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STUDIES ON THE FORMATION OF METHOXYCHLOR–PROTEIN ADDUCT IN RAT AND HUMAN LIVER MICROSOMES

IS DEMETHYLATION OF METHOXYCHLOR ESSENTIAL FOR CYTOCHROME P450 CATALYZED COVALENT BINDING?

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Abstract— Previous studies demonstrated that liver microsomal monooxygenases metabolize the pesticide methoxychlor into phenolic estrogenic derivatives. Additionally, methoxychlor is activated by the hepatic cytochrome P450 monooxygenase to bind covalently to microsomal proteins (Bulger WH, Temple JE and Kupfer D, *Toxicol Appl Pharmacol* **68**: 367–374, 1983). The current study examines, in liver microsomes from control and phenobarbital-treated rats and humans, whether demethylation of methoxychlor is essential for covalent binding and whether demethylated methoxychlor metabolites are on the pathway of formation of the reactive intermediate and protein adduct. Using ^{3}H-methoxyl-labeled and ^{14}C-ring-labeled methoxychlor, it was demonstrated that demethylation is not essential for covalent binding. Namely, the major portion of the methoxychlor moiety in the protein adduct was found to contain intact methoxyls. Nevertheless, in the absence of methoxychlor, both the mono- and bis-demethylated methoxychlor metabolites could undergo monooxygenase-mediated covalent binding to proteins. This was demonstrated in incubations of purified ^{14}C-labeled mono- and bis-demethylated methoxychlor metabolites with liver microsomes, in the presence of NADPH. Additionally, the dehydrochlorinated metabolite of methoxychlor, containing a double bond, underwent covalent binding, which exhibited characteristics similar to those of methoxychlor. These findings demonstrated that the protein adduct from relatively brief incubation periods contains a methoxychlor derivative with intact methoxyls. The possibility that the activation of methoxychlor involves modification of the side chain, which is the active site that binds to proteins, is discussed.

Methoxychlor† [1,1,1-trichloro-2,2-bis(4-methoxyphenyl)ethane] is currently being used as a substitute for certain pesticidal activities of DDT, which essentially has been banned in the industrially developed countries. The beneficial features of methoxychlor are its low acute toxicity in mammals and its short half-life in animals and in the biosphere in general, classifying it as a biodegradable pesticide [2–7]. However, the rapid metabolism of methoxychlor into phenolic estrogenic products [8–11] may have undesirable effects, particularly in animals exposed to that pesticide at early stages of development [12]. Recent studies in mature female rats demonstrated that methoxychlor causes changes in sexual behavior and anti-fertility [13, 14]. Whether anti-fertility is caused by methoxychlor *per se* or by its estrogenic metabolites remains to be established.

Of concern to potential long-term toxicity is the observation that methoxychlor undergoes a monooxygenase-mediated activation, resulting in covalent binding to hepatic proteins‡ and that phenobarbital (PB) treatment of rats markedly stimulates covalent binding [15, 16]. Furthermore, based on the potent inhibition of covalent binding in liver microsomes from PB-treated rats (PB-microsomes) by antibodies raised against the major PB-induced cytochrome P450b [16], it was concluded that the major portion of covalent binding is catalyzed by P450b (P450IIB1) and/or P450e (P450IIB2). The nature of the reactive oxygen involved in generating the reactive intermediate of methoxychlor was examined previously. Our studies suggested that the P450-mediated activation of methoxychlor involves a reaction with superoxide anions and/or hydroxyl radicals [17]. It was observed that a nonenzymatic reaction between methoxychlor and superoxide anions in aprotic medium consumes one superoxide with a loss of one chlorine [18]. By contrast, with DDT, there was an uptake of three superoxide anions and a loss of three chlorines, resulting in more extensive metabolism. Interestingly, marked differences between methoxy-

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† 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; MDDE, 1,1-dichloro-2,2′-bis(4-methoxyphenyl)ethene; SDS, sodium dodecyl sulfate; and DDT, 1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane. P450b and P450e have the genetic notations of P450IIB1 and P450IIB2 respectively [1].

‡ Irreversible binding was assumed to be due to covalent linkage when the proteins retained the radiolabel despite numerous washings with nonpolar and polar solvents and the radiolabel was retained during sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Furthermore, the covalent linkage to protein thiols was demonstrated by release of the radioactivity from the adduct during treatment of the adduct with Raney nickel.

chlor and DDT were observed in their monooxygenase-mediated covalent binding. Whereas the microsomal activation of methoxychlor absolutely requires the presence of oxygen [15], binding of DDT to microsomal proteins is increased substantially under anaerobic conditions [19], suggesting mechanistic differences in generating the reactive intermediates of methoxychlor and DDT respectively.

The adduct between the reactive intermediate of methoxychlor and microsomal proteins, which appears to involve a reaction with the cysteine thiols [16, 20], has not been identified hitherto. Methoxychlor is metabolized by cytochrome P450 monooxygenases into the mono- and bis-demethylated products (mono-OH-M and bis-OH-M respectively) [3, 20, 21] followed by ring hydroxylation into a tris-OH-M. The latter reaction is highly pronounced in PB-microsomes, and preliminary evidence, based on inhibition studies, suggests that different isoforms of cytochrome P450 may be involved in ring hydroxylation and in the demethylation reactions.

Our previous study indicated that PB treatment in rats stimulates methoxychlor demethylation only marginally when examined by formaldehyde formation, but that PB treatment significantly stimulates demethylation when evaluated by formation of phenolic metabolites [21]*. Nevertheless, the stimulation of covalent binding by PB treatment was much more pronounced than demethylation. This suggested that demethylation is probably not on the pathway of covalent binding or that demethylation is not the rate-determining step in covalent binding. The current study examines the question whether the demethylated metabolites are on the pathway of covalent binding and whether demethylation is essential for the formation of the reactive intermediate and covalent binding.

MATERIALS AND METHODS

EDTA disodium salt, NADPH, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were from the Sigma Chemical Co. (St. Louis, MO). Phenobarbital sodium and reagent grade solvents were purchased from Mallinckrodt (St. Louis, MO). Insta-Gel was from Packard (Downers Grove, IL). Liquifluor was from New England Nuclear (Boston, MA). All other chemicals were of reagent grade quality. [Ring-UL-^{14}C]Methoxychlor (5.85 mCi/mmol) was obtained from the Sigma Chemical Co. and was used at the original specific activity or was diluted (as indicated in text) with radioinert methoxychlor obtained from Chem Service (West Chester, PA). Routinely, the [^{14}C]methoxychlor was purified to a radiochemical purity of 99+% as previously described [11]. [^{3}H-*methoxyl*]Methoxychlor (4.4 Ci/mmol) was obtained by custom synthesis from New England Nuclear (Boston, MA) and was used as described in the text.

Male Sprague–Dawley CD rats (90–100 g), unless otherwise specified in text, were purchased from the Charles River Breeding Laboratories (Wilmington, MA) and were kept in a room with controlled temperature (22°) and light (12 hr light/dark cycle; lights off at 7:00 p.m. EDT). PB treatment (37.5 mg/kg i.p. in 0.2 mL H_2O twice daily) was for 4 days. Livers were removed 12 hr after the last injection. Human liver samples from eight kidney transplant donors were provided by Prof. Urs Meyer (Basel, Switzerland). Microsomes were prepared from a homogenate of 1 g of liver per 5 mL of 0.25 M aqueous sucrose, as previously described [22], and represented a pool of four to eight rat or human livers. The resulting microsomal pellets were washed by resuspension in 1.15% aqueous KCl followed by centrifugation at 105,000 *g* for 1 hr. The supernatant fraction was discarded, and the microsomal pellet was covered with 2 mL of 1.15% KCl and stored at −70°, until used.

Incubation procedure. Incubations were carried out in 20-mL glass scintillation vials containing: 0.6 mL of sodium phosphate buffer (pH 7.4, 60 μmol); microsomal suspension (0.8 to 1 mg of protein in 0.1 mL of 1.15% aqueous KCl); EDTA (1 μmol); [^{14}C]methoxychlor or [^{3}H]methoxychlor, usually 25 nmol (about 100,000 and 250,000 dpm respectively) added in 10 μL ethanol; NADPH-regenerating system (glucose-6-phosphate, 10 μmol; NADPH, 0.5 μmol; glucose-6-phosphate dehydrogenase, 2 I.U.) in 0.1 mL of phosphate buffer (pH 7.4, 10 μmol), and 0.1 mL of H_2O for a final volume of 1.0 mL. After a 2-min warm-up period at 37°, the reaction was initiated by adding the NADPH-regenerating system, and the vials were incubated at 37° in a water bath shaker; see text for duration of incubation. The reaction was terminated by adding 10 mL of ethanol, and the resulting ethanolic-incubation mixture suspension was processed by the following modification of a previously described method [15]. To trap the proteins, the suspension was filtered through a 2.4 cm Whatman GF/C glass microfiber filter (Whatman Ltd., Maidstone, England) in a filter holder (Schleicher & Schuell, Inc., Keene, NH) attached to a vacuum filter flask. The filtrate was processed as described below. The protein on the filter was washed with a variety of polar and nonpolar solvents, to remove unbound radiolabeled compounds, as previously described [23]. The protein residue was eluted by suspending the glass filters in 3 mL of 2% aqueous SDS solution, and an aliquot was used for radioactive determination using scintillation counting in a Packard 460C spectrometer and for protein determination, as previously described [24, 25].

The filtrate from the ethanolic-incubation mixture suspension was evaporated at room temperature to dryness under a stream of nitrogen, and the residue was stored under argon, at 4° until used for chromatography. The residue was subjected to TLC, using

* We observed a marked increase in demethylated methoxychlor products with PB microsomes as compared with control microsomes; however, this increase in demethylation was not evident when formaldehyde was assayed by the Nash procedure. We attribute this discrepancy to the use of low concentrations of methoxychlor (20–25 μM), the K_m for demethylation being about 9 μM (unpublished), and to the low sensitivity of the Nash formaldehyde assay.

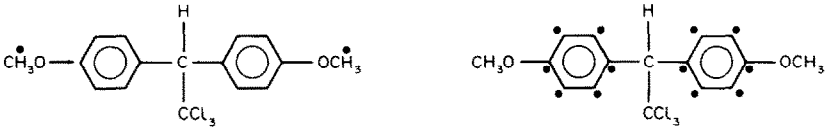


Fig. 1. Radiolabeled methoxychlor. Dots mark the location of the label. Left: ^{3}H-labeled-methoxychlor; right: ^{14}C-labeled methoxychlor.

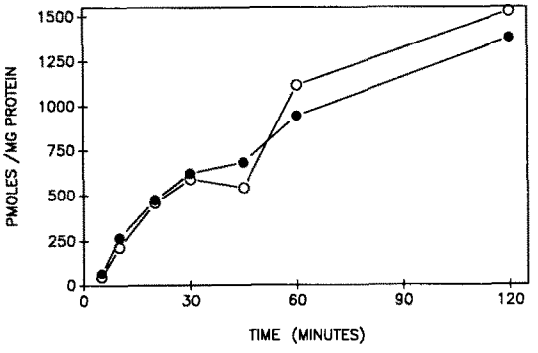


Fig. 2. Time course of covalent binding of radioactive metabolite(s) derived from [$OC^{3}H_{3}$]methoxychlor versus [^{14}C-ring]methoxychlor to liver microsomal proteins from control rats. Key: (●) ^{14}C; (○) ^{3}H.

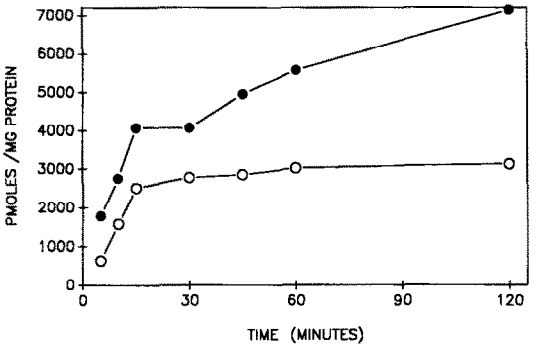


Fig. 3. Time course of covalent binding of radioactive metabolite(s) derived from [$OC^{3}H_{3}$]methoxychlor versus [^{14}C-ring]methoxychlor to liver microsomal proteins from PB-treated rats. Key: (●) ^{14}C; (○) ^{3}H.

a system previously described [26], and the radioactivity was detected with a Vanguard 930 radioactive scanner. The gel in the radioactive zones was scraped with a razor blade into individual vials, to which 1.0 mL methanol and 5 mL Liquifluor were added. The radioactivity was determined by scintillation counting, and quantitation of products was obtained from their respective ratio on TLC, assuming comparable loss of the various compounds. The compounds in radioactive peaks 1, 2 and 3 on TLC were identified by GC/MS and LC/MS as being mono-OH-M, bis-OH-M and tris-OH-M [21].

Preparation of [^{14}C]*mono-OH-M and* [^{14}C]*bis-OH-M.* These compounds were obtained by incubation of [^{14}C]methoxychlor with liver microsomes from control rats for various time intervals, using a short incubation time for enrichment of the mono-OH-M amd longer incubations for the bis-OH-M, essentially as described above under "Incubation procedure". An aliquot of the incubation extract, between 3000 and 9000 dpm, was usually subjected to TLC analysis, and the metabolites were detected by radioscanning on a Vangard 930 autoscanner. The rest of the ethanolic extract was evaporated to dryness, and the residue was dissolved in 60% acetonitrile/water and passed through a Sep-Pak (Waters) or through a filter (Gilman). The filtrate either was used as such or was evaporated to dryness and reconstituted in the appropriate mobile phase for chromatography on HPLC, using a 10μ-C8 (RAC_1) semiprep Whatman column. The mobile phase was 45% acetonitrile/water for isolating the bis-OH-M or 60% acetonitrile/water for the mono-OH-M, at 2.5 mL/min. Detection was achieved at 228 and 280 nm. The collected eluant fractions, containing UV peaks corresponding in retention time to the authentic compounds, were evaporated at room temperature under a stream of nitrogen. An aliquot of the residue was subjected to HPLC on a 10μ-C8 analytical column (Whatman). Usually the residue was found to contain some impurities; hence the whole sample was chromatographed again on the analytical column utilizing the above mobile phases, but at 2.0 mL/min, and narrow cuts were taken. The appropriate UV absorbing fractions were collected, and radioactivity in a small aliquot was determined. The purity of each of the products was determined to be 95% by analyzing an aliquot by HPLC and TLC. The rest of the sample was evaporated to dryness under a stream of nitrogen and stored under Argon gas at −20°, and the compounds were found to be stable for at least 2 years. Additional evidence for the identity of these compounds was obtained by demonstrating that the incubation of aliquots of each of these compounds with PB-liver microsomes yielded on TLC the expected metabolites, i.e. the mono-OH-M generated radiolabeled products with R_f values corresponding to bis-OH-M and tris-OH-M; the bis-OH-M formed a compound with an R_f value corresponding to tris-OH-M.

[^{14}C]*MDDE* [*1,1-dichloro-2,2'-bis(4-methoxyphenyl)ethene*]. This compound was synthesized from [^{14}C]methoxychlor by Dr. Vernon J. Feil (USDA, Fargo, ND). The specific radioactivity was 19.7 mCi/mmol. On HPLC, using a C18 Novapack RCM column, 70% acetonitrile/H_2O, the major radioactive peak eluted at about 10 min; under the same conditions the starting material (radiolabeled methoxychlor) exhibited a 7-min retention. The radiolabeled MDDE was found to yield a single radioactive peak on silica gel TLC.

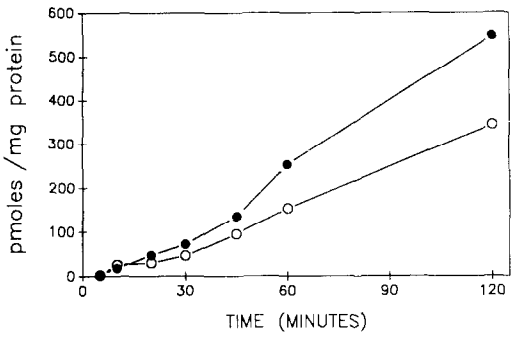
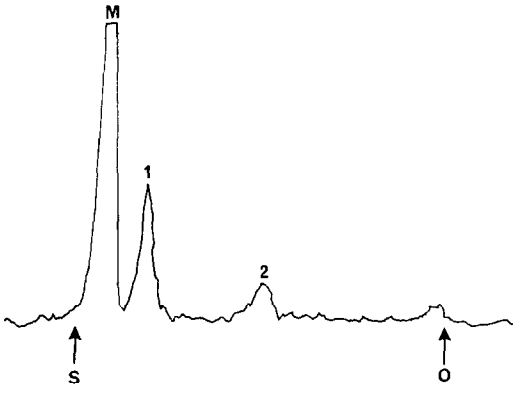


Fig. 4. Time course of covalent binding of radioactive metabolite(s) derived from [OC^{3}H$_3$]methoxychlor versus [^{14}C-ring]methoxychlor to liver microsomal proteins from humans. Key: (●) ^{14}C: (○) ^{3}H.



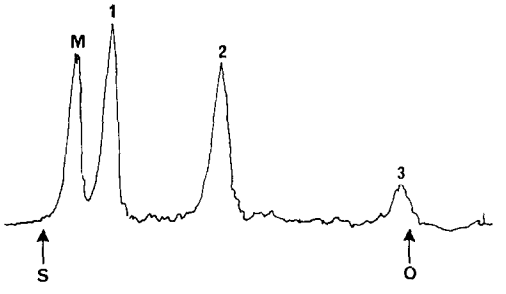


Fig. 5. Radioscan of TLC of an extract from incubation of [^{14}C]methoxychlor with control rat liver microsomes, in the presence of 1 mM semicarbazide, as described in Materials and Methods. Top panel: 10-min incubation; bottom panel: 30-min incubation. Key: M = methoxychlor; peak 1 = mono-OH-M; peak 2 = bis-OH-M; peak 3 = tris-OH-M (possibly containing a small amount of unidentified metabolite); O = origin; and S = solvent front.

RESULTS AND DISCUSSION

Is demethylation of methoxychlor required for covalent binding? To determine whether demethylation of methoxychlor is involved in the course of formation of the reactive intermediate and covalent binding, [^{3}H]methoxychlor (*methoxyl*-labeled) (Fig. 1) was incubated with liver microsomes from control rats (control-microsomes), PB-treated rats (PB-microsomes) and human microsomes. Parallel incubations were conducted with [^{14}C]methoxychlor (*ring*-labeled). Figures 2–4 demonstrate the time–course of covalent binding in control-, PB- and human microsomes respectively. The protein adducts from the various incubations with [^{3}H]methoxychlor were found to contain ^{3}H. However, a significant decrease in the ^{3}H-label as compared with the ^{14}C-label appeared to occur over the incubation period. The decrease in ^{3}H was particularly evident with PB microsomes and to a lesser extent with human-microsomes. By contrast, with control-rat microsomes, there was little or no difference between the two isotopes. These findings indicated that though methoxychlor could undergo activation and covalent binding without demethylation, in certain liver preparations, some demethylated methoxychlor could also participate in that reaction. Another possible explanation of these findings is that the lower rate of binding of [^{3}H]methoxychlor versus that of the [^{14}C]methoxychlor was due to the bulkiness of the ^{3}H-methoxyls, thus either hindering the formation of the reactive intermediate or yielding a slower rate of covalent binding of the reactive intermediate. This explanation appears unlikely, since in control-microsomes the ^{3}H-label and ^{14}C-label curves closely paralleled each other (Fig. 2), indicating that when the rate of formation of the bis- and tris-OH metabolites was low, as was the case with control-microsomes (Fig. 5), the rates of covalent binding with the two labels were similar. By contrast, in PB-microsomes where the rates of formation of the bis-OH-M and tris-OH-M were much higher (Fig. 6), the divergence of the ^{3}H and ^{14}C curves was pronounced (Fig. 3). The reason for the time-dependent divergence of the ^{3}H and ^{14}C curves in human microsomes, even though the rate of demethylation was relatively low (not shown), is not understood.

The possibility that the ^{3}H-label in the protein from incubations with [^{3}H]methoxychlor was due to binding of the [^{3}H]HCHO, generated by demethylation of [^{3}H]methoxychlor, was examined. The incubation of [^{3}H]HCHO with liver microsomes, in the presence or absence of NADPH, followed by the usual work-up involving solvent wash of the precipitated proteins, did not generate a significant amount of radiolabeled proteins. Also, the incorporation of semicarbazide (1 mM) in the incubation to trap formaldehyde did not alter covalent binding with either [^{3}H]- or [^{14}C]methoxychlor (not shown). This indicated that the ^{3}H-label in proteins was not derived from formaldehyde. Alternatively, the possibility that the progressive decrease in covalent binding with [^{3}H]methoxychlor in PB-microsomes and human-microsomes might be due to competition for the P450-mediated activation and/or for acceptor binding sites by the radioinert bis-OH-M, formed by demethylation of [^{3}H]methoxychlor, was considered (Table 1).

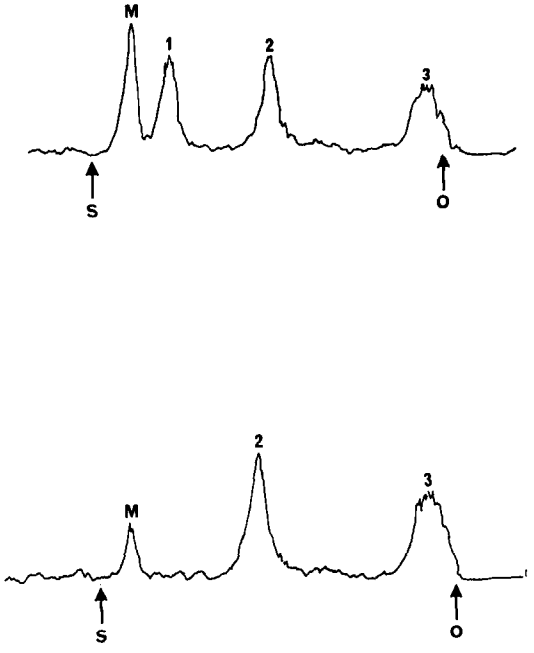


Fig. 6. Radioscan of TLC of an extract from incubation of [^{14}C]methoxychlor with PB-rat liver microsomes, in the presence of 1 mM semicarbazide. Top panel: 10-min incubation; bottom panel: 30-min incubation. Conditions and notations are as described in the legend of Fig. 5.

Results indicate that radioinert bis-OH-M causes an inhibition of the covalent binding of [^{14}C]methoxychlor. However, a relatively high ratio of the bis-OH-M to methoxychlor was needed for substantial inhibition to be elicited, a situation expected to occur only when the concentration of methoxychlor becomes limiting. It appears unlikely that the mono-OH-M would interfere significantly with the covalent binding of methoxychlor, since in control-microsomes which primarily produce the mono-OH-M, there was no decrease in ^{3}H-label versus ^{14}C-label during the time-course of the incubation. Unfortunately, the lack of a sufficient amount of pure radioinert mono-OH-M did not permit the direct determination of whether this metabolite can interfere with the covalent binding of radiolabeled methoxychlor.

The above observations suggested that the metabolites of methoxychlor may be able to undergo metabolic activation. Thus, the ability of demethylated methoxychlor metabolites to bind covalently to microsomal proteins was examined: [^{14}C]mono-OH-M and [^{14}C]bis-OH-M were incubated with PB- and control-rat and human liver microsomes (Table 2). Results demonstrate that both metabolites were activated by the microsomal monooxygenase and bound covalently to proteins; there was no binding in the absence of NADPH. Mono-OH-M exhibited a similar rate of binding to that of methoxychlor, whereas bis-OH-M was somewhat more sluggish. The observation that *N*-acetylcysteine (5 mM) strongly inhibited the binding of bis-OH-M and methoxychlor (not shown) supports the speculation that both compounds are activated by similar mechanisms.

Does tris-OH-M participate in covalent binding? An additional question requiring resolution is whether the tri-hydroxy metabolite, tris-OH-M, could undergo metabolic activation. This catechol derivative is a major metabolite in incubations with PB-microsomes [21]. It is conceivable that tris-OH-M could undergo metabolic oxidation by a mechanism similar to that of methoxychlor or its demethylated metabolites. However, it is also possible that tris-OH-M is oxidized to the semiquinone radical which would be expected to bind covalently to macromolecules. In fact, monooxygenase- and peroxidase-mediated reactions with other catechols have been observed previously [27–29]. However, studies with tris-OH-M must await the isolation of sufficient amounts of pure radiolabeled tris-OH-M, which hitherto has not been attained, due to major losses incurred during purification.

Table 1. Effect of bis-OH-M on the covalent binding of [^{14}C]methoxychlor to liver microsomal proteins from PB-treated rats

Microsomes	Methoxychlor (μM)	Bis-OH-M (μM)	Ratio bis-OH-M/ methoxychlor	Bound (nmol/mg protein/10 min)	Polar metabolites* (% of control)
Expt. 1					
	25			1.26 (100)†	100‡
	25	10	0.4	1.05 (83.1)	92.6
	25	25	1.0	0.90 (71.4)	87.0
Expt. 2					
	2.5			0.74 (100)	
	2.5	25	10	0.55 (74.3)	
	2.5	50	20	0.40 (54.1)	
Expt. 3					
	25			1.55 (100)	
	25	25	1.0	1.18 (76.1)	
	25	50	2.0	0.97 (62.5)	
	25	100	4.0	0.48 (31.0)	

* Total polar metabolites representing a composite of mono-hydroxy-, bis-hydroxy- and tris-hydroxy-metabolites.
† Percent of control.
‡ Control (100%) = 15.0 nmol/10 min.

Table 2. Covalent binding of [^{14}C]methoxychlor, [^{14}C]mono-OH-M and [^{14}C]bis-OH-M to liver microsomes from control rats, PB-treated rats and humans

Compound	Source of microsomes	Incubation (min)	Bound (nmol/mg protein/time interval)*
Methoxychlor	Control-rat	60	1.15
Mono-OH-M	Control-rat	60	0.85
Bis-OH-M	Control-rat	60	0.87
Methoxychlor	PB-rat	10	1.91
Mono-OH-M	PB-rat	10	2.46
Bis-OH-M	PB-rat	10	0.73†
Methoxychlor	PB-rat	30	2.95
Mono-OH-M	PB-rat	30	3.23
Bis-OH-M	PB-rat	30	2.24‡
Methoxychlor	Human	60	0.48
Mono-OH-M	Human	60	0.46
Bis-OH-M	Human	60	0.24

* The time interval is given in the column labeled "Incubation". The value represents the mean of 2–3 incubations from which the minor, non-specific binding, obtained in the absence of NADPH, was subtracted.

† In this experiment, methoxychlor yielded binding of 1.71 nmol/mg protein/10 min.

‡ In this experiment, methoxychlor yielded binding of 2.91 nmol/mg protein/30 min.

Table 3. Covalent binding of [^{14}C]MDDE to liver microsomal proteins in PB-treated rats

	MDDE (nmol/incubation)	Additions/Deletions	Bound (nmol/mg protein/10 min)	Polar metabolites* (% of control)
Expt. 1	0.6		0.07	ND†
	6.0		0.63	ND
	12.0		1.20	ND
Expt. 2	12.0		1.26	100‡
	12.0	Minus NADPH	0.04 (3.2)§	ND
	12.0	Plus SKF-525A (0.5 mM)	0.03 (2.6)	33.5
	12.0	Plus GSH (5 mM)	0.52 (41.0)	100.9
	12.0	Plus TCPO‖ (0.1 mM)	1.08 (85.5)	96.9
	12.0	Plus TCPO (0.5 mM)	0.92 (73.3)	100.2
	12.0	Plus TCPO (1.0 mM)	0.75 (60.0)	97.9
	12.0	Plus TCPO (2.0 mM)	0.53 (42.4)	85.2

* Composite of total polar metabolites in the aqueous phase after extraction of the aqueous incubation mixture made basic (pH 11.0) with hexane.

† ND = not determined.

‡ Control (100%) was 10.0 nmol products/10 min.

§ Numbers in parentheses represent percent of control.

‖ Trichloropropylene oxide.

Covalent binding of MDDE. The dehydrochlorinated methoxychlor [1,1-dichloro-2,2′-bis(4-methoxyphenyl)ethene], referred to as MDDE, appears to be an *in vivo* metabolite of methoxychlor [30, 31]. This compound is also a major contaminant of the commercial pesticidal preparation of methoxychlor [32]. Like methoxychlor, MDDE has been found to readily undergo monooxygenase-mediated demethylation to mono- and bis-hydroxy estrogenic metabolites [10, 20]. In fact, these metabolites are more potent estrogens than the corresponding methoxychlor metabolites. These findings and the fact that MDDE contains an accessible double bond, which potentially could form reactive epoxides, suggested that it would be of interest to determine whether MDDE undergoes metabolic activation and binds covalently to microsomal proteins. Preliminary evidence suggested that MDDE could bind to microsomal proteins [20, 33]. Table 3 demonstrates that indeed [^{14}C]MDDE bound covalently to microsomal proteins and that a typical monooxygenase catalyzed that reaction. Also, like with methoxychlor, MDDE covalent binding to proteins was inhibited by glutathione (GSH), which probably reacts with the intermediate; GSH had no inhibitory effect on the formation of the polar demethylated metabolites. Furthermore, trichloropropylene oxide (TCPO), an inhibitor of epoxide hydratase, did not potentiate covalent binding of MDDE and, in fact, inhibited binding at concentrations which did not inhibit the P450-mediated demethylation of methoxycholor into polar compounds, suggesting that the reactive intermediate is not an epoxide.

Does covalent binding of methoxychlor inactivate

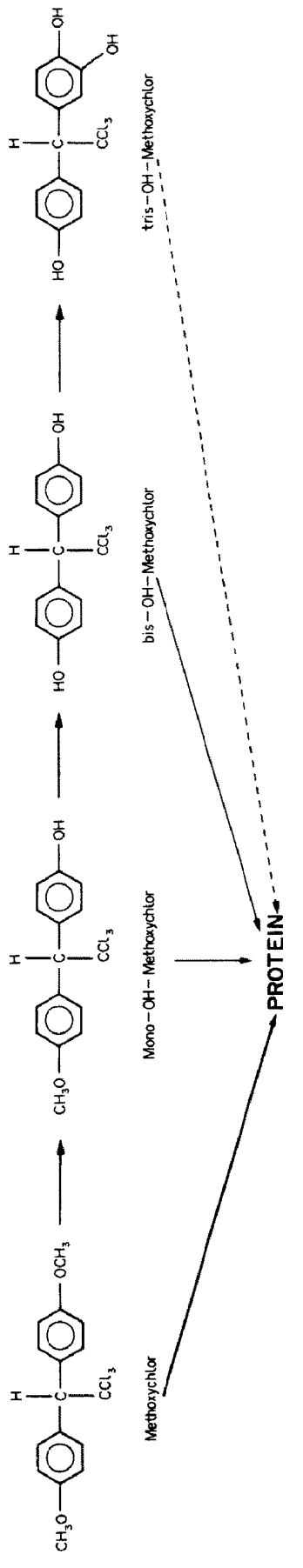


Fig. 7. Proposed pathway of monooxygenase-mediated metabolism of methoxychlor and covalent binding to liver microsomal proteins. Thick line arrow = major pathway; thin line = minor pathway; and broken line = hypothetical pathway.

cytochrome P450? The possibility that the covalent binding of methoxychlor inactivates the P450(s), which catalyze the formation of the reactive intermediate, in a time-dependent fashion was considered. If that were the case, this would indicate that methoxychlor might act as a mechanism-based inhibitor (suicide substrate). The incubation of methoxychlor (25 μM) with PB liver microsomes in the presence of NADPH for 30 min at 37° did not cause a decrease in cytochrome P450, as measured spectrally by the carbon monoxide difference spectra. Values of P450 (nmol/mg protein) were: (a) 2.04 ± 0.6 (plus NADPH plus methoxychlor), (b) 2.01 ± 0.02 (plus NADPH minus methoxychlor), and (c) 2.14 ± 0.22 (minus NADPH minus methoxychlor). Since in PB-microsomes P450b (P450IIB1) is responsible for most of the covalent binding of methoxychlor [16], the lack of decrease in total P450 indicates that the heme of the major PB-inducible P450b was not affected or if affected was not altered at a catalytically critical position. That finding, however, did not exclude the possibility that the reactive intermediate of methoxychlor binds to the apo-P450b and thus diminishes its catalytic activity. However, P450b does not appear to be inactivated, as evidenced by the observation that covalent binding of methoxychlor by PB-microsomes proceeds linearly over a relatively long period of time. In fact, in earlier studies, we observed linearity of covalent binding for about 60 min or longer with control-rat and human liver microsomes and for 30 min with PB-rat liver microsomes [15, 16]. The shorter linearity with PB microsomes apparently was due to the more rapid metabolism of methoxychlor by these microsomes and not due to P450 inactivation. It is conceivable, however, that other P450s not involved in methoxychlor covalent binding are inactivated by covalent binding of methoxychlor. Preliminary results suggest that this may be the case with certain P-450 isozyme(s) catalyzing the 6β-hydroxylation of testosterone. Such studies will be the subject of future investigations.

Characteristics of the reactive intermediate. The current study demonstrates that the major portion of the adduct derived from the incubation of methoxychlor with hepatic microsomal proteins represents a reaction of methoxychlor metabolite containing *intact* methoxyls. In addition, the demethylated metabolites may also contribute to some extent to that reaction (Fig. 7). Previous findings suggested that the reactive intermediate is a free radical and that superoxide anion participates in its formation [17]. Also, nonenzymatic studies in aprotic medium demonstrated that superoxide releases a single chlorine from methoxychlor [18]. Collectively, these findings suggest that the reactive intermediate represents methoxychlor modified in the side chain, possibly by homolytic cleavage of the C—H or of the C—Cl bond. It is conceivable that two or more different reactive species are formed. Such seems to be the case in the covalent binding of bromobenzene, where two reactive intermediates, epoxide and orthoquinone, have been observed [34]. The above observations that demethylated metabolites can undergo metabolic activation and covalent binding indicate that attempts to identify the adduct will be complicated by the presence of multiple forms of adducts. This problem might be overcome by utilizing brief incubations, which should produce essentially a single reactive species, i.e. containing intact methoxyls, and facilitate the eventual characterization of the protein–methoxychlor adduct. However, our observation that MDDE, an *in vivo* metabolite of methoxychlor, also bound covalently to microsomal proteins adds substantial complexity to characterization of adducts *in vivo*. This complexity is likely to make it impractical to characterize these adducts *in vivo*.

Are protein sulfhydryls involved in adduct formation? Although indirect evidence indicates that covalent binding of methoxychlor occurs primarily with sulfhydryl groups of proteins [16], unequivocal proof of this reaction must await more direct evidence involving characterization of the amino acid in the protein–methoxychlor adduct. Such evidence will require enzymatic and chemical hydrolysis of the purified radiolabeled protein adduct and the identification of the amino acid bound to the labeled methoxychlor derivative. Additional, albeit indirect, evidence for the participation of protein sulfhydryls in covalent binding could be obtained through the identification of the adducts formed with glutathione or *N*-acetylcysteine. We obtained preliminary evidence that such water-soluble adducts are formed during incubation of methoxychlor with microsomes in the presence of these sulfhydryl agents (not shown). Though identification of RSH-adducts will not provide unequivocal evidence concerning the participation of sulfhydryls in the protein adduct, these adducts could be useful in the characterization of the reactive intermediate of methoxychlor. Studies using both approaches are being pursued.

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