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Role of Hepatic Monooxygenases in Generating Estrogenic Metabolites from Methoxychlor and from Its Identified Contaminants

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SUMMARY

Previous investigations demonstrated that methoxychlor [1,1,1-trichloro-2,2-bis(4-methoxyphenyl)ethane] contains estrogenic contaminants and that methoxychlor *per se* is not an estrogen but is a *pro*estrogen being metabolized *in vivo* into estrogenic products. The present study examined structurally identified methoxychlor contaminants as to their estrogenic or proestrogenic properties. Also, the estrogenic activity of demethylated metabolites of methoxychlor and of one contaminant was determined. To examine these properties, we utilized an assay developed by us that monitors whether a given compound, incubated with isolated rat uteri, can diminish the uterine cytosolic estrogen receptor and elevate the nuclear estrogen receptor and whether metabolic intervention by hepatic microsomal monooxygenase(s) is required by the respective compound for this cellular redistribution of the receptor. Of the 15 compounds examined which constitute with methoxychlor 99.5% of total technical grade methoxychlor, two compounds, 1,1-dichloro-2-(4-hydroxyphenyl)-2-(4-methoxyphenyl)ethene (mono-OH-MDDE) and 1,1,1-trichloro-2-(4-hydroxyphenyl)-2-(4-methoxyphenyl)ethane (mono-OH-methoxychlor), were active *per se* and two compounds, 1,1-dichloro-2,2-bis(4-methoxyphenyl)ethene (MDDE) and methoxychlor, required metabolic transformation for estrogenic activity to be manifested. Subsequently, it was shown that the mono- and bis-OH metabolites of MDDE and of methoxychlor were active estrogens and that the order of activity, either by the above procedure or in terms of relative binding affinity to rat uterine cytosolic receptor, was as follows: bis-OH-MDDE ≫ bis-OH-methoxychlor > mono-OH-MDDE > mono-OH-methoxychlor. Following the *in vitro* observations, the activity of MDDE and bis-OH-MDDE was determined *in vivo* in immature rats. It appears that both compounds are estrogenic, yielding marked elevation in ornithine decarboxylase (EC 4.1.1.17) levels and moderate increase in uterine weight. A comparison with methoxychlor and bis-OH-methoxychlor [1,1,1-trichloro-2,2-bis(*p*-hydroxyphenyl)ethane] demonstrates that the order of potencies is similar to that observed in the *in vitro* determinations. These studies demonstrate the usefulness of the *in vitro* assay for determining the estrogenic and proestrogenic properties of compounds of which limited quantities are available, often insufficient for *in vivo* determination. Also, whereas the *in vitro* assay is simple and rapid, a lengthy investigation might be required to determine *in vivo* whether a given compound is an estrogen or a proestrogen. Lastly, this investigation underlines the necessity for using ultrapure materials when examining highly sensitive biological endpoints, such as estrogenic action. Of additional importance is the availability of structurally characterized compounds, particularly when the compound in question is further metabolized into a biologically active substance.

INTRODUCTION

Earlier studies, both *in vivo* and *in vitro*, demonstrated that technical grade methoxychlor, a widely used pesticide, is a more potent estrogen than purified methoxychlor[1] (1–3). Moreover, even the laboratory grade, 99% pure methoxychlor was found to contain

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[1] Trivial names and abbreviations used are: methoxychlor, compound 36, 1,1,1-trichloro-2,2-bis(4-methoxyphenyl)ethane; compound 20, 1,1,1,2-tetrachloro-2-(2-methoxyphenyl)ethane; compound 21A, 2,2,2-trichloro-1-(2-methoxyphenyl)ethanol; compound 22, 1,1,1,2-te-

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base-soluble contaminants which appear estrogenic since they inhibit the binding of [^{3}H]E_2 to the uterine cytosolic 8 S estrogen receptor (4). Additionally, we demonstrated that base extraction and recrystallization of the laboratory grade methoxychlor yielded a preparation that *in vitro* has no estrogen-like properties. By contrast, *in vivo*, this purified methoxychlor has estrogenic activity in elevating uterine ODC and exhibiting uterotropic activity (5). Subsequent experiments demonstrated that methoxychlor could be metabolized by rat hepatic microsomes into at least three potent estrogenic products that *in vitro* inhibit the binding of [^{3}H]E_2 to the uterine cytosolic receptor (5). One of these products resulting from demethylation was identified as the bisphenolic derivative of methoxychlor (bis-OH-methoxychlor); this compound was *in vivo* about 50-fold more potent an estrogen than the purified methoxychlor (5). Additionally, other workers reported that incubation of methoxychlor with rat liver microsomes and NADPH increased the ability of methoxychlor to inhibit [^{3}H]estradiol binding to rat uterine estrogen receptor *in vitro* (6). Subsequently, the mono- and bishydroxy derivatives of methoxychlor were identified as metabolites in the microsomal mixture (5–7).[2]

Our attempts to determine whether a given compound is an estrogen *per se* or a *pro*estrogen led to the development of an *in vitro* assay which permits a relatively easy approach to that question (8). This assay combines a metabolizing system (rat liver microsomes, NADPH, and oxygen) with an estrogen-detecting system (isolated rat uteri) (see Fig. 1). A compound is considered to be an estrogen when, in the absence of metabolism (using heat-inactivated liver microsomes or deleting NADPH), the compound causes translocation of the cytosolic receptor into the nucleus.[3] Conversely, if an *active* liver microsomal monooxygenase system is needed for the translocation of the receptor by the respective compound, that compound is assumed to be a proestrogen. Using this method in conjunction with *in vivo* findings, we concluded that methoxychlor is a proestrogen (8).

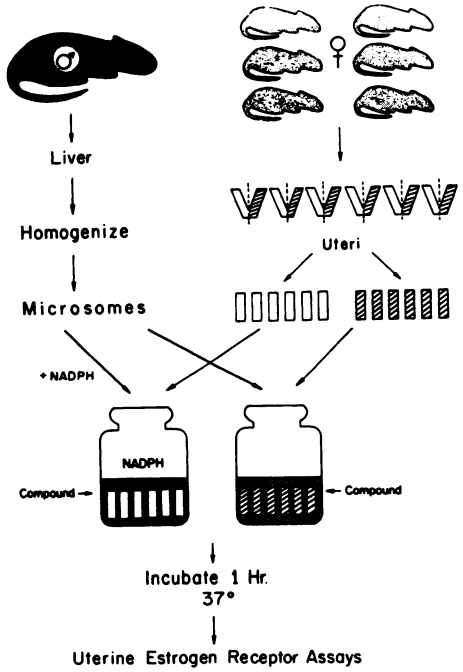

FIG. 1. *Flow diagram representing methodology for the in vitro determination of estrogenic and proestrogenic activity*

The present investigation utilized the above *in vitro* assay to examine which of the major structurally identified contaminants of technical grade methoxychlor are estrogenic *per se*, or proestrogenic, and which of the contaminating compounds exhibit no activity altogether. Also, a comparison was conducted of *in vitro* and *in vivo* estrogenic assays to establish whether potency correlates well among the different assays. This study provides further affirmation for the usefulness of the *in vitro* method to help ascertain whether a given compound is an estrogen or a proestrogen.

EXPERIMENTAL PROCEDURES

Materials

The NADPH, chemically reduced (Sigma Chemical Co., St. Louis, MO), [2,4,6,7-^{3}H]estradiol, 85–100 Ci/mmol (New Engand Nuclear Corp., Boston, MA), 17β-estradiol (Steraloids, Inc., Wilton, NH), dimethylformamide and HPLC solvents (Burdick and Jackson Laboratories, Inc., Muskegon, MI), and corn oil (Matheson Coleman and Bell, Norwood, OH) were purchased from their respective suppliers. All common laboratory chemicals were reagent quality.

Test Compounds

Methoxychlor (99% pure) was purchased from Chem Service, Inc. (West Chester, PA) and was repurified by base-washing as previously described (4) and recrystallized from hexane. The recrystallized methoxychlor was subjected to additional purification by preparative HPLC as described below. The bis-OH-methoxychlor was a generous gift from Drs. Toshio Fujita (Kyoto University, Japan) and James R. Sanborn (Illinois Natural History Survey, Urbana, IL). Compounds 20, 21A, 22, 23, 24, 26, 30, 31, 34, and 35 (Fig. 2) were prepared synthetically as described by Lamoureux and Feil (9). Other compounds prepared synthetically were 35A, 37, and the bis-OH-MDDE (10). Compound 44, 1,1,2,2-tetrakis(4-methoxyphenyl)ethene, was obtained during an

trachloro-2-(4-methoxyphenyl)ethane; compound 23, 2,2,2-trichloro-1-(4-methoxyphenyl)ethanol; compound 24, (2-methoxyphenyl)(4-methoxyphenyl)methane; compound 26, 1,1-bis(4-methoxyphenyl)ethane; compound 30, 1-chloro-2-(2-methoxyphenyl)-2-(4-methoxyphenyl)ethene; compound 31, *o,p'*-MDDE, 1,1-dichloro-2-(2-methoxyphenyl)-2-(4-methoxyphenyl)ethene; compound 33, *o,o'*-methoxychlor, 1,1,1-trichloro-2,2-bis(2-methoxyphenyl)ethane; compound 34, MDDE, 1,1-dichloro-2,2-bis(4-methoxyphenyl)ethene; compound 35, *o,p'*-methoxychlor, 1,1,1-trichloro-2-(2-methoxyphenyl)-2-(4-methoxyphenyl)ethane; compound 35A, mono-OH-MDDE, 1,1-dichloro-2-(4-hydroxyphenyl)-2-(4-methoxyphenyl)ethene; compound 37, mono-OH-methoxychlor, 1,1,1-trichloro-2-(4-hydroxyphenyl)-2-(4-methoxyphenyl)ethane; compound 44, 1,1,2,2-tetrakis(4-methoxyphenyl)ethene; bis-OH-methoxychlor, 1,1,1-trichloro-2,2-bis(4-hydroxyphenyl)ethane; bis-OH-MDDE, 1,1-dichloro-2,2-bis(4-hydroxyphenyl)ethene; E_2, estradiol; ER_c, cytosolic estrogen receptor; ER_n; nuclear estrogen receptor; HPLC, high performance liquid chromatography; MS, mass spectrometry; EIMS, electron impact MS; CIMS, chemical ionization MS; GC, gas chromatography; ODC, ornithine decarboxylase; RBA, relative binding affinity; KRS, Krebs-Ringer solution; DMF, dimethylformamide.

[2] Our laboratory has characterized mono-OH- and bis-OH-methoxychlor by GC/MS as microsomal metabolites of methoxychlor in the rat (A. D. Theoharides and D. Kupfer, unpublished).

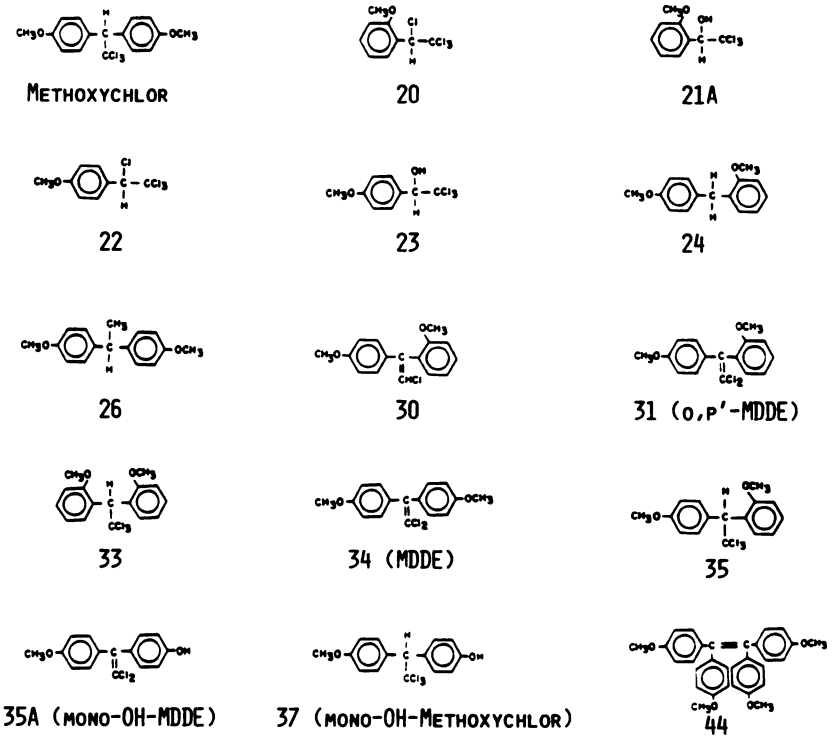

FIG. 2. *Structures of 15 of more than 50 compounds contained in technical grade methoxychlor*
Numbers assigned to each compound are the original numbers representing the peak in the gas chromatography-mass spectrometry total ion chromatogram in a study by Lamoureux and Feil (9). Compound 44 was also identified as a contaminant of technical grade methoxychlor by Mazuch *et al.* (34).

attempted Clemmensen reduction of 4,4′-dimethoxybenzophenone by the procedure in Fieser and Fieser (35). The oily residue obtained by extraction with ether was crystallized from hexane and ethanol and recrystallized from ethanol to yield product m.p. 174–179° (literature, 185°) (34). HPLC (C_{18} NOVAPAK, RCM, Waters Associates, 60–100% acetonitrile in water, 10-min linear program, 3 ml/min) yielded two peaks. The major peak was consistent with 1,1,2,2-tetrakis(4-methoxyphenyl)ethene; EIMS, *m/z* (relative intensity) 452, M^+ (100); NMR ($CDCl_3$) δ 3.73 (s, OCH_3; literature, 3.7) (34), 6.63 (d, $J = 8.9$ Hz, 3,5-ArH, literature, q, 6.76) (34), 6.93 (d, $J = 8.9$ Hz, 2,6-ArH). The minor HPLC peak (approximately 5%) yielded a fragment at *m/z* 227 (100) but no M^+ by EIMS and peaks at *m/z* 227 (72), 347 (100), and 455, $M^+ + 1$ by CIMS, suggesting that the impurity was 1,1,2,2-tetrakis(4-methoxyphenyl)ethane. All compounds were used at their initial level of purity (Table 1). Compounds demonstrating activity were repurified by semipreparative HPLC (see below) and assayed a second time to confirm their estrogenic properties. Structural identity of the HPLC-purified compounds was confirmed by NMR analysis and by mass spectrometry.

High Pressure Liquid Chromatography

A Waters Associates model ALC-GPC 204 high performance liquid chromatograph equipped with a dual wavelength UV detector (model 440), solvent delivery system (model 6000A), injector (model U6K), and solvent flow programmer (model 660) was employed. Analytical HPLC was carried out with a Whatman (Clifton, NJ) C_8 column (reversed phase) and an eluting solvent of 60% CH_3CN and 40% H_2O run at 2 ml/min. Compounds were monitored with the fixed wavelength detector at 280 nm and with a Schoeffel model SF770 variable wavelength detector, usually at 228 nm. Preparative HPLC was used to further purify certain compounds of interest, employing a reversed phase Whatman C_8 RAC column and 70% CH_3CN/30% H_2O run at 2.5 ml/min.

Animals

Sprague-Dawley CD rats were obtained from Charles River Breeding Laboratories (Wilmington, MA) and were maintained in our animal colony under conditions of controlled light (12-hr light:12-hr dark; lights off at 7:00 p.m. EDT) and temperature (22.2°C). Animals were allowed free access to water and Purina rat formula.

Preparation of Microsomes

Male rats (80–90 g) were treated with phenobarbital as previously described (11), and microsomes were prepared from the livers. The procedure was as described earlier (12) with the exception that the initial 105,000 × *g* microsomal pellets were washed by resuspension in 1.15% aqueous KCl and repelleted by centrifugation for 1 hr at 105,000 × *g*. Phenobarbital-treated microsomes were used to enhance metabolism of test compounds.

Assay I: In Vitro Determination of Proestrogenic and Estrogenic Activity

Fig. 1 depicts an assay based on the incubation of a potential proestrogen with hepatic microsomes in the presence of isolated immature rat uteri. The microsomes, when fortified with NADPH, are utilized to transform the proestrogen into an active estrogen and the isolated uteri serve as a target monitor for estrogenic activity. The estrogen causes a measurable increase in uterine ER_n and a decrease in uterine ER_c.[3] In this procedure, each test compound (potential proestroen) is assayed in two vials. NADPH is added to the microsomes

[3] For the purpose of this article, we refer to the phenomenon of increased nuclear estrogen receptor and decreased cytosolic receptor as *translocation*, even though the question whether an estrogen actually translocates the cytosolic receptor into the nucleus or merely prevents the exit of nuclear receptor into the cytosol during homogenization has not yet been resolved (32). For possible mechanisms describing this phenomenon, see Ref. 33.

TABLE 1

Effect of incubation of identified contaminants in technical grade methoxychlor with rat hepatic microsomes and with rat uteri on the subcellular distribution of uterine estrogen receptor

Experimentation was carried out as described under Experimental Procedures (assay I) and in Fig. 1. Each value represents a single observation. Compound numbers are those assigned by Lamoureux and Feil (9) and are identified as contaminants of technical grade methoxychlor.

Compound	Relative amount[a]	Microsomes[b]	Distribution of uterine estrogen receptor ([^{3}H]E_2 bound)		
			Cytosolic	Nuclear	Total receptor
μM	%		*fmol/uterus*		
Methoxychlor	93.10				
2		Active	940	289	1229
		Inactive	1135	107	1242
2[c]		Active	1031	234	1265
		Inactive	1287	70	1357
2[c]		Active	384	239	623
		Inactive	478	101	579
No. 20	0.011				
4		Active	778	101	879
		Inactive	875	119	994
		Active	835	101	936
		Inactive	774	97	871
No. 21A					
4		Active	633	95	728
		Inactive	641	94	735
		Active	794	116	910
		Inactive	877	116	993
No. 22	1.430				
4		Active	730	98	828
		Inactive	874	109	983
		Active	755	101	856
		Inactive	637	125	762
No. 23	0.363				
4		Active	474	137	611
		Inactive	844	111	955
		Active	633	117	750
		Inactive	717	113	830
No. 24	0.019				
4		Active	857	106	963
		Inactive	781	152	933
		Active	807	102	909
		Inactive	780	212	992
No. 26	0.003				
4		Active	902	162	1064
		Inactive	803	110	913
		Active	871	77	948
		Inactive	1039	142	1181
No. 30	0.004				
4		Active	777	112	889
		Inactive	755	112	867
		Active	702	83	785
		Inactive	878	96	974
No. 31	0.161				
4		Active	696	232	928
		Inactive	821	159	980
		Active	755	208	963
		Inactive	802	196	998
2		Active	579	128	707
		Inactive	683	95	778
No. 33	0.087				
4		Active	663	99	762
		Inactive	587	108	695
2		Active	663	68	731
		Inactive	652	101	753
No. 34[d]	0.761				
4		Active	299	460	759
		Inactive	751	112	863
		Active	579	373	952
		Inactive	617	99	716
2		Active	300	492	792
		Inactive	960	111	1071
No. 35	3.057				
4		Active	784	102	886
		Inactive	747	79	826
		Active	642	81	723
		Inactive	708	91	798
No. 44					
4		Active	724	108	832
		Inactive	735	95	830
		Active	842	126	968
		Inactive	883	118	1001
		Active	688	87	775
		Inactive	823	105	928

[a] Relative amount in technical grade methoxychlor (9).
[b] Inactive refers to microsomes boiled for 10 min.
[c] Compound was added in 10 μl of ethanol.
[d] No. 34 = MDDE.

in one vial. The other vial, as indicated in Fig. 1, contains microsomes without NADPH and serves as a control.[4] Because the microsomes in the control vial do not metabolize the given compound, the above changes in levels of ER_n and ER_c which occur only in the uteri from the vial containing a *complete* incubation mixture are indicative of a proestrogen. When no change in ER_n and ER_c levels takes place in the uteri from either vial, the test compound is judged inactive. An increase in ER_n and a decrease in ER_c in both vials indicate that the test compound is intrinsically estrogenic and requires additional analysis (see assay II below). The assays were performed with liver microsomes (0.5–0.7 mg of protein/incubation) suspended in KRS (8) which were added to two 30-ml serum vials (3 ml/vial). Uteri from six 20-day-old rats were transected through the fused region and the halves were divided equally between the two vials. Test compounds were added in 10 μl of DMF. Oxygen was bubbled through the incubation mixture for 2 min; the vials were sealed with rubber stoppers and flushed with additional oxygen. Incubation was initiated by injecting NADPH (120 μM final concentration) in 0.1 ml of KRS through a rubber septum into the vials (control vials received 0.1 ml of KRS in place of NADPH) and by placing the vials in a water-bath shaker at 37°. After 1 hr, the vials were removed and placed on ice, and the uteri were removed and washed in cold KRS. Uterine levels of ER_n and ER_c were determined as described below.

[4] In an alternate procedure (not depicted in Fig. 1), the microsomes in the control vial were inactivated by boiling for 10 min and were resuspended with a hand-held Potter-Elvehjem homogenizer prior to addition of the uterine halves. Incubation was initiated by adding NADPH to both vials (Table 1). Both types of controls (microsomes minus NADPH versus boiled microsomes plus NADPH) yielded equivalent results (Table 4).

Assay II: In Vitro Determination of Estrogenic Activity

Test compounds suspected of intrinsic estrogenic activity were assayed as follows. Uteri from six 20-day-old rats were halved as in assay I and distributed between two vials. Each vial contained 3 ml of KRS; however, microsomes were *not* added to either vial. Test compounds were added in 10 μl of DMF to one vial. The second vial served as a control and received 10 μl of DMF only. The vials were exposed to an O_2 atmosphere, as described above, and were incubated for 1 hr at 37° in a water-bath shaker. After incubation, the uteri were removed, washed with cold KRS, and assayed for ER_n and ER_c content as described below. Compounds causing an increase in ER_n and a decrease in ER_c in uteri from the vial containing test compounds were considered estrogenic. Compounds causing no change in ER_n and ER_c in either vial were judged inactive.

Determination of Uterine Estrogen Receptor

The uterine halves from the above procedures (assays I and II) were fractionated into cytosolic and nuclear preparations as described previously (8), and estrogen receptor content of the two fractions was determined by an exchange assay with [^{3}H]estradiol employing a modification (13) of a procedure originally described by Anderson *et al.* (14) and Clark and Peck (15).

Evaluation of Data from Assays I and II

Interpretation of results from assays I and II relied primarily on the change in ER_n (rather than ER_c) levels in uteri from the two vials (assay I, active versus inactive microsomes; assay II, test vial versus control). Changes in ER_n levels proved to be a more sensitive parameter than changes in ER_c levels, since the latter measurement yields small differences between two large values. A change in ER_n value of 50 fmol or greater was considered to be a positive response. ER_c measurements provided useful supporting evidence only when the relative change in ER_c was substantial, which occurred with potent compounds (e.g., MDDE). All compounds were tested at least twice at the same concentration and at times at multiple levels. Compounds were judged inactive if negative both times. Active compounds were further purified by HPLC and recrystallized and were tested again several times by assay I or II. If judged positive, the compounds were considered active and were subjected to additional experimentation (see below).

Inhibition of Binding of [^{3}H]Estradiol to Rat Uterine Cytosolic Estrogen Receptor

Relative binding affinity of estrogenic derivatives of methoxychlor. Rat uterine cytosol (20,000 × *g*) prepared from 20-day-old rats was incubated (cytosol equivalent to ⅛ uterus/incubation) with [^{3}H]estradiol in the presence of various concentrations of a given test compound employing previously described methodology (16), with the exception that the cytosol was not stripped with dextran-coated charcoal prior to use. Each compound was tested at least twice to establish reproducibility.

Saturation analysis of inhibition of [^{3}H]estradiol binding to rat uterine cytosol by estrogenic derivatives of methoxychlor. Twenty-day-old rats were used to prepare uterine cytosol which was incubated with various concentrations of [^{3}H]estradiol in the presence of a given concentration of a test compound according to previously described methodology (13). Resulting data were analyzed by Scatchard (17) and double reciprocal plots.

Metabolism Studies

The conditions for incubation of MDDE (compound 34) with NADPH-fortified microsomes from phenobarbital-treated rats mimicked those described under assay I with the exception that the uteri were omitted and that the incubation time varied. The reaction was stopped by addition of 10 ml of ethanol. The resulting mixture was vacuum filtered through a Whatman GF/C glass microfiber filter to remove macromolecular aggregates and the filtrate was evaporated to dryness under a stream of N_2.The resulting residue was dissolved in 100 μl of CH_3CN and 5-μl aliquots were subjected to analytical HPLC (see above).

Demethylation was determined by analyzing formaldehyde evolution by the method of Nash (18). The incubation mixture, designed to resemble assay I, consisted of 3 ml of KRS, 1 mM semicarbazide, and microsomes from phenobarbital-treated rats equivalent to 0.56 mg of protein.[5] Test compounds were added in 10 μl of DMF. Prior to incubation, O_2 was bubbled through the mixture as described under assay I. Reaction was initiated by addition of NADPH (120 μM final concentration) in KRS and incubation was conducted for 1 hr at 37°. The reaction was terminated by addition of 0.2 ml of 100% trichloroacetic acid and, after centrifugation at 800 × *g* for 10 min, formaldehyde content was determined in the resulting supernatant.

Determination of Estrogenic Activity in Vivo

Twenty-day-old female rats were randomized according to weight into groups of 5–10 animals. Test compounds were administered intraperitoneally in 0.2 ml of corn oil between 3:00 and 3:30 a.m. (EDT). Control rats received 0.2 ml of corn oil intraperitoneally. Six hours after injection, the animals were weighed and sacrificed. The uteri were removed, weighed, and immediately frozen on dry ice. The ratio of uterine weight to 100 g of body weight was calculated as an indicator of uterotropic activity, and an elevation of uterine ornithine decarboxylase (EC 4.1.1.17) activity was employed as an additional marker for estrogenic activity (19, 20). Within 2 hr of freezing, each uterus was assayed for ODC activity by a previously described modification (21) of the procedures of Kaye *et al.* (20) and Kobayashi *et al.* (22).

Protein Determination

The method of Lowry *et al.* (23) employing modifications recommended by Stauffer (24) with bovine plasma albumin (Metrix, Chicago, IL) as a standard was used.

RESULTS AND DISCUSSION

Fifteen compounds (Fig. 2) that together with methoxychlor constitute about 99.5% of the total material present in technical grade methoxychlor (9) were examined with respect to their ability to translocate the cytosolic uterine estrogen receptor *in vitro* in the presence of active or inactive liver microsomal monooxygenase (Table 1). Compound 34 (MDDE), in addition to methoxychlor, was found to be active, albeit only in the presence of an active metabolizing system. Also, two base-soluble compounds, the monophenolic derivative of methoxychlor (compound 37) and the monophenolic derivative of MDDE (compound 35A), were active *per se* (Table 2). Surprisingly, both *o, p′* derivatives of methoxychlor (compound 35) and of MDDE (compound 31) were not active (Table 1). This is by contrast to *o, p′*-DDT, known to be estrogenic (21, 25), and which we previously showed to be active *per se* by the *in vitro* method (8). The possibility that the *o, p′* derivatives of methoxychlor and of MDDE are not metabolized by the hepatic monooxygenases was ruled out (Table 3). Under the conditions of the *in vitro* assay, the two compounds are as effectively demethylated as methoxychlor or MDDE. However, these findings did not rule out the unlikely possibility that the demethylated metabolites of compounds 31 and 35 are not active because of their inability to penetrate the uteri and reach the site of

[5] Semicarbazide was included in incubations only when the intention was to measure formaldehyde.

TABLE 2

In vitro effects of HPLC-purified mono- and bishydroxy derivatives of methoxychlor and MDDE (No. 34) on the subcellular distribution of rat uterine estrogen receptor (in the absence of liver microsomes)

Experimentation was as described under Experimental Procedures (assay II). Each value represents a single observation.

Compound	Distribution of uterine estrogen receptor ([^{3}H]E_2 bound)		
	Cytosolic	Nuclear	Total
μM	*fmol/uterus*		
Mono-OH-methoxychlor			
0.2	636	125	761
—[a]	700	33	733
2.0	233	424	657
—	702	68	770
Bis-OH-methoxychlor			
0.02	608	68	676
—	570	65	635
0.2	386	236	622
—	585	67	652
MDDE			
2.0	909	74	983
—	872	77	949
Mono-OH-MDDE			
0.2	527	192	719
—	666	78	744
2.0	477	460	937
—	809	78	887
Bis-OH-MDDE			
0.02	464	241	705
—	619	87	706
0.2	393	413	806
—	839	90	929
2.0	343	452	795
—	1055	94	1149

[a] —, compound not present.

TABLE 3

Demethylation of methoxychlor and analogues by rat hepatic microsomes

Incubations were under similar conditions as in Table 1; however, uteri were excluded and certain modifications were incorporated (see Experimental Procedures).

Substrate	Concentration	HCHO[a]	Conversion[b]
	μM	*nmol/60 min*	%
Methoxychlor	4	19.0	79.2
	16	17.8	18.5
o,p'-Methoxychlor (No. 35)	16	27.4	28.5
MDDE (No. 34)	4	23.8	99.2
	16	33.8	35.2
o,p'-MDDE (No. 31)	4	23.4	97.5
	16	23.2	24.2

[a] Each 3-ml incubation contained 0.56 mg of microsomal protein.
[b] Percentage of total methoxyl groups metabolized to HCHO in 1 hr.

action. Compound 24 appeared slightly active *per se* (higher ER_n observed with inactive microsomes) and apparently was converted into inactive metabolite(s) by active microsomes (Table 1). Compound 26 exhibited marginal or no activity (variable results in two assays); however, because of its low relative abundance (0.003%) in technical grade methoxychlor, it was not further investigated.

To minimize the possibility that the active compounds may have contained small amounts of highly estrogenic impurities, we subjected those compounds to further analysis and purification by HPLC. The eluted major component from HPLC was recrystallized and analyzed by NMR and by mass spectrometry. In all cases examined, positive structure identification was obtained. Using the purified compounds, we repeated the *in vitro* estrogenic assays (Table 4). The results with the HPLC-purified materials were identical to the original findings, i.e., methoxychlor and MDDE (compound 34) exhibited proestrogenic activity and *o,p'*-methoxychlor (compound 35) was inactive.

The above findings, that methoxychlor and MDDE behave like proestrogens, raised the question as to the nature of the metabolites which represent the estrogenic activity of these compounds. Two methoxychlor metabolites have been identified as mono-OH-methoxychlor and bis-OH-methoxychlor (5–7, 10, 27)[2] (Fig. 3). We also examined the metabolism of the highly active MDDE under metabolizing conditions similar to those used in our *in vitro* assay for estrogenic action (Fig. 4). Based on co-injections on HPLC with authentic mono- and bis-OH-MDDE derivatives, it appears that the metabolites of MDDE are, in fact, the expected demethylation products. Further evidence for that assignment is derived from the observations on generation of formaldehyde during metabolism (Table 3) and from the finding that the chromatographically less polar putative mono-OH-MDDE, found during 5-min incubations, disappeared on longer incubations (15 min), yielding the putative bis-OH-MDDE (Fig. 4).[6] Using the above information, we examined whether the mono-OH-MDDE and bis-OH-MDDE exhibit, as expected, estrogenic activity without need for further metabolic transformation, i.e., in the absence of a microsomal metabolizing system (Table 2). Additionally, we compared their activity to the corresponding mono- and bis-OH-methoxychlor derivatives. As can be seen, the bis-OH metabolites are more potent than the corresponding mono-OH metabolites. Also, as expected from the observations with the parent compounds (methoxychlor and MDDE), the metabolites of MDDE are more potent than the corresponding metabolites of methoxychlor.

Since in the described assay the relative activity of these metabolites could be a result of multiple factors, among these the intrinsic activity of the compound and its ability to reach the site of action, we examined the interaction of these compounds with the cytosolic estrogen receptor without the complicating factor of possible differences in organ uptake and transport ("pharmacokinetics"). Thus, the two sets of demethylated metabolites of MDDE and of methoxychlor were compared with respect to their RBA for the cytosolic estrogen receptor by examining their relative potencies as inhibitors of binding of [^{3}H]E_2 to the receptor (Fig. 5 and Table 5).

[6] An unequivocal characterization of the products will be attempted after isolation of larger quantities of these compounds and will be the subject of another study.

TABLE 4

Effect of incubation of HPLC-purified methoxychlor, MDDE (No. 34), and o,p'-methoxychlor (No. 35) with rat hepatic microsomes and rat uteri on the subcellular distribution of uterine estrogen receptor

Conditions were as in Table 1.

Compound	Microsomes	Distribution of uterine estrogen receptor ([^{3}H]E_2 bound)		
		Cytosolic	Nuclear	Total receptor
μM		*fmol/uterus*		
Methoxychlor				
2	Active	559	309	868
	Inactive	769	83	852
MDDE				
2	Active	356	384	740
	Inactive	806	99	905
MDDE				
2	Active	333	350	683
	Active (minus NADPH)[a]	766	88	854
MDDE				
2	Active	385	399	784
	Active (minus NADPH)[a]	626	106	732
MDDE				
0.2	Active	669	184	853
	Inactive	924	75	999
o,p'-Methoxychlor				
4	Active	783	156	939
	Inactive	885	154	1039

[a] Controls consisted of active microsomes; however, NADPH was deleted.

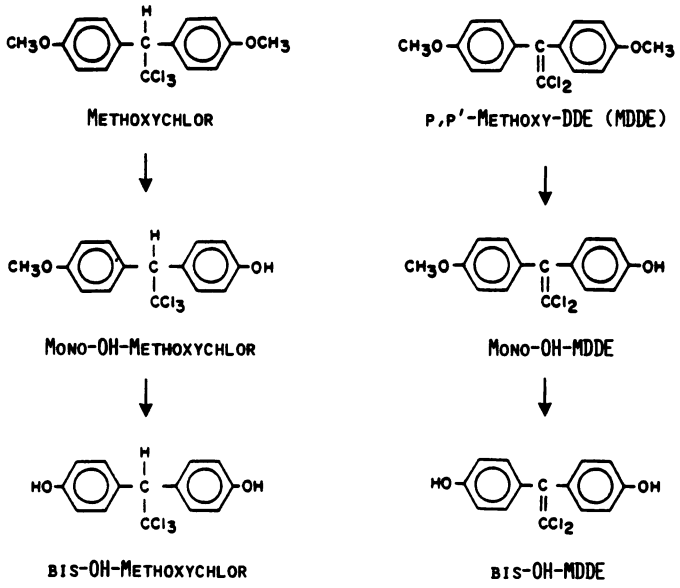

FIG. 3. *Proposed metabolic pathways for hepatic monooxygenase-mediated metabolism of methoxychlor and MDDE (compound 34) to their respective estrogenic metabolites*

The results demonstrate that the RBA to the estrogen receptor are in the following order: bis-OH-MDDE ≫ bis-OH-methoxychlor > mono-OH-MDOE > mono-OH-methoxychlor. Similar order of potency was observed with these compounds in the translocation of the uterine cytosolic estrogen receptor (Table 2). Additionally, based on competition experiments utilizing Scatchard and double reciprocal analysis, it appears that the mono- and bis-OH derivatives of MDDE and methoxychlor bind in a competitive manner to the same active site on the cytosolic estrogen receptor (Fig. 6).

To establish whether our *in vitro* findings with MDDE and its demethylated metabolites parallel *in vivo* estrogenic activity,[7] we administered HPLC-purified MDDE and bis-OH-MDDE to immature female rats and determined the effects of these compounds on uterine ODC levels and uterotropic activity (Table 6).[8] Results (Table 6) demonstrate that, as in previous observations with methoxychlor and bis-OH-methoxychlor (5, 8, 25), MDDE as well as its bis-OH metabolite are estrogenic *in vivo*. These findings, taken in conjunction with the above *in vitro* observations, classify MDDE as a potent proestrogen.

CONCLUSION

The present findings demonstrate that technical grade methoxychlor contains two estrogenic contaminants, mono-OH-methoxychlor and mono-OH-MDDE, and two proestrogens, methoxychlor and MDDE. Both methoxychlor and MDDE were found to be metabolized by the rat hepatic monooxygenase(s) into mono- and bishydroxy derivatives, yielding four estrogenic compounds. Furthermore, other studies reveal that methoxychlor is metabolized *in vivo* to bis-OH-MDDE and bis-OH-methoxychlor in the mouse (27) and the goat (10, 28). Also, MDDE and mono-OH-MDDE are *in vivo* metabolites of methoxychlor in goats (10, 28). These findings indicate

[7] Because of insufficient quantities of purified mono-OH-MDDE, this compound was not tested *in vivo*.

[8] Uterine ornithine decarboxylase levels in response to administered chlorinated hydrocarbon pesticides appear to correlate with their respective uterotropic activities (26).

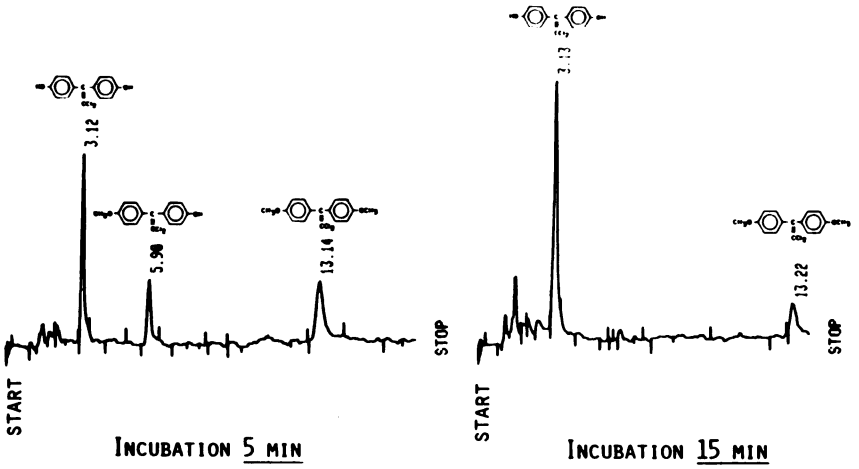

FIG. 4. *Analysis of HPLC of the in vitro metabolites of MDDE (compound 34) following incubation for 5 and 15 min*
See Experimental Procedures for methodology. Retention time and structure of metabolite or parent compound appear above the corresponding peaks; bis-OH-MDDE (3.12, 3.13 min), mono-OH-MDDE (5.90 min), and MDDE (13.14, 13.22 min).

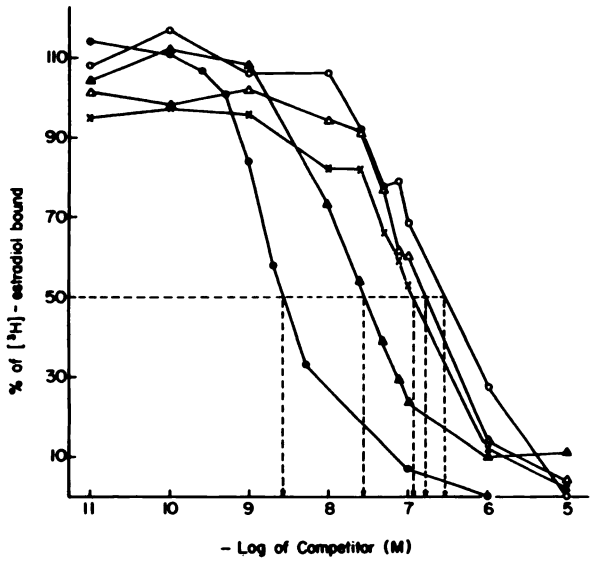

FIG. 5. *Relative binding affinity of bis-OH-MDDE, bis-OH-methoxychlor, mono-OH-MDDE (compound 35A), and mono-OH-methoxychlor (compound 37) versus unlabeled estradiol with respect to inhibition of [^{3}H]estradiol binding to rat uterine cytosol*
Conditions are as described in Experimental Procedures. ●, unlabeled estradiol; ▲, bis-OH-MDDE; ×, bis-OH-methoxychlor; △, mono-OH-MDDE; and ○, mono-OH-methoxychlor.

TABLE 5
Relative binding affinities of mono- and bisphenolic derivatives of methoxychlor and MDDE for rat uterine cytosol receptor

Compound	RBA[a]
Estradiol	100
Bis-OH-MDDE	10.0
Bis-OH-methoxychlor	2.2
Mono-OH-MDDE	1.4
Mono-OH-methoxychlor	0.8

[a] Values were calculated from Fig. 5.

a potential for *in vivo* transformation of methoxychlor into several potent estrogens and into a more potent proestrogen (MDDE). Clearly, the *in vivo* estrogenic properties of technical grade methoxychlor represent a

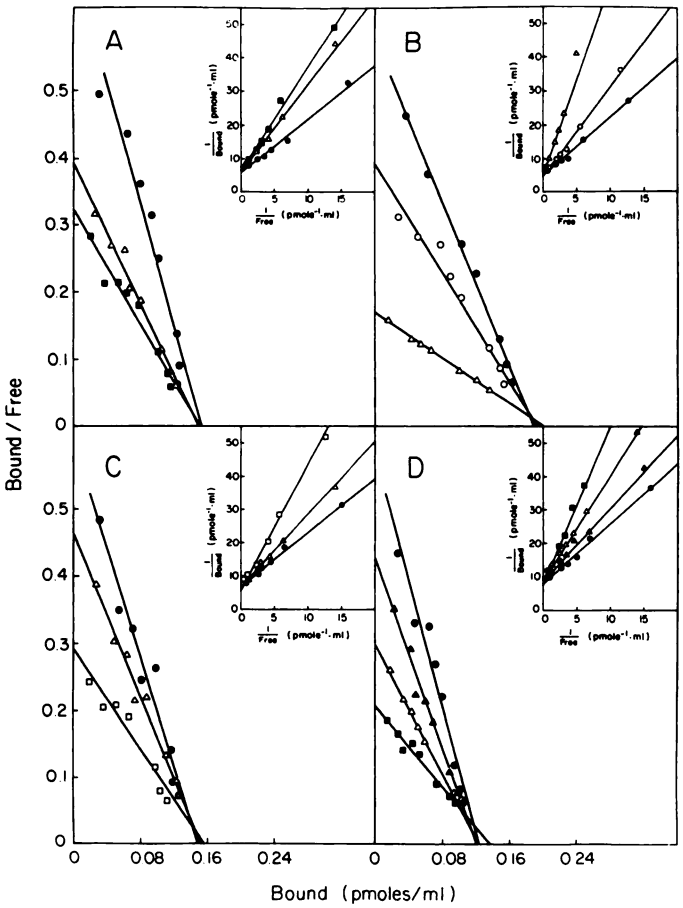

FIG. 6. *Saturation analysis of inhibition of [^{3}H]estradiol binding to rat uterine cytosol by A, mono-OH-MDDE (compound 35A); B, bis-OH-MDDE; C, mono-OH-methoxychlor (compound 37); and D, bis-OH-methoxychlor*
Each data point was determined in duplicate with subsequent analysis by Scatchard plot and double reciprocal plot (insets). Linear regression was used to fit the lines to the data. In all cases, the correlation coefficient was 0.9000 or greater. ●, control (no additions); ○, 7.7 nM; ▲, 15.4 nM; △, 38.9 nM; ■, 77.8 nM; and □, 155.6 nM.

TABLE 6

In vivo effects of HPLC-purified methoxychlor, bis-OH-methoxychlor, MDDE (No. 34), and bis-OH-MDDE on uterine ornithine decarboxylase activity and uterine weight in the immature rat

Values are means ± standard error, number of experiments in parentheses. Bis-OH-M, bis-OH-methoxychlor; ND, not determined.

Treatment		OCD activity				Uterotropic activity	
		$\bar{x}^b$	Treated/control	$\bar{x}$	Treated/control	$\bar{x}$	Treated/control
	nmol/animal	*pmol CO_2/hr/uterus*		*pmol CO_2/hr/mg protein*		*mg/100 g*[a]	
1. Control		15.8 ± 1.8 (10)		11.1 ± 1.2 (10)		75.0 ± 3.0 (10)	
Bis-OH-M	300	219 ± 49.3[g] (6)	13.8	128 ± 29.2[g] (6)	11.5	87.3 ± 3.1[e] (6)	1.2
Bis-OH-M	150	270 ± 80.6[f] (6)	17.1	136 ± 55.6[d] (6)	12.3	91.7 ± 5.4[e] (6)	1.2
Bis-OH-M	50	38.8 ± 7.2[f] (5)	2.5	23.6 ± 4.8[d] (5)	2.1	79.1 ± 4.6[c] (5)	1.1
Bis-OH-MDDE	150	329 ± 57.9[h] (5)	20.8	166 ± 32.6[h] (5)	15.0	100 ± 4.7[h] (6)	1.3
Bis-OH-MDDE	50	147 ± 20.9[h] (6)	9.3	82.2 ± 13.2[h] (6)	7.4	86.3 ± 3.0[e] (6)	1.2
Bis-OH-MDDE	10	73.7 ± 23.6[d] (6)	4.7	51.3 ± 16.9[d] (6)	4.6	76.3 ± 3.1[c] (6)	1.0
Estradiol	0.2	1980 ± 113[h] (3)	126	872 ± 74.0[h] (3)	78.5	125 ± 7.5[h] (3)	1.7
2. Control		3.8 ± 1.0 (6)		ND		77.5 ± 1.6 (7)	
Methoxychlor	8000	91.1 ± 34.3[d] (6)	23.8	ND		78.5 ± 6.1[c] (7)	1.0
Estradiol	0.2	1200 (2)	313	ND		114 ± 4.1[h] (3)	1.5
3. Control		4.8 ± 2.8 (8)		5.0 ± 3.2 (8)		78.7 ± 2.6 (8)	
MDDE	8000	205 ± 65.9[e] (6)	42.5	147 ± 45.5[f] (6)	29.2	87.3 ± 3.1[c] (6)	1.1
Estradiol	0.2	2890 ± 546[h] (4)	599	1620 ± 106[h] (4)	321	140 ± 13.5[g] (4)	1.8

[a] Uterine wt/body wt.

[b] Statistical analysis (experimental versus control) by Student's t test. p = probability that the means of treated and controls are identical; [c] not significant ($p > 0.050$); [d] $p \leq 0.050$; [e] $p \leq 0.025$; [f] $p \leq 0.010$; [g] $p \leq 0.005$; [h] $p \leq 0.001$.

complex interaction between the intrinsic estrogenic properties of the insecticide mixture and the biotransformation of specific components of that mixture. Other workers (29) have reported that, under simulated environmental conditions, some of the impurities in technical grade methoxychlor are transformed into new products, suggesting that, in an ecosystem, the estrogenic or proestrogenic properties of technical grade methoxychlor could be further modified. The *in vitro* assay which we utilized is highly sensitive and hence is particularly useful for providing information on the estrogenic or proestrogenic activities of compounds of which only small amounts are available. This assay eliminates the need for a lengthy and often equivocal determination of whether a compound is a proestrogen or an estrogen. However, whether these compounds are frank estrogens or antiestrogens cannot be determined with ease, particularly since these compounds are potential inducers of hepatic monooxygenases (30, 31) and thus could diminish estradiol action by a metabolic, nonreceptor mechanism. This study also demonstrates the difficulty encountered in trying to study environmental pollutants which are themselves contaminated by a variety of biologically active substances. Hence, it underlines the need to rigorously establish the purity of a compound under investigation.

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