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STUDIES ON THE *IN VIVO* AND *IN VITRO* ESTROGENIC ACTIVITIES OF METHOXYCHLOR AND ITS METABOLITES. ROLE OF HEPATIC MONO-OXYGENASE IN METHOXYCHLOR ACTIVATION*

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Abstract — The injection of rats with methoxychlor stimulated uterine ornithine decarboxylase (ODC) activity and caused an increase in uterine weight 7 hr after injection. The di-demethylated derivative of methoxychlor {[2,2-bis(p-hydroxyphenyl-1,1,1-trichloroethane] (HPTE)} markedly stimulated rat uterine ODC and enlarged uterine wet weight 6 hr after administration. Because we previously demonstrated that HPTE, but not methoxychlor, inhibited the binding of [^{3}H]estradiol-17β ([^{3}H]E_2) to uterine cytosolic estrogen receptor *in vitro*, we considered the possibility that the estrogenic activity of methoxychlor *in vivo* was due to biotransformation of methoxychlor. The evolution of formaldehyde occurred when methoxychlor was incubated with rat hepatic microsomes in the presence of NADPH, indicating that methoxychlor was *O*- demethylated *in vitro*. The demethylation of methoxychlor was inhibited when methoxychlor was incubated with microsomes in the presence of hexobarbital or 2-diethylaminoethyl diphenyl-propylacetate hydrochloride (SKF-525A), suggesting the involvement of mono-oxygenase. Furthermore, the demethylated products were resolved by thin-layer chromatography (t.l.c.) into three chromatographically distinct components more polar than methoxychlor. One of the products appears to be the di-demethylated derivative of methoxychlor, since it was chromatographically identical to HPTE in three t.l.c. systems. Each of the three components inhibited [^{3}H]E_2 binding to rat uterine cytosol *in vitro*; however, the metabolite with an R_f equal to that of HPTE demonstrated equal potency to HPTE with respect to suppression of [^{3}H]E_2 binding to uterine cytosol. The possible involvement of mono-oxygenase in biotransformation in methoxychlor into estrogenic metabolites *in vivo* is discussed.

Methoxychlor, the bis-*p*-methoxy derivative of DDT,† has certain properties which have led to its prosposed use as a substitute for DDT [1, 2]. Methoxychlor is less acutely toxic than DDT, has a short half-life in mammals, and is biodegradable [3–6]. In the mouse, methoxychlor is *O*-demethylated to polar mono- or bis-*p*-phenolic metabolites which are readily excreted and apparently result in a diminished toxicity [6]. The biotransformation of methoxychlor to more polar products by a variety of life forms probably also accounts for its favorable performance, with respect to DDT, in a model ecosystem [6]. However, methoxychlor appears to have certain estrogen-like properties in mammals which might be undesirable. The estrogenic activity of DDT derivatives, among these *o,p'*-DDT and methoxychlor, has been well documented [7, 8]. The administration of methoxychlor and other DDT derivatives was found to increase rat uterine glycogen [9]. Also, methoxychlor decreased [^{3}H]E_2 uptake by rat uteri *in vivo* [10] and Nelson [11] observed that methoxychlor inhibited [^{3}H]E_2 binding to rat uterine cytosol *in vitro*. However, in the studies of Bitman and Cecil [9] and of Nelson [11] the technical grade of methoxychlor was more active than the pure compound, suggesting that methoxychlor was contaminated with a material possessing estrogenic properties. We observed that even laboratory-grade methoxychlor (99 per cent pure) contained a base-soluble contaminant which interfered with *in vitro* binding of [^{3}H]E_2 to the 8S estrogen receptor in rat uterine cytosol [12]. Furthermore, we found that highly purified methoxychlor failed to suppress [^{3}H]E_2 binding to the cytosolic 8S receptor in rat uteri [12]. However, the di-demethylated metabolite of methoxychlor, 2,2-bis-(*p*-hydroxyphenyl)-1,1,1-trichloroethane (HPTE), was a potent inhibitor of [^{3}H]E_2 binding to the uterine estrogen receptor. These data suggested that methoxychlor is not active *per se* but that its estrogenic activity might be primarily due to its biotransformation to a highly active metabolite(s). Recently it has been briefly reported that incubation of methoxychlor with a liver microsomal system yielded estrogenic derivatives [13]. Although these investigators did not characterize the estrogenic derivatives, previous

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† Abbreviations used in the text are as follows: DDT, (commercial) primarily *p,p'*-DDT; *p,p'*-DDT, 2,2-bis-(*p*-chlorophenyl)-1,1,1,-trichloroethane; [^{3}H]E_2, [^{3}H]-estradiol-17β; HPTE, 2,2-bis-(*p*-hydroxyphenyl)-1,1,1-trichloroethane; ODC, ornithine decarboxylase; t.l.c., thin-layer chromatography; DCC, dextran-coated charcoal; DES, diethylstilbestrol; 3-MC, 3-methylcholanthrene; and SKF-525A, 2-diethylaminoethyl diphenylpropylacetate hydrochloride.

studies in the mouse by Kapoor *et al.* [6] suggest that these metabolites might be the demethylated derivatives of methoxychlor.

To examine the estrogenic characteristics of methoxychlor and its metabolites formed *in vitro*, we determined the binding of these compounds to the rat uterine cytosolic receptor *in vitro* and the induction of uterine ornithine decarboxylase *in vivo*.

The binding of an estrogen to the uterine cytosolic receptor has been proposed to be the initial step leading to estrogenic expression [14–16]. To demonstrate binding of a non-radiolabeled compound to the estrogen receptor, it is common to employ inhibition of [^{3}H]E_2 binding as a monitor of binding by the respective compound [17]. This procedure was employed in this study.

Ornithine decarboxylase (EC 4.1.1.17) catalyzes the metabolism of ornithine to putrescine, which is the initial and rate-limiting step in the biosynthesis of spermidine and spermine [18, 19]. In turn, it has been suggested that an alteration in ODC activity might represent an important event in biological control at the subcellular level [18, 19]. The advantage in utilizing the induction of uterine ODC as a monitor for estrogenic activities is due to the dramatic response of elevation of uterine ODC as compared with other indicators of estrogenic activity [9, 20–23].

MATERIALS AND METHODS

Animals. Female 40–50 g (20- to 28-days-old) and 200–250 g male Sprague–Dawley-CD rats were obtained from Charles River Breeding Laboratories Inc., and were fed Charles River Rat Formula and allowed water *ad lib.* Bilateral ovariectomy was accomplished under ether anaesthesia 9–10 days before use. Prior to experimentation, animals were randomized according to weight, so that the weight range was controlled within each group.

Materials. The following compounds were purchased: Tris (hydroxymethyl) aminomethane (Fisher); NADP, glucose 6-phosphate, glucose 6-phosphate dehydrogenase and diethylstilbestrol (Sigma); semicarbazide hydrochloride (Aldrich); 3-methylcholanthrene (Eastman); hexobarbital sodium (Winthorp Laboratories); ethylmorphine hydrochloride (Merck); DL[1-^{14}C]ornithine hydrochloride (54.08 mCi/m-mole); [2,4,6,7-^{3}H]estradiol-17β (100 Ci/m-mole); Liquifluor and Aquasol (New England Nuclear, Boston, MA); and [ring-UL-^{14}C]methoxychlor (4.48 mCi/m-mole) (California Bionuclear Corp.). All radiolabeled compounds had a radiochemical purity of 99+ per cent. The SKF-525A hydrochloride was a gift from Smith Kline & French Laboratories. All other compounds utilized in this study were of reagent grade quality.

DDT analogs. The 2,2-bis-(*p*-hydroxyphenyl)-1,1,1-trichloroethane (HPTE) was generously supplied by Drs. Toshio Fujita (Kyoto University, Japan) and James R. Sanborn (Illinois Natural History Survey, Urbana, IL). The 2,2-bis-(*p*-chlorophenyl)-1,1-dichloroethylene (*p,p'*-DDE) was from Aldrich Chemical Co. (99+ per cent pure). Laboratory grade 2,2-bis-(*p*-methoxyphenyl)-1,1,1-trichloroethane (methoxychlor) was purchased from Chem Service, Inc., West Chester, PA, and was employed as obtained from the supplier (99 per cent pure) or was subjected to the following purification procedure.

Purification of methoxychlor. A mixture of 5% NaOH (w/v) and methoxychlor (5 mg/ml) was subjected to vigorous vortexing for 5 min and was extracted three times with hexane. Each extraction used a volume of hexane equivalent to twice the volume of 5% sodium hydroxide. The hexane extracts were pooled and washed with distilled water until the water fractions were no longer basic. Anhydrous sodium sulfate was added to the washed hexane and 30 min later the hexane was decanted and evaporated under a stream of nitrogen. The resulting material was redissolved in hexane, and the methoxychlor was recovered by recrystallization from hexane. The authenticity of the methoxychlor thus obtained was established by nuclear magnetic resonance (n.m.r.) analysis. Methoxychlor subjected to base-washing by the above procedure is referred to as purified methoxychlor in the text. The [^{14}C]-methoxychlor was also base-washed prior to use in this study. When the [^{14}C]methoxychlor was chromatographed in the three solvent systems described below and the localization of radioactivity was determined by radiochromatogram scanning with confirmation by liquid scintillation counting, it was found to have a radiochemical purity of 99+ per cent.

Thin-layer chromatography. Pre-coated, 5 × 20 cm glass supported, silica gel 60, t.l.c. plates (EM reagents) with a layer thickness of 0.25 mm were employed. Development was in three solvent systems: (1) ether–hexane (3 : 1); (2) petroleum ether–chloroform–methanol (3 : 2 : 1); and (3) hexane–acetone (4 : 1). Solvent systems 1 and 2 were previously described by Kapoor *et al.* [6]. Solvent system 3 was originally suggested by the supplier of the [^{14}C]methoxychlor and was modified by us to include two developments of the t.l.c. plate to achieve optimal separation of metabolites. A radiochomatogram scanner (Vangard 930) employing a scanning rate of 8 in./hr was used to detect the radiolabeled products. Occasionally, products were visualized by placing the chromatogram in a tank of iodine vapor.

ODC determination. The ODC assay was by a previously described modification [22] of the methods of Kaye *et al.* [21] and Kobayashi *et al.* [24]. Ovariectomized rats were employed because they were found to have a lower basal level of uterine ODC than 21-day intact immature rats [22]. All uterine ODC assays were performed at the time of peak ODC activity for the compound under study. Peak ODC activities for estradiol, methoxychlor and HPTE, when injected i.p. in corn oil, were determined in preliminary experiments and were found to occur 5, 6 and 7 hr after administration respectively. The injection of corn oil alone was reported earlier to have no effect on uterine ODC activity [22]. Because uterine ODC activity was determined in the ovariectomized rat, ovariectomized rats were also used to prepare microsomes for *in vitro* metabolism studies.

Preparation of microsomes. Ovariectomized female

rats were injected i.p. with a daily dose of 100 mg/kg of *p,p'*-DDE in 0.2 ml corn oil for 2 days. Male rats (90–100 g) were injected i.p. with 3-methylcholanthrene, 25 mg/kg in corn oil, daily for 3 days. All rats were sacrificed 48 hr after the last injection. Control rats were given corn oil only. Liver microsomes were prepared as previously described [25] from ovariectomized female and intact male rats. (Male rat liver microsomes were occasionally used because they generate greater amounts of metabolites.)

Metabolism of methoxychlor. A 2-ml incubation mixture containing Tris buffer (pH 7.5, 50 mM), $MgCl_2$ (5 mM), glucose-6-phosphate (12 mM), NADP (0.4 mM), glucose-6-phosphate dehydrogenase (2 units), semicarbazide (1 mM) and a microsomal preparation equivalent to 5 mg protein was utilized. The incubation was started by adding 1 mM purified methoxychlor in 10 μl acetone and the mixture was allowed to shake in an incubator (Dubnoff) at 37° for 30 min. When ethylmorphine (8 mM) was used as a substrate, incubation was for 10 min. When formaldehyde evolution was to be measured, the reaction was stopped by adding 0.6 ml of 20% $ZnSO_4$ (w/v) and 0.6 ml of saturated $Ba(OH)_2$ to the incubation mixture and centrifuging for 10 min. The resulting supernatant fraction was used for formaldehyde determination by the method of Nash [26]. When demethylated derivatives of methoxychlor were to be extracted, the reaction was stopped by adding 2 ml of 5 N NaOH and the resulting mixture was immediately extracted three times with 8 ml hexane to remove the unmetabolized methoxychlor. The resulting aqueous fraction was acidified with concentrated HCl (to pH 1–2) and extracted twice with 8 ml of anhydrous ethyl ether. The ether extracts were pooled and evaporated to dryness under a stream of nitrogen; the resulting material was taken up in an appropriate volume of ethanol and employed in studies of inhibition of $[^3H]E_2$ binding to uterine cytosolic proteins.

Metabolism of [^{14}C]methoxychlor. A 2-ml mixture containing sodium phosphate buffer (pH 7.4, 50 mM), $MgCl_2$ (11.2 mM), glucose 6-phosphate (12 mM), NADP (0.4 mM), glucose 6-phosphate dehydrogenase (2 units) and microsomal preparations equivalent to 8 mg protein was employed. The reaction was started by adding methoxychlor (2 μmoles/200,000 dis./min) in 10 μl acetone and the mixture was allowed to shake in an incubator at 37° in an atmosphere of air for 30 min. The reaction was terminated by adding 2 ml of 5 N NaOH and the resulting mixture was immediately extracted as follows. The unmetabolized methoxychlor was removed from the incubation mixture by extracting three times with 8 ml hexane and the remaining aqueous fraction was acidified with concentrated HCl (to pH 1–2) and extracted twice with 8 ml of anhydrous ether. The ether extracts were pooled and evaporated to dryness under a stream of nitrogen. The resulting material was taken up in acetone and subjected to thin-layer chromatography. In preliminary experiments the localization of metabolites on the thin-layer plates was determined by radiochromatogram scanning with confirmation by subsequent liquid scintillation counting in which appropriate regions of the chromatograms were scraped off and the radioactivity in the silica gel was counted in 10 ml Liquifluor containing 1 ml methanol. To isolate metabolites, the radioactive bands were localized with a radiochromatogram scanner and the silica gel was scraped into a conical test tube. The silica gel was extracted four times with 4 ml ethanol. The ethanol extracts were pooled, evaporated under a stream of nitrogen and redissolved in the desired volume of ethanol. Quantification of the metabolites was by liquid scintillation counting of aliquots of ethanolic extracts and was based on the specific activity of the methoxychlor (substrate).

Determination of [^3H]E_2 binding. The determination of $[^3H]E_2$ binding to immature rat uterine cytosol was by sucrose density gradient sedimentation analysis and a dextra-coated charcoal assay as previously modified [17] from the method of McGuire *et al.* [27, 28].

Protein determination. Protein was determined by the method of Lowry *et al.* [29] employing the modifications recommended by Stauffer [30]. Bovine serum albumin was used as a standard.

Table 1. Effect of methoxychlor on uterine ornithine decarboxylase activity (ODC) and uterine size in the ovariectomized rat*

Treatment (methoxychlor) (mg/animal)	ODC activity (pmoles CO_2/hr/mg protein)		Uterotropic activity [Uterine wt (mg)/body wt (100 g)]	
	$\bar{X} \pm$ S.E.	T/C†	$\bar{X} \pm$ S.E.	T/C
	3.8 ± 0.6 (6)		27.2 ± 0.7 (33)	
3	58.5 ± 23.7‡ (7)	15.3	28.5 ± 1.1§ (28)	1.0
10	521.6 ± 125.0‖ (6)	136.0	41.7 ± 1.7¶ (24)	1.5

* Rats were injected i.p. with laboratory grade methoxychlor in 0.2 ml corn oil (controls received 0.2 ml corn oil) and were sacrificed 7 hr later. The uteri were removed, weighed and employed in the determination of ODC activity. ODC values represent the mean of six to seven determinations (N) with each determination representing a pool of three to six uteri. Uterotropic values represent the mean of the individual values for each animal (N). ODC and uterotropic activities were determined from the same animals. Student's *t*-test was employed.

† Ratio of treated values to control values.

‡ $P \leq 0.05$, with respect to control.

§ Not significant.

‖ $P \leq 0.005$, with respect to control.

¶ $P \leq 0.001$, with respect to control.

Table 2. Effect of laboratory grade methoxychlor and purified methoxychlor on uterine ornithine decarboxylase activity (ODC) and uterine size in the ovariectomized rat*

Treatment (methoxychlor)	ODC activity (pmoles CO_2/hr/mg protein)		Uterotropic activity [uterine wt (mg)/body wt (100 g)]	
	$\bar{X} \pm$ S. E.	T/C†	$\bar{X} \pm$ S. E.	T/C
	11.4 ± 3.5 (5)		35.8 ± 1.5 (21)	
Laboratory grade	161.8 ± 39.3‡ (5)	14.3	58.0 ± 3.8§ (15)	1.6
Purified	78.8 ± 20.2‖,¶ (5)	6.9	50.2 ± 2.4§,¶ (15)	1.4

* Rats were injected i.p. with laboratory grade or purified methoxychlor (5 mg) in 0.2 ml corn oil (controls received 0.2 ml corn oil) and were sacrificed 7 hr later. Uteri were removed, weighed and employed in the determination of ODC activity. ODC values represent the mean of five determinations (N) with each determination representing a pool of three to six uteri. Uterotopic values represent the mean of the individual values for each animal (N). ODC and uterotropic activities were determined from the same animals. Student's *t*-test was employed.

† Ratio of treated to control values.

‡ $P \leq 0.005$ with respect to control.

§ $P \leq 0.001$, with respect to control.

‖ $P \leq 0.025$, with respect to control.

¶ P value with respect to laboratory grade methoxychlor > 0.05 (not significant).

RESULTS AND DISCUSSION

As depicted in Table 1, the administration of 3 and 10 mg of laboratory grade methoxychlor/rat caused a 15- and 136-fold increase (T/C) in uterine ODC activity respectively. In the same animals, both doses of methoxychlor also caused an increase in uterine weight. However, only the higher dose (10 mg) caused a significant increase in uterine weight, suggesting that ODC activity was a better measure of marginal estrogenic activity than the uterotropic activity. The report from earlier studies that technical grade methoxychlor was more active as an estrogen than the pure insecticide [9, 11] and the observation in our laboratory that even 99 per cent pure (laboratory grade) methoxychlor contained an impurity that inhibited [^{3}H]E_2 binding to uterine cytosol *in vitro* [12], prompted us to compare the effect of laboratory grade and purified methoxychlor on uterine ODC activity and uterotropic activity. The results presented in Table 2 demonstrated that both preparations of methoxychlor markedly elevated uterine ODC activity with respect to control values. In both cases the uterotropic response was also elevated with respect to the control. The laboratory grade methoxychlor appeared to be more active than the purified methoxychlor in elevating both uterine ODC and the uterine to body weight ratio. However these differences were not statistically significant. Most importantly, these results indicate that purified methoxychlor is estrogenic *in vivo* itself. The effect of HPTE, the di-demethylated metabolite of methoxychlor [6], on rat uterine (ODC) activity is shown in Table 3. A dose as low as 100 μg/rat of HPTE markedly elevated uterine ODC activity. Uterotropic activity was also elevated. However, lower doses of HPTE (10 and 1 μg) failed to elevate uterine ODC and uterotropic activity; estradiol (0.1 μg) markedly elevated uterine ODC and uterotropic activity. Our results with HPTE *in vivo* are in agreement with an earlier observation by Fisher *et al.* [31] that HPTE maintained estrus in ovariectomized rats. However, by contrast, these investigators observed no such activity with methoxychlor.

From these results, we conclude that both methoxychlor and HPTE are estrogenic *in vivo*. However,

Table 3. Effect of 2,2-bis-(*p*-hydroxyphenyl)-1,1,1-trichloroethane (HPTE) on uterine ornithine decarboxylase activity (ODC) and uterine size in the ovariectomized rat*

Treatment (HPTE) (μg/animal)	ODC activity (pmoles CO_2/hr/mg protein)		Uterotropic activity [uterine wt (mg)/body wt (100 g)]	
	$\bar{X} \pm$ S. E.	T/C	$\bar{X} \pm$ S. E.	T/C
	11.0 ± 3.7(5)		34.2 ± 1.5(19)	
500	607.5 ± 55.0‡ (6)	55.1	45.0 ± 2.1‡ (18)	1.3
100	185.6 ± 40.4‡ (10)	16.8	39.5 ± 1.4§ (30)	1.2
10	10.9 ± 3.6† (4)	1.0	35.9 ± 2.4† (12)	1.0
1	6.5 ± 1.9† (3)	0.6	36.0 ± 1.9† (9)	1.0
E_2,0.1	635.1 ± 96.0‡ (4)	57.6	60.8 ± 4.9‡ (11)	1.8

* Animals were injected i.p. with HPTE in 0.2 ml corn oil (controls received 0.2 ml corn oil) and were sacrificed 6 hr later. Uteri were removed, weighed and employed in ODC determination. ODC values represent the mean of three to ten determinations (N) with each determination representing a pool of three to six uteri. Conditions and notations are as in Table 1.

† Not significant.

‡ $P \leq 0.001$, with respect to control.

§ $P \leq 0.010$, with respect to control.

Table 4. Demethylation of methoxychlor and ethylmorphine by liver microsomes from ovariectomized rat

	Pretreatment	Substrate	Inhibitor	Activity (nmoles formaldehyde/mg protein)
Experiment 1*	*p,p'*-DDE	Methoxychlor		14.5
	p,p'-DDE	Methoxychlor	Hexobarbital	3.2
	p,p'-DDE	Methoxychlor	SKF-525A	1.0
Experiment 2†		Methoxychlor		11.1 ± 0.8
	p,p'-DDE	Methoxychlor		21.7 ± 1.0‡
		Ethylmorphine		19.5 ± 2.7
	p,p'-DDE	Ethylmorphine		69.6 ± 3.3§

* Microsomes were prepared from three pooled livers from *p,p'*-DDE-pretreated rats as described in Materials and Methods. Duplicate incubations were conducted with the same microsomal preparation in the absence or presence of hexobarbital (1.0 mM) or SKF-525A (1.0 mM); methoxychlor (0.1 mM).

† Values represent the mean ± S. E. of duplicate incubations of microsomes from three animals/group. Procedure is described in Materials and Methods.

‡ $P \leq 0.005$, with respect to control.

§ $P \leq 0.001$, with respect to control.

we previously observed that HPTE, but not methoxychlor, markedly suppressed [^{3}H]E_2 binding to the 8S estrogen receptor from rat uterine cytosol [12]. These differences between the *in vivo* and *in vitro* activity of methoxychlor suggest that, *in vivo*, methoxychlor is bio-activated to an estrogenic metabolite. The observations in this study that HPTE is a more potent estrogen *in vivo* than methoxychlor and that the peak of ODC activity for HPTE is 1 hr earlier (6 hr) than for methoxychlor (7 hr) suggest that HPTE is an active metabolite of methoxychlor.* This proposition is further supported by the report that HPTE as well as the mono-demethylated derivative of methoxychlor [2-(*p*-hydroxyphenyl)-2-(*p*-methoxyPhenyl)-1,1,1-trichloroethane is a metabolite of methoxychlor in the mouse [6].

To further study this problem, we incubated methoxychlor with rat liver microsomes in the presence of NADPH and measured the formaldehyde formation. The results depicted in Table 4, experiment 2, indicate that methoxychlor is *O*-demethylated *in vitro* by rat liver microsomes and that methoxychlor demethylase activity is enhanced by using microsomes prepared from animals pretreated with *p,p'*-DDE, a non-estrogenic DDT analog known to induce hepatic mono-oxygenase activity [32, 33]. In the same experiment, ethylmorphine demethylase activity increased when microsomes from *p,p'*-DDE-pretreated rats were employed, indicating that the control and *p,p'*-DDE-induced microsomes employed in this experiment were active toward a classical substrate of mono-oxygenase. Under identical incubation conditions, *p*-nitroanisole was *O*-demethylated and the demethylase activity was enhanced by employing *p,p'*-DDE-induced microsomes (data not shown).

In experiment 1 (Table 4), the addition of hexobarbital or SKF-525A to the incubation mixture inhibited methoxychlor demethylase activity by 77.9 and 93.1 per cent respectively. This is consistent with the observation that methoxychlor inhibited hexobarbital hydroxylase activity [34] of male rat liver microsomes in a kinetically competitive manner.† From these experiments it was concluded that methoxychlor is *O*-demethylated *in vitro* and that this biotransformation probably involves the mono-oxygenase system. However, the nature of the methoxychlor demethylase activity must be further characterized to establish the involvement of mono-oxygenase with certainty.

The effect of the demethylated derivative(s) of methoxychlor on the *in vitro* binding of [^{3}H]E_2 to rat uterine cytosolic estrogen receptors is depicted in Table 5. Aliquots of the ether extracts (acidic metabolites) of the various incubation mixtures were incubated with rat uterine cytosol in the presence of [^{3}H]E_2 and were subjected to sucrose gradient sedimentation or DCC analysis. An extract of the complete incubation mixture (microsomes, an NADPH-generating system, and methoxychlor), representing a 400 nM concentration of demethylated derivative(s) of methoxychlor, caused a 20.4 per cent inhibition of [^{3}H]E_2 binding to the 8S (estrogen receptor) region of the sucrose gradient (Table 5, column A). However, extracts of equivalent volumes of incubation mixtures lacking methoxychlor or an NADPH-generating system or microsomes failed to suppress 8S [^{3}H]E_2 binding. A 200 nM concentration of demethylated derivative(s) of methoxychlor (extracted from the complete incubation mixture) caused a 52.2 per cent inhibition of [^{3}H]E_2 binding as determined by DCC analysis (Table 5, column B).

Extracts of equivalent volumes of incubation mixtures lacking either methoxychlor or an NADPH-generating system inhibited [^{3}H]E_2 binding by 7.4 and 8.7 per cent respectively (Table 5, column B). The difference in the percent of inhibition of [^{3}H]E_2 binding between the sucrose gradient sedimentation analysis and the DCC analysis can be explained by the different concentrations of [^{3}H]E_2 employed in the two assays. The sucrose gradient sedimentation analysis was performed under saturating conditions with respect to [^{3}H]E_2 concentration (4 nM), whereas the DCC assay employed nonsaturating concentrations of [^{3}H]E_2 (0.2 nM). Therefore, the lower concentration of [^{3}H]E_2 employed in the DCC assay permitted a more facile competition for

* The differences in peak ODC activities could have other explanations, such as marked differences in physical-chemical properties.

† D. Kupfer and J. Navarro, unpublished observations.

Table 5. Inhibition of [^{3}H]estradiol binding to rat uterine cytosolic estrogen receptor by demethylated derivative(s) of purified methoxychlor*

Incubation conditions	% Inhibition of [^{3}H]estradiol binding A†	B‡
Complete mixture	20.4 (400 nM) §	52.2 (200 nM) §
Minus methoxychlor	0	7.4
Minus NADPH-generating system	0	8.7
Minus microsomes	0	0

* Derivatives were isolated from incubations of purified methoxychlor with liver microsomes from 3-MC-pretreated male rats as described in Materials and Methods.

† Inhibition of [^{3}H]E_2 binding was determined by sucrose density gradient sedimentation analysis in the presence of 4 nM [^{3}H]E_2.

‡ Inhibition of [^{3}H]E_2 binding was determined by DCC analysis in the presence of 0.2 nM [^{3}H]E_2.

§ Concentration of methoxychlor derivative(s) was based on formaldehyde formation.

[^{3}H]E_2-binding sites by the demethylated derivatives of methoxychlor and resulted in a greater inhibition (52.2 per cent) of [^{3}H]E_2 binding to uterine cytosol at a lower concentration (200 nM) of demethylated derivative(s) of methoxychlor (Table 5, column B). In preliminary experiments employing sucrose gradient sedimentation analysis, it was found that higher concentrations of the extracts lacking either methoxychlor or an NADPH-generating system also suppressed [^{3}H]E_2 binding and that it was necessary to perform serial dilutions on the extracts until a volume of extract equivalent to the concentration shown in Table 5, column A (400 nM) was reached before a distinction could be made between the inhibition of [^{3}H]E_2 binding caused by a "factor" in the microsomes and the demethylated derivative(s) of methoxychlor.

In column B of Table 5, the inhibition of [^{3}H]E_2 binding caused by the extracts lacking methoxychlor or an NADPH-generating system probably represents a residual microsomal factor which was detected because the DCC assay was more sensitive, being carried out under nonsaturating conditions with respect to the concentration [^{3}H]E_2. It was concluded that the demethylation products of methoxychlor inhibit [^{3}H]E_2 binding to the 8S estrogen receptor of rat uterine cytosol and that the inhibition of binding takes place under both saturating and nonsaturating conditions with respect to [^{3}H]E_2 concentration. It was also found, unexpectedly, that rat liver microsomes contain a factor, the nature of which is unknown, that can inhibit [^{3}H]E_2 binding to rat uterine cytosol.

To obtain preliminary information on the nature of the demethylated derivatives of methoxychlor, we determined the chromatographic behavior, by t.l.c., of the acidic derivatives (ether extract) of the complete microsomal incubation mixture. In three different t.l.c. solvent systems the extract was resolved into three chromatographically distinct compounds.* The R_f values of the compounds in solvent system 3 are shown in Table 6. Compound 2 and HPTE were chromatographically identical in this solvent system. This was also the case with the other two chromatography systems: solvent system 1 R_f (HPTE, 0.27; compound 2, 0.30); and solvent system 2, R_f (HPTE, 0.37; compound 2, 0.36). In a given incubation, under the conditions described in Materials and Methods, about 10 per cent (200 nmoles product) of the methoxychlor was converted to compounds 1, 2 and 3 which were formed in 62, 16 and 21 per cent respectively.

When the three compounds were eluted from the t.l.c. plate and 100 and 400 nM concentrations of each compound were incubated with rat uterine cytosol in the presence of [^{3}H]E_2, the binding characteristics depicted in Table 6 were obtained. All three compounds suppressed [^{3}H]E_2 binding to rat uterine cytosol under nonsaturating conditions with

Table 6. Inhibition of [^{3}H]estradiol binding to rat uterine cytosolic estrogen receptor by isolated metabolites of [^{14}C]methoxychlor

Compound*	R_f†	% Inhibition of [^{3}H]estradiol binding‡
1	0.33	37.7 § (100)
		56.6 (400)
2	0.13	32.6 (100)
		78.7 (400)
3	0 (Origin)	4.4 (100)
		42.0 (400)
Methoxychor	0.63	0 (800)
HPTE	0.10	35.4 (100)
		81.3 (400)

* Compounds 1–3 were isolated from incubations of [^{14}C]methoxychlor with liver microsomes from male rats as described in Materials and Methods.

† Values were determined by t.l.c. in solvent system 3 described in Materials and Methods.

‡ Binding inhibition was determined by DCC analysis in the presence of 0.2 nM [^{3}H]E_2. DES (400 nM) gave 94.9 per cent inhibition of [^{3}H]E_2 binding.

§ All values represent an average of duplicate determinations. Values in parentheses represent concentrations (nM) of the compound used.

* It has not been established whether each radioactive zone represents a single compound; this term is used for operational convenience.

respect to $[^3H]E_2$ concentration. Of the three metabolites, compound 3 (most polar) was the least potent inhibitor of $[^3H]E_2$ cytosolic binding. Compound 2 and HPTE at two concentrations (100 and 400 nM) were equally potent in suppressing $[^3H]E_2$ binding to uterine cytosol. The failure of 800 nM methoxychlor to suppress $[^3H]E_2$ binding to rat uterine cytosol (Table 6) confirms our earlier observation that methoxychlor is inactive with respect to the *in vitro* inhibition of $[^3H]E_2$ binding [12]. The identical chromatographic behavior of compound 2 and HPTE, the similar binding characteristics of the two compounds, and the report that HPTE is a methoxychlor metabolite in the mouse (6) suggest that compound 2 and HPTE are identical. The chromatographic polarity of compound 1 (least polar of the three metabolites) and the stoichiometric relationship between formaldehyde formation and the total amount of polar ^{14}C-labeled metabolites generated by microsomal incubation support the speculation that compound 1 is the mono-demethylated derivative of methoxychlor. The mono-demethylated derivative of methoxychlor is also an *in vitro* metabolite of methoxychlor in the mouse [6]. The polarity of compound 3 (most polar of the three metabolites) suggests that it possesses three hydroxyl groups. The hydroxyl groups could result from a combination of *O*-demethylation and direct hydroxylation of the ring systems.* However, the metabolites shown in Table 6 must be further characterized before their identity can be established. The suppression of $[^3H]E_2$ binding to uterine cytosol by the isolated metabolites of methoxychlor (Table 6) diminishes the possibility that the suppression of $[^3H]E_2$ binding shown in Table 5 by the demethylated derivatives from the *in vitro* incubation of methoxychlor is an artifact of the binding factor present in rat liver microsomes. Because the metabolites shown in Table 6 were generated by liver microsomes prepared from male rats, it was necessary to rule out the possibility of a sex-dependent difference in the nature of the metabolites. When $[^{14}C]$methoxychlor was incubated with liver microsomes prepared from control or *p,p'*-DDE-pretreated ovariectomized rats, the acidic metabolites isolated from the incubation mixtures were chromatographically identical to the metabolites shown in Table 6, indicating that the *in vitro* metabolites of methoxychlor are the same in both sexes of the rat.

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*The ultraviolet spectrum of HPTE in ethanol showed an elevated plateau at 270–278 nm. Similar spectra were also obtained with the three compounds, further indicating that they are phenolic derivatives.

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