‡	Expt. 3‡
CuDIPS, 1	94.7	ND	ND	ND	ND
10	65.3	69.1	62.5	90.0	90.3
20	ND	58.9	54.7	85.6	80.0
ZnDIPS, 1	100.4	ND	ND	ND	ND
10	97.0	97.4	94.4	102.2	94.6
20	ND	91.5	97.8	97.3	94.9

Incubation conditions were as in Table 1 for 10 min, using NADPH as the cofactor.

* Control incubations contained the vehicle 10 μl DMSO; this amount of DMSO had little or no effect on binding or on the formation of polar metabolites. ND, not determined.

† Control (100%) in Experiments 1, 2 and 3 yielded 131.2, 131.4 and 142.9 pmoles [^{14}C]methoxychlor equivalents bound/min/mg protein respectively.

‡ In control incubations, formation of polar metabolites in Experiments 2 and 3 was 12.9 nmoles and 18.8 nmoles per 10 min respectively.

tyrosine complex [29], and consequently lower P-450 monooxygenase activity, the effect of CuDIPS on the demethylation of methoxychlor into acidic (phenolic) products was examined (Table 2). Neither CuDIPS nor ZnDIPS (at 10 μM) affected methoxychlor metabolism, and at 20 μM CuDIPS only slightly inhibited metabolism, indicating that the inhibitory effect by CuDIPS on covalent binding did not involve a general inhibition of the P-450 monooxygenases. Additionally, $CuSO_4$ (10 μM) did not affect covalent binding (not shown), indicating that the inhibition of covalent binding by CuDIPS was not a mere consequence of inhibition of reductase mediated electron transport. Based on the effect by CuDIPS on covalent binding, it appears that O_2^- was involved in the covalent binding of methoxychlor; albeit for an unknown reason, possibly due to the inaccessibility of SOD to O_2^- [30], SOD did not cause the inactivation of this species of oxygen. Nevertheless, the less likely possibility that CuDIPS may have inhibited a specific P-450 involved in generation of the reactive intermediate of methoxychlor by a non O_2^--mediated process was not ruled out.

Additional support for the possible involvement of O_2^- in covalent binding of methoxychlor stems from our observation that α-tocopheryl acid succinate (α-tocopherol) inhibited covalent binding; at 1 and 2 mM, α-tocopherol caused a 67.7 and 59.6% inhibition of methoxychlor binding respectively (not shown). α-Tocopherol has been observed to react effectively with O_2^- and by itself to undergo oxidation [31–34]. However, α-tocopherol has also been shown to readily react with ·OH [32] and to react only at a slow rate with $^1O_2$ [35]; hence, the possibility that the inhibition of covalent binding of methoxychlor by α-tocopherol involves scavenging of ·OH cannot be ruled out.

Since H_2O_2 is known to be formed by hepatic microsomes in the presence of NADPH [14, 15], and H_2O_2 can support microsomal monooxygenases [36–38], the possibility that the formation of the methoxychlor reactive intermediate involves an H_2O_2-supported monooxygenase reaction was examined (Table 1). However, the addition of catalase or of SOD plus catalase to the incubation did not inhibit covalent binding. Also the addition of H_2O_2 instead of NADPH to liver microsomes did not yield covalent binding of [^{14}C]methoxychlor (Table 1). These findings demonstrate that H_2O_2 was not involved in covalent binding. In view of these findings, it seems unlikely that ·OH radicals, generated by the reaction of O_2^- plus H_2O_2, were involved in the covalent binding of methoxychlor. Nevertheless, it is conceivable that ·OH produced by a reaction involving H_2O_2 which is not readily accessible to catalase has mediated the formation of the reactive intermediate of methoxychlor. This possibility was examined with the help of ·OH radical scavengers (Table 3). Among several ·OH scavengers examined, ethanol, *t*-butanol and benzoate inhibited covalent binding. By contrast, other ·OH scavengers (DMSO and mannitol) did not inhibit covalent binding. Incidentally, the inhibition of covalent binding by benzoate could have been due to general inhibition of monooxygenase (see diminished levels of polar metabolites) and not due to depletion of ·OH. Thus, these findings provided, at best, only equivocal evidence for the involvement of ·OH in the generation of the reactive intermediate.

To probe for the involvement of ·OH in a given

Table 3. Effects of scavengers of hydroxyl radical (·OH) on covalent binding of [^{14}C]methoxychlor metabolite(s) to hepatic microsomal proteins and on the formation of polar methoxychlor metabolites

		Covalent binding (% control)			Polar metabolites (% control)
Addition (mM)		Expt. 1*	Expt. 2†	Expt. 3‡	Expt. 1*
Ethanol,	150		81.1		
	173	66.2	68.0		85.1
	200	61.3	64.0		79.4
	400		42.7		
DMSO,	141	88.4			91.9
	150		91.5		
	300		84.1		
t-Butanol,	50	60.9			86.2
	100	41.7			75.6
Mannitol,	100			102.2	
	200			96.0	
Benzoate,	60	68.5[a]			87.1[b]
	120	32.6			59.7

* Expt. 1: Control: 149.0 pmoles bound/min/mg protein; 16.9 nmoles polar metabolites/10 min.

† Expt. 2: Control: 140.1 pmoles bound/min/mg protein.

‡ Expt. 3: Control: 142.0 pmoles bound/min/mg protein.

[a] Control: 162.2 pmoles bound/min/mg protein.

[b] Control: 14.7 nmoles/10-min incubation.

Table 4. Effect of chelating agents on covalent binding of [^{14}C]methoxychlor metabolite(s) to liver microsomal proteins

Additions/Omissions (mM)		Covalent binding of [^{14}C]methoxychlor equivalents (pmoles/min/mg protein) (% control)	
		Expt. 1	Expt. 2
None (control *minus* EDTA)		145.2 (100)*	147.5 (100)*
EDTA,	0.1	162.7 (112.0)	179.2 (121.5)
	2.0	178.0 (122.6)	187.3 (127.0)
	5.0	143.2 (98.7)	160.0 (108.5)
Desferal,	0.2	155.5 (107.0)	143.3 (97.2)
	1.0	128.7 (88.6)	132.7 (90.0)
	5.0	123.7 (85.1)	145.7 (98.8)
DETAPAC	2.0	140.1 (96.4)	170.3 (111.5)
	5.0	74.0 (51.0)	83.4 (56.6)

* In these experiments, control incubations were conducted without EDTA. All buffers were depleted of iron by passing through columns of Chelex-100; however, the possibility that small amounts of spurious iron were present in the incubations was not excluded.

reaction, it is informative to utilize specific metal chelators which stimulate or inhibit ·OH formation. It has been observed that EDTA-chelated iron effectively catalyzes the production of ·OH [39, 40] and, in turn, supports alcohol oxidation by liver microsomes [41]. By contrast, desferal (desferrioxamine) was found to inhibit ·OH-mediated microsomal oxidation of alcohol [41]. Using these chelators, we observed that, whereas EDTA stimulated covalent binding of methoxychlor, desferal had no such effect (Table 4). Also of interest is our finding that DETAPAC strongly inhibited covalent binding. A possible explanation for these findings is that, while desferal is a specific and stoichiometric chelator of Fe^{3+} [42], DETAPAC also chelates other metals. Additionally, since different chelates alter the redox potential of the bound ion to different extents, the activity of the iron would markedly depend upon the chelate [43]. In fact, it has been shown that DETAPAC (1 mM) prevents ·OH formation and that iron–DETAPAC complex cannot catalyze ·OH generation from H_2O_2 and O_2^- [44, 45]. Thus, our findings suggest that ·OH is indeed involved in supporting covalent binding of methoxychlor, possibly via participation of a metal other than iron.

Lastly, the possibility that $^1O_2$ is involved in supporting covalent binding of [^{14}C]methoxychlor was examined (Table 5). Scavengers of $^1O_2$ [21, 46–50] were found to inhibit covalent binding without having a marked effect on polar metabolite formation.

In conclusion, the above findings suggest that several active oxygen species, probably derived from O_2^-, are involved in the generation of the reactive intermediate of methoxychlor. The lack of activity of certain scavengers of active oxygen species could be attributed to the possibility that the covalent

Table 5. Effects of *singlet* oxygen scavengers on covalent binding of [^{14}C]methoxychlor metabolite(s) to hepatic microsomal proteins

Addition (mM)		Binding of [^{14}C]methoxychlor equivalents (% control)			Polar metabolites (% control)		
		Expt. 1*	Expt. 2†	Expt. 3‡	Expt. 1*	Expt. 2†	Expt. 3‡
β-Carotene,	0.10§	79.1			105.7		
	0.30§	72.5			100.7		
L-Histidine,	20.0		70.4			90.6	
L-Tryptophan,	10.0	95.1			86.9		
	20.0	86.3			92.5		
Sodium azide,	1.0			78.9			85.1
	10.0			46.9			72.3

* Expt. 1: Control (100%) containing [^{14}C]methoxychlor in 5 μl ethanol yielded 128.2 pmoles bound/min/mg protein and 13.4 nmoles polar metabolites/10 min.

† Expt. 2: Control (100%) containing [^{14}C]methoxychlor in 5 μl ethanol yielded 162.2 pmoles bound/min/mg protein and 16.9 nmoles polar metabolites/10 min.

‡ Expt. 3: Control (100%) containing [^{14}C]methoxychlor in 5 μl acetone yielded 161.1 pmoles bound/min/mg protein and 19.3 nmoles polar metabolites/10 min. In the absence of acetone, there was 157.4 pmoles bound/mg protein and 19.6 nmoles polar metabolites/10 min.

§ The concentration of β-carotene is approximate because of solubility problems.

binding of methoxychlor occurs by two pathways, one involving O_2^- and/or ·OH and the other involving a different oxygen species generated by a specific P-450. Such a two-pathway oxidation mode has been proposed to explain the oxidation of alcohols by liver microsomes [51]. The possibility that the lack of SOD effect on the covalent binding of methoxychlor is merely due to inaccessibility of the O_2^- in the microsomes [30] could be explored further by using purified cytochrome P-450 in reconstituted systems and by incorporating SOD into the lipid vesicles. Such experiments will be conducted at a later date.

Preliminary studies provide evidence that the reactive intermediate of methoxychlor is a free radical or is derived from a free radical (D. Kupfer and W. H. Bulger, unpublished observations). Since phenols are effective scavengers of free radicals [29], it is tempting to speculate that the phenolic metabolites of methoxychlor (the mono- and bis-phenol derivatives) generated by the action of microsomal cytochrome P-450 monooxygenases [4, 6] could inhibit the covalent binding of methoxychlor to macromolecules and thus minimize the potential toxicity associated with such covalent binding. These studies are currently being pursued.

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