Allergy Division ELLIOTT MIDDLETON, JR.*
Departments of Medicine and Pediatrics
State University of New York at Buffalo
Buffalo, NY 14214, *U.S.A.*

Allergy Division GARY DRZEWIECKI
State University of New York at Buffalo
Buffalo, NY 14214, *U.S.A.*

Department of Biochemical Pharmacology DAVID TRIGGLE
School of Pharmacy
State University of New York at Buffalo
Buffalo, NY 14214, *U.S.A.*

REFERENCES

1. A. Fleckenstein, *A. Rev. Pharmac. Toxic.* **17**, 149 (1977).
2. L. Rosenberger and D. J. Triggle, in *Calcium, Calcium Translocation and Specific Calcium Antagonists* (Eds. G. B. Weiss), pp. 3–31. Plenum Press, New York (1978).
3. T. B. Bolton, *Physiol. Rev.* **59**, 666 (1979).
4. G. Haeusler, *J. Pharmac. exp. Ther.* **180**, 672 (1972).
5. H. J. Schumann, B. D. Gorlitz and T. Wagner, *Naunyn-Schmiedeberg's Archs. Pharmac.* **289**, 409 (1975).
6. L. B. Rosenberger, M. K. Ticku and D. J. Triggle, *Can. J. Physiol. Pharmac.* **57**, 333 (1979).
7. D. J. Triggle and V. C. Swamy, *Chest* **78**, (Suppl.), 174 (1980).
8. C. Y. Chiou and M. H. Malagodi, *Br. J. Pharmac.* **53**, 278 (1978).
9. S. Graves, K. L. Dretchen and G. O. Kruger, *Eur. J. Pharmac.* **47**, 29 (1978).
10. J. C. Foreman, J. L. Mongar and B. D. Gomperts, *Nature, Lond.* **245**, 249 (1973).
11. W. W. Douglas, *Biochem. Soc. Symp.* **39**, 1 (1974).
12. B. D. Gomperts, in *Receptors and Recognition* (Eds. P. Cuatrecasas and M. F. Greaves), pp. 45–102. Chapman & Hall, London (1976).
13. H. R. Bourne, R. I. Lehrer, L. M. Lichtenstein, G. Weissman and R. Zurier, *J. clin. Invest.* **52**, 698 (1973).
14. M. H. Malagodi and C. Y. Chiou, *Eur. J. Pharmac.* **25**, 33 (1974).
15. A. Payne, *Ph.D. Thesis*, Chap. 8, London University, (1977).
16. C. M. S. Fewtrell and B. D. Gomperts, *Nature, Lond.* **265**, 635 (1977).
17. C. M. S. Fewtrell and B. D. Gomperts, *Biochim. biophys. Acta* **469**, 52 (1977).
18. E. Middleton, Jr., G. Drzewiecki and D. KrishnaRao, *J. Immun.*, in press.
19. C. Robinson and B. V. R. Sastry, *J. med. Chem.* **15**, 870 (1972).
20. L. M. Lichtenstein and A. G. Osler, *J. exp. Med.* **120**, 507 (1964).
21. C. D. May, M. Lyman, R. Alberto and J. Cheng, *J. Allergy* **46**, 12 (1970).
22. P. A. Shore, A. Burkhalter and V. H. Cohen, Jr., *J. Pharmac. exp. Ther.* **127**, 182 (1959).
23. L. Hurwitz and A. Suria, *A. Rev. Pharmac.* **11**, 303 (1971).
24. R. Casteels, T. Godfraind and J. C. Ruegg, in *Excitation–contraction Coupling in Smooth Muscle.* Elsevier, North-Holland, Amsterdam (1978).
25. J. W. Putney, *Pharmac. Rev.* **30**, 209 (1978).

* Correspondence should be sent to: Elliott Middleton Jr., M.D., Allergy Division, Buffalo General Hospital, 100 High St, Buffalo, NY 14203, U.S.A.

Biochemical Pharmacology, Vol. 30, No. 20, pp. 2869–2871, 1981.
Printed in Great Britain.

0006-2952/81/202869-03 $02.00/0

Estrogenic activities of methoxychlor metabolites

(*Received* 5 *February* 1981; *accepted* 22 *April* 1981)

The DDT substitute, methoxychlor [2,2-bis(*p*-methoxyphenyl)-1,1,1-trichloroethane], has been shown to have weak estrogenic activity *in vivo* in experimental animals [1–3]. The parent compound does not have significant affinity for the estrogen receptor in *in vitro* assays [4–7]; however, incubation of methoxychlor with rat liver microsomal preparations in the presence of NADPH increases the ability of methoxychlor to inhibit [^{3}H]estradiol binding to rat uterine receptor [6–9] or to elicit translocation of the receptor to the nucleus [7]. These observations strongly suggest that a metabolite(s) of methoxychlor might account for its *in vivo* estrogenic activity. Two principal metabolites of methoxychlor are the demethylated derivatives [10]: (1) 2-(*p*-hydroxyphenyl)-2-(*p*-methoxyphenyl)-1,1,1-trichloroethane (mono-phenol), and (2) 2,2-bis(*p*-hydroxyphenyl)-1,1,1-trichloroethane (bis-phenol). This communication describes the *in vitro* affinities of these metabolites for the rat uterine estrogen receptor. A preliminary report of this work has appeared [11].

Materials and methods

With the exception of the chemical synthesis of the major methoxychlor metabolites, the materials were as described previously [4]. Following the procedure of Kapoor *et al.* [10], freshly distilled chloral (5 ml) and 7.4 g of aluminum chloride were added to 9.4 g of phenol in 250 ml of chloroform cooled to 0°. The mixture was stirred for 5 hr at 0°,

overnight at room temperature, and washed with 200 ml of water. The chloroform layer was dried over sodium sulfate, and concentrated *in vacuo* to 20 ml. A crystalline precipitate was collected by filtration and washed with benzene. Recrystallization from benzene yielded 550 mg of the bis-phenol (m.p. 203°), as confirmed by mass spectral analysis. The bis-phenol (450 mg) in 10 ml of methanol was treated dropwise with stirring at 0° with an ether solution of diazomethane (prepared from 1 g of Diazald, Aldrich Chemical Co., Milwaukee, WI). Stirring was continued for 30 min at 0°, after which the ether was removed by evaporation. The residue was separated on a preparative silica gel thin-layer plate (Analtech, Newark, DE) in chloroform–acetone (9:1). A band at R_f 0.4 was eluted with methanol, filtered, and evaporated, yielding 56 mg of the mono-phenol as determined by mass spectral analysis. The starting material (230 mg) was recovered at R_f 0.2. Ring, uniformly labeled [^{14}C]methoxychlor (4.0 mCi/mmole) was obtained from California Bionuclear Corp. (Sun Valley, CA).

The methods for measuring 17β-[6,7-^{3}H]estradiol ([^{3}H]estradiol) binding to rat uterine supernatant protein using the dextran-coated charcoal procedure were described previously [4]. The [^{3}H]estradiol concentration was 2 nM in the binding assay. Alcoholic solutions of methoxychlor (recrystallized from ethanol), the mono- and bis-phenol derivatives, and diethylstilbestrol (DES) were added to the [^{3}H]estradiol binding assay to give a final ethanol concentration of 4%.

Microsomal pellets from fresh liver (100–200 g female, Sprague–Dawley rats) were prepared by homogenizing the tissue (20%) in 0.25 M sucrose, 10 mM Tris–HCl, and 1.5 mM EDTA (pH 7.4). The supernatant fraction obtained after centrifugation at 9,000 *g* at 4° for 20 min was further separated by centrifugation at 105,000 *g* for 45 min. The 105,000 *g* pellet was resuspended in sucrose–Tris solution and 25 mg equivalent wet weight of liver was incubated for 1 hr at 37° in the presence or absence of 2.25 mM NADPH. [^{14}C]Methoxychlor in ethanol was added at a concentration of 50 ppm (0.14 mM) in a final volume of 2 ml of 100 mM phosphate buffer (pH 7.4). The reaction was stopped by boiling for 5 min, and the precipitated proteins were removed by centrifugation. The solution was cooled, acidified, and extracted with an equal volume of chloroform. The extraction of methoxychlor and the mono- and bis-phenols was virtually complete using this procedure. The extract was evaporated under nitrogen and analyzed by high pressure liquid chromatography (h.p.l.c.) or thin-layer chromatography (t.l.c.).

Methoxychlor was separated from its mono- and bis-phenolic metabolites using a Waters Associates model 204 HPLC with a model 660 solvent gradient programmer and a model 440 UV absorbance detector. Chloroform, at a flow rate of 1 ml/min, eluted methoxychlor at 3 min and the mono-phenol at 5 min from a μPorasil column (4.6 mm × 25 cm; Waters Associates, Milford, MA). After 5 min, elution with 5% methanol in chloroform at a flow rate of 1 ml/min was begun, and the bis-phenol eluted with a retention time of 14.5 min. Absorbance at 254 nm was recorded with a Hewlett–Packard model 3380A digital electronic integrator. Fractions of the eluate were collected at 1-min intervals with a Gilson Medical Electronics fraction collector. Radioactivity was determined in ScintiVerse (Fisher Chemical Co., Houston, TX) using a Searle Analytic Isocap 300 Liquid Scintillation Counter.

Methoxychlor and its metabolites were also separated by t.l.c. on plastic-backed analytical plates (Silica gel 60 F-254, E. Merck Co., Darmstadt, Germany) in chloroform–acetone (9:1). The R_f values obtained were 0.8 for methoxychlor, 0.5 for the mono-phenol, and 0.2 for the bis-phenol. Compounds were visualized by their quenching under short-wave u.v. light or by spraying the plate with 0.6% silver nitrate in acetone and exposing it to u.v. light for 10 min. Radioactive spots were removed and counted in a toluene scintillation fluid.

Results and discussion

The abilities of methoxychlor and two of its major metabolities, the mono- and bis-phenolic derivatives, to inhibit [^{3}H]estradiol binding to the rat uterine receptor are illustrated in Fig. 1. Purified methoxychlor is relatively inactive as an inhibitor of [^{3}H]estradiol binding [4–7]. On the other hand, the mono- and bis-phenolic metabolites are among the most active chlorinated hydrocarbons thus far examined for this property. The relative affinities of prototype chlorinated hydrocarbons for the estrogen receptor are given in Table 1. The bis-phenolic metabolite was approximately 10 times more potent as an inhibitor of [^{3}H]estradiol binding than was 1,1,1-trichloro-2-(*p*-chlorophenyl)-2-(*o*-chlorophenyl)ethane (*o,p'*-DDT), and it was about 100 times less active than DES. This is similar to the relative affinity of the bis-phenolic compound compared with that of estradiol itself reported by Bulger *et al.* [5–7]. The affinity of the mono-phenolic metabolite for the receptor has not been reported previously. The mono- and bis-phenolic metabolites were probably interacting with the [^{3}H]estradiol binding site in a manner similar to that of DES since neither metabolite added significantly to a maximally effective level of DES (data not shown).

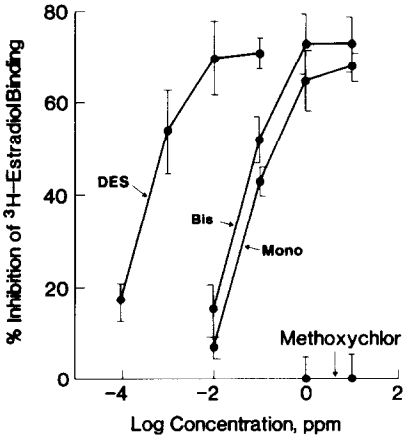

Fig. 1. Inhibition of [^{3}H]estradiol binding to rat uterine supernatant protein. The potential antagonists were added at the concentrations shown. The amount of [^{3}H]estradiol bound was calculated after a 1-hr incubation at 4°. DES = diethylstilbestrol. Bis and mono = the bis- and mono-phenolic metabolites of methoxychlor. Mean values ± S.E. for three to five separate determinations are shown.

Table 1. Relative affinities of methoxychlor metabolites for the rat uterine estrogen receptor *in vitro*

Compound	I_{50}* ppm	I_{50}* μM
Mono-phenol	0.2	0.6
Bis-phenol	0.08	0.2
DES	0.0007	0.002
o,p'-DDT†	0.6	2.0

* I_{50} = concentration required to produce 50 per cent inhibition of [^{3}H]estradiol binding to rat uterine supernatant protein *in vitro*.

† I_{50} value from Ref. 4. Other values were obtained from Fig. 1.

In an attempt to determine which, if either, of the methoxychlor metabolites might account for the activation of methoxychlor observed upon incubating with rat liver microsomes in the presence of NADPH [9], the products of *in vitro* incubations were measured as illustrated in Fig. 2. Quantitation of metabolites was achieved by measurement of the fractions when [^{14}C]methoxychlor was used in the incubation. The mono- and bis-phenolic metabolites found were 2.6 and 0.4 per cent respectively, of the parent drug (three separate experiments, each determined by h.p.l.c. and t.l.c.). These amounts can adequately explain the activation of methoxychlor observed earlier. That is, following incubation of rat liver microsomes with methoxychlor (50 ppm), the percent inhibition of [^{3}H]estradiol binding after a 5-fold diultion in the binding assay was approximately 50 per cent (Table 1 of Ref. 9). The amount of mono-phenolic metabolite formed, therefore, is adequate to explain the activation, i.e. it is present at its I_{50} concentration in the binding assay under these *in vitro* incubation conditions. When fractions of the h.p.l.c. separation were collected and evaluated for abilities to inhibit [^{3}H]estradiol binding in the *in vitro* assay, similar results were obtained, i.e. formation of the mono-phenolic derivative was adequate to explain the activation.

In summary, these results confirm earlier speculations from this laboratory [8, 9] that metabolites of methoxychlor might account for its estrogenic activity *in vivo*. The relative importance of the mono- and bis-phenolic derivatives in the intact animal in this regard is uncertain. Thus, when considering estrogenic activity alone, methoxychlor serves as another example of a drug for which metabolism leads to a more active agent. On the other hand and as illustrated by Kapoor *et al.* [10], metabolism to phenolic metabolites, which are more water soluble and readily excreted, serves as a more rapid means to eliminate the drug from biological systems.

Department of Pharmacology and Toxicology,
University of Texas Medical Branch,
Galveston, TX 77550, U.S.A.
JULIA OUSTERHOUT

Biochemistry Research Department,
Southern Research Institute,
Birmingham, AL 35205, U.S.A.
ROBERT F. STRUCK

Pharmacology Laboratory, Department of Experimental Pediatrics,
University of Texas System Cancer Center,
M.D. Anderson Hospital and Tumor Institute,
Houston, TX 77030, U.S.A.
J. ARLY NELSON*

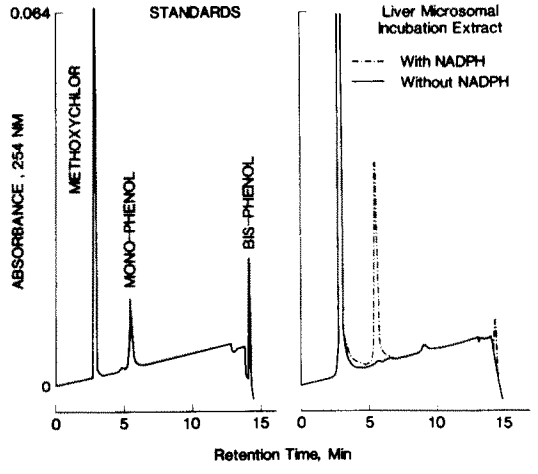

Fig. 2. Separation of methoxychlor from two of its major metabolites following incubation with rat liver microsomes *in vitro*. Metabolites were measured in chloroform extracts of the microsomal incubation mixture as described in the text. Methoxychlor and its two major metabolites were measured by integration of the peak areas and by radioactivity in collected fractions.

REFERENCES

1. R. M. Welch, W. Levin and A. H. Conney, *Toxic. appl. Pharmac.* **14**, 358 (1969).
2. J. Bitman and H. C. Cecil, *J. agric. Fd Chem.* **18**, 1108 (1970).
3. D. Kupfer, *CRC Crit. Rev. Toxic.* **4**, 83 (1975).
4. J. A. Nelson, *Biochem. Pharmac.* **23**, 447 (1974).
5. W. H. Bulger, R. M. Muccitelli and D. Kupfer, *J. Toxic. environ. Hlth.* **4**, 881 (1978).
6. W. H. Bulger, R. M. Muccitelli and D. Kupfer, *Biochem. Pharmac.* **27**, 2417 (1978).
7. D. Kupfer and W. H. Bulger, *Life Sci.* **25**, 975 (1979).
8. J. A. Nelson, *Fedn. Proc.* **32**, 236 (1973).
9. J. A. Nelson, R. F. Struck and R. James, *J. Toxic. environ. Hlth.* **4**, 325 (1978).
10. I. P. Kapoor, R. L. Metcalf, R. F. Nystrom and G. K. Sangha, *J. agric. Fd Chem.* **18**, 1145 (1970).
11. J. M. Ousterhout, R. F. Struck and J. A. Nelson, *Fedn Proc.* **38**, 537 (1979).

* Address correspondence to: J. Arly Nelson, Ph.D., Pharmacology Laboratory, Department of Experimental Pediatrics, M.D. Anderson Hospital and Tumor Institute, Houston, TX 77030, U.S.A.