Estrogenic Effects and Liver Microsomal Enzyme Activity of Technical Methoxychlor and Technical 1,1,1-Trichloro-2,2-bis(*p*-chlorophenyl)ethane in Sheep

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Crossbred yearling ewes were fed ad libitum for 10 or 16 weeks a control diet or a pelleted ration containing 250 ppm of technical 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT) or 250, 1000, or 2500 ppm of technical methoxychlor (MeOCl). There were no differences in food consumption, body weight, liver weight, or uterine weight, water and glycogen. Examination of ovaries indicated that all animals were cycling. All treatments significantly increased liver microsomal enzyme activity. Although MeOCl was fed at 10× the DDT level, liver enzyme activity was less than with DDT. Demethylase activity was 5× control in DDT-treated sheep and 2–3× control in MeOCl-treated sheep. Hydroxylase activity was 2× control in DDT-treated sheep and 1.5× control in MeOCl-treated sheep.

Organochlorine pesticides are potent inducers of liver microsomal enzyme activity in laboratory animals (Conney, 1967; Durham, 1967; Kupfer, 1967). However, there are no reports of organochlorine insecticide effects on liver enzyme activity in ruminants, and little information about liver enzyme activity of domestic meat animals is available. The barbiturate pentobarbital induced the liver microsomal *N*-demethylase in sheep (Cook and Wilson, 1970), and Fries et al. (1971) suggested that barbiturates be used to reduce body burdens of organochlorine pesticides.

The estrogenic effects of technical 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT), *o,p'*-DDT, and technical methoxychlor (MeOCl) have been shown in small laboratory animals (Bitman and Cecil, 1970; Harris et al., 1974; Tullner, 1961; Welch et al., 1969). However, 10 ppm of *o,p'*-DDT fed to sheep had no effect on reproductive function or on estrogen sensitive uterine and ovarian factors (Wrenn et al., 1971).

The present study determines MeOCl and DDT effects on the liver microsomal enzymes (aniline hydroxylase and aminopyrine demethylase) of sheep and rats and examines the estrogenic effects of MeOCl in sheep. Pesticide residue analyses will be presented elsewhere.

MATERIALS AND METHODS

Treatment of Animals. *Experiment 1.* Sixteen crossbred yearling nonpregnant ewes were divided into four treatment groups and individually fed ad libitum for 17 weeks a pelleted control ration or ration containing 250 ppm of technical DDT, 250 ppm of technical MeOCl, or 2500 ppm of technical MeOCl. The source and stated purity of the compounds used were: technical DDT, Olin-Matheson; technical MeOCl, Sigma Corporation, Sigma Class II, 90% active ingredient, 10% petroleum hydrocarbon. We found the actual purity determined by GLC analysis to be: (A) technical DDT: 61.3% *p,p'*-DDT [1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane]; 27.2% *o,p'*-DDT [1,1,1-trichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)ethane]; 0.7% *p,p'*-DDE [1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene]; and no detectable amounts of *p,p'*-DDD [1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethane]; (B) technical MeOCl: 62% *p,p'*-methoxychlor [1,1,1-trichloro-2,2-bis(*p*-methoxyphenyl)ethane] and seven unidentified organochlorine chromatographic peaks. The insecticide was premixed with barley; then the premix was combined with the remaining dietary ingredients, mixed, and pelleted. Experimental diets were fed during January, February, March, and April with no regulation of the light received.

Experiment 2. Thirty-two crossbred yearling ewes were divided into five treatment groups and fed ad libitum for 10 weeks a control diet or a pelleted ration containing 250 ppm of technical DDT, or 250, 1000, or 2500 ppm of technical MeOCl. Diets were prepared as in experiment 1. Sheep were housed in controlled long daylight, 18-hr light and 6-hr dark, to maintain the animals in an anestrous stage of the estrous cycle, thus eliminating the effects of endogenous ovarian hormone secretion.

Sheep were shot with a captive bolt pistol, and immediately bled from the jugular vein. The liver, ovaries, and uterus were quickly excised, wrapped in foil, and kept on ice. Tissue samples were taken for chemical analysis within 0.5 to 1 hr after slaughter.

Experiment 3. Adult female albino rats (60 to 90 days old) on a schedule of 12-hr light and 12-hr dark were fed diets containing 0 and 100 ppm of technical DDT for 20 weeks and 0, 1000, and 2500 ppm of technical MeOCl for 12 weeks. The rats were killed by decapitation, and the liver was quickly excised for enzyme analysis.

Liver Microsomal Enzyme Assay (Experiments 1, 2, and 3). Duplicate 10-g samples of sheep liver were homogenized in 20 ml of ice-cold 0.25 *M* sucrose in all-glass Potter-Elvehjem tissue grinders. Two rat livers were combined to make a 10- to 14-g liver sample for enzyme analysis. Homogenates were centrifuged at 10,000*g* for 30 min at 4°. The postmitochondrial supernatant fractions were then centrifuged at 102,000*g* and 4° for 60 min. The supernatant was discarded and the microsomal pellet covered with 1 ml of 0.1 *M* phosphate buffer (pH 7.4) and frozen at −20° until enzyme analysis.

Activities of aniline hydroxylase and *N*-demethylase were determined as described by Schenkman et al. (1967) using TES [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid] buffer, 50 m*M* and pH 7.4, instead of Tris buffer. The enzyme assay system was incubated aerobically at 37° for 60 min; then trichloroacetic acid was added to stop the reaction. Hydroxylase activity was determined by the amount of *p*-aminophenol formed from aniline and *N*-demethylase activity was determined by the amount of formaldehyde released from aminopyrine.

Protein concentration in the microsomal preparations was determined by the method of Lowry et al. (1951).

Ovarian and Uterine Assay (Experiment 2). Ovaries were examined for corpora lutea development. After weighing the whole uterus, a cross section of uterus was weighed, and the endometrium removed from the myometrium and the proportionate weight of each tissue deter-

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Table I. Liver Weight, Microsomal Protein, and Microsomal Enzyme Activity of Sheep Fed Technical DDT and Technical Methoxychlor for 10 and 17 Weeks (Experiments 1 and 2)

					Liver microsomal enzyme assay			
					Aminopyrine demethylase		Aniline hydroxylase	
Treatment	ppm	Sheep, no.	Liver wt, g ± SE	Microsomal protein, mg of protein/g of liver ± SE	μg of HCHO formed/ mg of micro-somal protein per hr	% control[a]	μg of PAP formed/ mg of micro-somal protein per hr	% control[a]
Control	0	11	951 ± 54	12.7 ● 1.1	0.40	100	2.24	100
DDT	250	9	973 ± 50	14.2 ± 1.8	2.10[d]	544[d]	5.28[d]	221[d]
MeOCl	250	10	951 ± 42	14.6 ± 2.0	0.76[b]	198[b]	3.78[c]	162[b]
MeOCl	1000	8	1023 ± 57	17.2 ± 0.8[c]	0.65	172[c]	3.38[b]	143[c]
MeOCl	2500	7	972 ± 79	15.0 ± 1.7	1.37[d]	351[c]	4.15[c]	172[d]

[a] Each time an enzyme analysis was performed, controls were analyzed and the mean control value for these control samples was used to calculate percent control for each sample within that set of analyses. The mean percent control for all samples is presented in the table. [b-d] Treated vs. control: [b] $P < 0.05$, [c] $P < 0.025$, [d] $P < 0.001$.

Table II. Liver Weight, Microsomal Protein, and Microsomal Enzyme Activity of Adult Female Rats Fed Technical DDT and Technical Methoxychlor for 20 Weeks

					Liver microsomal enzyme assay			
					Aminopyrine demethylase		Aniline hydroxylase	
Treatment	ppm	Rats, no.	Liver wt, g ± SE	Microsomal protein, mg of protein/g of liver ± SE	μg of HCHO formed/mg of microsomal protein per hr	% control[a]	μg of PAP formed/mg of microsomal protein per hr	% control[a]
Control	0	5	8.2 ± 0.6	13.5 ± 1.1	0.044 ± 0.015	100	0.52 ± 0.10	100
DDT	100	5	8.9 ± 0.2	14.8 ● 0.7	0.253 ± 0.031[b]	577[b]	0.50 ± 0.07	96
Control	0	17	7.9 ± 0.2	15.5 ± 1.0	0.048 ± 0.007	100	0.153 ● 0.008	100
MeOCl	1000	10	7.3 ± 0.2	17.0 ± 1.4	0.057 ± 0.008	120	0.171 ± 0.019	103
MeOCl	2500	10	6.5 ● 0.3	17.5 ± 0.9	0.128 ± 0.003[b]	317[b]	0.150 ± 0.025	125

[a] Each time an enzyme analysis was performed, controls were analyzed and the mean control value for these control samples was used to calculate percent control for each sample within that set of analyses. The mean percent control for all samples is presented in the table. [b] Treated vs. control: $P < 0.005$.

Table III. Composition of Uteri from Sheep Fed Technical DDT and Technical Methoxychlor for 10 Weeks and Housed in an 18-hr Light:6-hr Dark Environment (Experiment 2)

						% H_2O ± SE			μg of glycogen/100 mg wet weight ± SE		
Treat-ment	ppm	Sheep, no.	Uterus wt, g ± SE	Endo,[a] % ± SE	Whole	Endo[a]	Myo[a]	Whole	Endo	Myo
Control	0	7	33.5 ± 2.9	42 ± 3	84.3 ± 0.3	85.4 ± 0.3	83.0 ± 0.4	94 ± 17	112 ± 8	81 ± 9
DDT	250	6	28.3 ± 2.8	42 ± 3	83.9 ± 0.3	85.7 ± 0.3	83.1 ± 0.3	103 ± 20	146 ± 34	97 ± 16
MeOCl	250	6	38.4 ± 2.1	39 ± 2	85.0 ± 0.2	86.1 ± 0.3	83.7 ± 0.3	76 ± 6	126 ± 8	55 ± 7
MeOCl	1000	8	34.8 ± 2.6	38 ± 2	84.2 ● 0.2	85.9 ± 0.4	83.2 ± 0.4	90 ± 14	114 ± 15	81 ± 21
MeOCl	2500	7	34.7 ± 3.1	44 ± 4	84.9 ± 0.3	86.0 ± 0.4	83.4 ± 0.3	82 ± 10	96 ± 8	65 ± 9

[a] Endo = endometrium; Myo = myometrium.

mined. Myometrial samples included serosal covering as well as muscle.

Uterine glycogen was determined by the anthrone method of Seifter et al. (1950). Total H_2O was determined by difference in weight of uterine samples dried overnight at 100° in vacuo.

Experimental data were analyzed by using the Student "*t*" test with correction for unequal group size.

RESULTS AND DISCUSSION

None of the treatments produced any toxic symptoms in the sheep. The sheep consumed approximately 1100 g/day; thus, the daily consumption of insecticide was 0.2, 1.1, and 2.7 g for the 250-, 1000-, and 2500-ppm diets, respectively. Other workers reported no toxic symptoms in sheep fed 4.5 g of MeOCl daily for 60 days while nervous disorders developed in sheep fed 4.5 g of DDT (Welch, 1948).

Liver Weight, Microsomal Protein, and Microsomal Enzyme. The sheep experiments had comparable results and the data were combined. Although liver weight and microsomal protein of sheep appear to be increased by the pesticide treatments, none of the increases were statistically significant (Table I). However, all treatments increased liver microsomal enzyme activity. Although the highest MeOCl treatment (2500 ppm of technical MeOCl) was 10× the concentration of the DDT treatment, liver enzyme activity was less than with DDT. Demethylase activity was 5× control in DDT-treated sheep and 2 to 3× control in MeOCl-treated sheep. Hydroxylase activity was 2× control for DDT-treated sheep and 1.5× control for MeOCl-treated sheep.

In the rat (Table II), the two enzyme systems were differentially affected by organochlorine treatments. Demethylase activity increased 5.8× with DDT and 3× with MeOCl feeding, comparable to increases observed in the sheep. However, these organochlorines had no effect on hepatic hydroxylase activity in the rat, whereas hydroxylase activity increased in the sheep. A similar pattern in aminopyrine demethylase of hepatic microsomes of rats was reported by Hart and Fouts (1965), who found that both DDT and MeOCl increased the hepatic enzymic activity in rats, and the DDT response was 3× that of MeOCl. The variations in the hepatic enzyme-inducing capacities of DDT and MeOCl are probably due to the variability in the rate of their in vivo metabolism. MeOCl accumulates in the animal body to a lesser extent than DDT (Lehman, 1956), and the rate of metabolism of MeOCl is greater than that of DDT (Kapoor et al., 1970; Woodward et al., 1948).

We also found species differences in the normal hepatic enzyme activity of sheep and rats. Control sheep liver had much more hepatic demethylase and hydroxylase activity than the control rat liver ($p < 0.001$). Cook and Wilson (1970), however, reported that the rat had the same hepatic *N*-demethylase activity as the sheep.

Estrogenic Effects of DDT and Methoxychlor (Table III). Although the lighting sequence selected for experiment 2 should have produced an anestrous state, we found that all of the sheep ovaries had corpora lutea. Thus, the uteri of these animals would have been under the influence of endogenous hormones. Estrogen treatment increases uterine water and glycogen content in mammals (rabbits and sheep, Bitman et al., 1959, 1967; cow, Hawk et al., 1961; rat, Cecil et al., 1964) and in the chicken (Cecil et al., 1969, 1970). There were no effects of any of the treatments on the endocrine parameters studied (uterine weight, H_2O, and glycogen, Table III). Although the dosages of MeOCl used in this experiment did not show an overt action of estrogen upon the sheep uterus, these amounts do interfere with fertility and conception in rats (Harris et al., 1974).

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