data also provided some evidence for suggesting that both pathways for 4-chloro-*o*-toluidine formation were operable in EIL, but to a much lesser degree than in HIL. With respect to NDL, 4-chloro-*o*-toluidine apparently was formed from 4-chloro-*o*-formotoluidide by the conventional pathway.

The reason for this apparent difference in chlordimeform metabolism by NDL, EIL, and HIL remains unknown. Chippendale (1979) has discussed certain of the biochemical characteristics of NDL, EIL, and HIL. For example, EIL and HIL accumulated significantly larger amounts of lipids and proteins than did NDL. Moreover, application of a juvenile hormone mimic to early last instar NDL resulted in their transformation to HIL which were physiologically similar to EIL (Yin and Chippendale, 1974). The juvenile hormone mimic treatment apparently elevated the functional juvenile hormone titer of the larval tissues, thereby causing the larvae to enter a diapause-like state. In contrast, the juvenile hormone titer in the hemolymph of last instar NDL declined to very low levels immediately after ecdysis (Yin and Chippendale, 1976).

It seems probable that treatment of the SWCB larvae with the juvenile hormone mimic resulted in the induction of an enzyme that effected the "direct" conversion of chlordimeform to 4-chloro-*o*-toluidine. However, other possibilities also exist. For example, chlordimeform degradation in HIL may have been shunted to a different metabolic pool. This appears unlikely since demethylchlordimeform and 4-chloro-*o*-formotoluidide seemed to be degraded similarly in both NDL and HIL. These studies have established a probable relationship between xenobiotic metabolism and juvenile hormone mediated changes in SWCB larvae.

ACKNOWLEDGMENT

We thank Dr. G. M. Chippendale of the Department of Entomology for supplying the insects, for valuable discussions, and for reading this manuscript.

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Received for review May 29, 1981. Accepted September 25, 1981. This research was supported, in part, by a grant from the National Institutes of Health (ES01316) and is a contribution from the Missouri Agricultural Experiment Station, Columbia. Journal Series No. 8882.

Toxicity of *O,O,S*-Trialkyl Phosphorothioates to the Rat

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A series of simple *O,O*-dimethyl and *O,O*-diethyl *S*-alkyl phosphorothioate esters were prepared and examined for acute and delayed toxicity to rats. Several of the compounds were highly toxic to the rat, with death occurring over a period of 1–11 days. Most of the compounds caused symptoms of delayed intoxication, i.e., weight loss, diarrhea, uncontrolled urination, and hemorrhaging. All of the esters were poor inhibitors of bovine erythrocyte acetylcholinesterase and rat plasma cholinesterase, the *O,O*-dimethyl esters being virtually devoid of inhibitory activity. The poor anticholinesterase activity of these compounds suggests a noncholinergic mechanism of action.

In recent communications (Mallipudi et al., 1979; Umetsu et al., 1979) we described the unexpectedly high delayed toxicity of *O,O,S*-trimethyl phosphorothioate (LD_{50} 15–20 mg/kg) and *O,O,S*-triethyl phosphorothioate (LD_{50} 45–90 mg/kg) following administration of single oral doses to rats. In a previous study (Umetsu et al., 1977), *O,O,S*-trimethyl phosphorothioate was demonstrated to be a trace contaminant in technical samples of malathion and acephate. Intoxication of rats by this simple compound was markedly different from that caused by typical organophosphorus insecticides in that death occurred over a substantially longer time frame, e.g., up to 3 weeks following treatment (Mallipudi et al., 1979). At the LD_{50} dose of 15–20 mg/kg, rats treated with *O,O,S*-trimethyl phosphorothioate showed no visible sign of distress but refused food and water, resulting in the loss of weight. Of four rats dosed orally at 20 mg/kg, one died on day 6, the second on day 8, and the third on day 17.

Because of the unusual mode of action and signs of poisoning by this compound, other structural analogues were synthesized and examined for acute and delayed toxicity to rats and for anticholinesterase activity.

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MATERIALS AND METHODS

Chemicals. Except for *O,O*-diethyl *S*-(*tert*-butyl) phosphorothioate (**13**), all compounds examined for toxicological properties were synthesized by reaction of the appropriate alkylsulfenyl chloride and trimethyl or triethyl phosphite (Morrison, 1955). Compound **13** was prepared by reaction of *tert*-butylsulfenyl chloride with sodium diethyl phosphonate at 0 °C. All products were vacuum distilled and purified by either preparative TLC, (solvent system was 1:1 benzene–ethyl acetate) or silica gel column chromatography (solvent system was hexane–ethyl acetate gradient). Structures were verified by NMR and elemental analyses (see Table I); the latter were carried out by C. F. Geiger, Ontario, CA. Product purity was determined by

0021-8561/82/1430-0126$01.25/0

Table I. Physical Properties and Elemental Analyses of O,O-Dimethyl and O,O-Diethyl S-Alkyl Phosphorothioates

			$(RO)_2P(O)SR'$				
						analysis	
compd	R	R'	bp, °C/mmHg	$n^{23}D$	R_f[a]	calcd	found
2	CH_3	C_2H_5	67-69/1.25	1.4631	0.42	C, 28.23; H, 6.52	C, 28.63; H, 6.82
3	CH_3	n-C_3H_7	76-79/0.125	1.4621	0.50	C, 32.60; H, 7.11	C, 32.96; H, 7.10
4	CH_3	n-C_4H_9	75-78/0.75	1.4618	0.64	C, 36.61; H, 7.62	C, 36.37; H, 7.34
5	CH_3	n-C_5H_{11}	97-100/1.25	1.4622	0.54	C, 39.61; H, 8.07	C, 39.94; H, 8.31
6	CH_3	n-C_6H_{13}	105-107/0.1	1.4626	0.58	C, 42.46; H, 8.46	C, 42.79; H, 8.68
7	CH_3	i-C_3H_7	67-69/0.8	1.4612	0.58	C, 32.60; H, 7.11	C, 32.67; H, 7.21
8	CH_3	t-C_4H_9	48-50/0.07	1.4641	0.50	C, 36.61; H, 7.62	C, 36.95; H, 7.88
9	C_2H_5	CH_3	97-99/1.0	1.4572	0.45	C, 32.60; H, 7.11	C, 32.58; H, 7.41
11	C_2H_5	n-C_3H_7	93-96/0.3	1.4542	0.40	C, 39.61; H, 8.07	C, 39.74; H, 8.26
12	C_2H_5	i-C_3H_7	70-73/0.75	1.4540	0.57	C, 39.61; H, 8.07	C, 39.84; H, 8.38
13	C_2H_5	t-C_4H_9	69-72/0.21	1.4568	0.59	C, 42.47; H, 8.46	C, 42.49; H, 8.48
14	C_2H_5	n-C_6H_{13}	125-127/0.25	1.4561	0.43	C, 47.23; H, 9.12	C, 47.40; H, 9.41

[a] Thin-layer chromatography; solvent system was benzene–ethyl acetate (1:1).

Table II. Toxicological Properties of O,O-Dimethyl and O,O-Diethyl S-Alkyl Phosphorothioates

			$(RO)_2P(O)SR'$				
						cholinesterase inhibition (k_i), M^{-1} min^{-1}	
compd	R	R'	toxicity to rats, time frame for death, days after treatment	LD_{50}, mg/kg	housefly LD_{50}, μg/g	BAChE	RPChE
1	CH_3	CH_3	4-22	15-20[a]	~95	0.43	5.16
2	CH_3	C_2H_5	3-11	18.2 (7.3-26.7)[b]	103	0.44	3.34
3	CH_3	n-C_3H_7	1-3	11.0 (7.7-13.9)	150	0.39	2.34
4	CH_3	n-C_4H_9	1-2	53.6 (25.6-64.5)	162	0.51	2.04
5	CH_3	n-C_5H_{11}	1-3	311.0 (283-335)	165	0.80	1.73
6	CH_3	n-C_6H_{13}		>750	260	0.44	2.76
7	CH_3	i-C_3H_7	1-7	108.0 (101-115)	239	0.36	0.20
8	CH_3	t-C_4H_9	2-6	63.4 (49.2-104)	301	17.8	28.7
9	C_2H_5	CH_3	1-7	47.1 (42.6-50.3)	126	105.0	65.3
10	C_2H_5	C_2H_5	1-8	50-85[a]			
11	C_2H_5	n-C_3H_7	1-3	48.5 (32.5-71.6)	208	89.9	78.0
12	C_2H_5	i-C_3H_7	1	179.0 (166-192)	223	144.0	99.8
13	C_2H_5	t-C_4H_9	1-3	127 (112-230)	247	1490.0	14.4
14	C_2H_5	n-C_6H_{13}		>350	180	377.0	23.0

[a] Data from Mallipudi et al. (1979). [b] Parenthetical values are 95% confidence limits.

analytical TLC (solvent system was 1:1 benzene–ethyl acetate). Spots on TLC plates were detected by ultraviolet light and by spraying with 0.75% 2,6-dibromoquinone-4-chloroimide (DBQ) in ether (Menn et al., 1957).

Analytical and preparative TLC utilized silica gel 60 PF-254 plates of 0.25- and 1.0-mm thickness (EM Laboratories, Inc.). Silica gel (Mallinckrodt CC-7) was used for column chromatography. NMR spectra were recorded on a Varian EM-390 instrument with tetramethylsilane as the internal standard.

Toxicological Evaluation. Acute and delayed rat toxicity was determined with 120–140-g female albino rats (Sprague-Dawley derived) from Simonsen Laboratories, Gilroy, CA. Solutions of the toxicants in corn oil were administered orally at 0.1 mL/100 g of rat to animals fasted for 6 h before treatment. Animals were kept under observation for 25 days. At least five different doses with a minimum of four rats per dose were used to determine LD_{50} values.

Insecticidal activity was determined with the susceptible S_{NAIDM} strain of houseflies, *Musca domestica*, at 72 °F according to usual procedures (March and Metcalf, 1949). LD_{50} values were based on 24-h mortality by using six dosages per compound.

Bimolecular rate constants (k_i) for the inhibition of bovine erythrocyte acetylcholinesterase (BAChE) (Sigma Chemical Co.) and rat plasma cholinesterase (RPChE) were determined at 30 °C as previously described (Lee et al., 1978) by using acetylthiocholine as the substrate (Ellman et al., 1961). Rat plasma was prepared from blood drawn from the orbital sinus of 250–300-g female rats as described by Riley (1960). Duplicate measurements were made for each inhibitor at each concentration. The resulting data, i.e., $\ln A_0/A_t$ vs. time, where A_0 is the activity of the enzyme at time zero and A_t is the activity after reaction with the inhibitor for time t, were subjected to least-squares analysis. The $\log A_0/A_t$ vs. time plots were reasonably linear with correlation coefficients (r) greater than 0.92.

RESULTS

Rat Toxicity. Toxicological data presented in Table II for the different S-alkyl O,O-dimethyl and O,O-diethyl phosphorothioates reveals wide variability in the toxicity of these compounds to rats, with LD_{50} values ranging from 11 to >750 mg/kg. The most toxic compound was O,O-dimethyl S-propyl phosphorothioate (3), followed by either the O,O-dimethyl S-methyl (1) or O,O-dimethyl S-ethyl analogue (2). A change in the size of the S-alkyl moiety had a profound effect on rat toxicity, and the S-hexyl analogue, (6) showed no toxicity at 750 mg/kg. Thus, increasing the number of carbon atoms of the S-propyl chain by three, i.e., from S-propyl to S-hexyl, reduced rat toxicity more than 68-fold.

The S-alkyl O,O-diethyl phosphorothioates were generally less toxic to rats than the O,O-dimethyl esters, and in those cases where the S-alkyl moiety was the same, the O,O-diethyl esters were 1.6–4.4-fold less toxic than the

Table III. Time of Death of Rats Treated Orally with Different Amounts of *O,O*-Dimethyl *S*-Isopropyl Phosphorothioate

dose mg/kg	no. of rats treated	time, days, of occurrence of death								total rats killed	% killed
		1	2	3	4	5	6	7	8-25		
90	5										0
100	7					1				1	14.28
110	7	1		1	1	1	1			5	71.42
120	5	2					1	1		4	80
130	5	3		1			1			5	100
140	5	3				1	1			5	100

corresponding *O,O*-dimethyl ester. As in the case of the dimethyl esters, compounds with small *S*-alkyl groups showed the highest rat toxicities.

In general, rats treated with the compounds listed in Table II showed signs of delayed intoxication as previously described for *O,O,S*-trimethyl phosphorothioate (Mallipudi et al., 1979). For example, typical symptoms of poisoning were weight loss, diarrhea, uncontrolled urination, and bleeding from the mouth and nose. Delayed toxicity is evident in the data presented in Tables III and IV for *O,O*-dimethyl *S*-isopropyl phosphorothioate (**7**) where death occurred over a period of 7 days. Symptoms of intoxication of rats treated with **7** are indicated in Table IV. Extended weight loss was the most obvious sign of poisoning, and even at the sublethal dose of 90 mg/kg, substantial loss in weight was observed over 7 days. However, these animals eventually gained weight and survived the treatment. Weight loss, diarrhea, and bleeding were most apparent on the fourth and fifth days following treatment. At the high dosages of 130 and 140 mg/kg, most of the animals died within 24 h after treatment. At these dosages it was difficult to distinguish between delayed and acute intoxication. At 120 mg/kg one animal died on the sixth day and a second on the seventh day.

While delayed toxicity was observed in rats treated with virtually all of the compounds listed in Table II, particularly at dosages near the LD_{50} value, in some cases death was rapid with rats showing typical cholinergic signs of poisoning. For example, out of five rats treated with 15 mg/kg *O,O*-dimethyl *S*-propyl phosphorothioate (**3**), two rats died within 24 h and another on the second day (Table V). Symptoms of poisoning appeared to be cholinergic with animals showing salivation, tremors, difficulty in breathing, and eventually tetany. At 10 mg/kg, one rat died on the second day and two on the third day.

Cholinesterase Inhibition. Bimolecular rate constants (k_i) for the inhibition of bovine erythrocyte acetylcholinesterase (BAChE) and rat plasma cholinesterase (RPChE) are given in Table II. All of the trialkyl phosphorothioate esters were poor inhibitors of these two cholinesterases, and, further, the *O,O*-dimethyl esters were essentially devoid of anticholinesterase activity. The absence of anticholinesterase activity for the dimethyl esters is particularly significant because of the high rat toxicity of some of these compounds.

For BAChE inhibition, the *O,O*-diethyl phosphorothioates were slightly stronger anticholinesterases than the dimethyl analogues with highest activity observed for *O,O*-diethyl *S*-(*tert*-butyl) phosphorothioate (**13**). This compound, however, was among those with lowest rat toxicity. Overall, the *O,O*-diethyl esters appeared to be somewhat stronger inhibitors of BAChE than RPChE. The difference in the inhibitory potency for a given compound between BAChE (acetylcholinesterase) and RPChE (psuedocholinesterase) may be attributed to differences in the active centers and hydrophobic areas adjacent to the centers (Kabachnik et al., 1970).

Housefly Toxicity. None of the phosphorothioate esters was particularly toxic to houseflies with LD_{50} values ranging from about 95 to 300 μg/g.

DISCUSSION

The *O,O*-dimethyl *S*-alkyl phosphorothioate esters containing small *S*-alkyl moieties (methyl; ethyl; *n*-propyl) were highly toxic to the rat. However, an increase in the *S*-alkyl chain length beyond propyl caused a precipitous drop in rat toxicity. Of the smaller *S*-alkyl *O,O*-dimethyl esters, the *S*-methyl and *S*-ethyl analogues clearly caused delayed toxic effects with death occurring at long time intervals after treatment. In contrast, rats treated with the *S*-(*n*-propyl) analogue, the most toxic ester, died relatively quickly, and it was difficult to differentiate acute from delayed effects.

It is apparent from anticholinesterase measurements that the dimethyl esters are inactive as cholinesterase inhibitors, being 10^5–10^6-fold less effective than such strong inhibitors as paraoxon and tetraethyl pyrophosphate (Aldridge and Davison, 1952). Further, the bimolecular rate constants (k_i) for the inhibition of either BAChE or RPChE were essentially the same for all of the straight-chain *O,O*-dimethyl *S*-alkyl esters (CH_3 to *n*-C_6H_{13}). In contrast, the range in rat toxicity for these compounds was 11 to >750 mg/kg. The striking difference between rat toxicity and anticholinesterase activity suggests a noncholinergic mechanism of intoxication for these compounds.

The possibility exists that these compounds may be activated in vivo to a derivative of stronger anticholinesterase activity which may be responsible for intoxication. This is improbable from a chemical standpoint since the only possible place where metabolic activation may occur is the thiolate sulfur atom, e.g., oxidation to –S(O)– (sulfoxide), and the existence of an organophosphorus ester of this type has not been demonstrated. It has been suggested that methamidophos (*O,S*-dimethyl phosphoramidothioate) is activated in vivo to the corresponding sulfoxide (Eto et al., 1977), but evidence for its formation is indirect.

The toxicological properties of a number of *O,O*-diethyl *S*-alkyl phosphorothioate esters have been described previously (Bracha and O'Brien, 1968a,b; Gazzard et al., 1974; Langel and Järv, 1978), and there is general agreement that simple esters of this series, i.e., esters with small *S*-alkyl groups, are relatively poor anticholinesterases. Bracha and O'Brien (1968a) reported relatively high mouse toxicities for a number of *O,O*-diethyl *S*-alkyl phosphorothioates where the *S*-alkyl moiety was relatively large and branched, e.g., LD_{50} as low as 4.5 mg/kg. However, samples used in their study may have contained a contaminant which caused anomalous results in both cholinesterase inhibition and mouse toxicity (Gazzard et al., 1974).

In the limited number of diethyl phosphorothioate esters examined in this study, anticholinesterase activity appeared to increase with increasing size of the *S*-alkyl moiety. This is in agreement with the results of Langel

Table IV. Symptoms of Intoxication in Rats Treated Orally with *O,O*-Dimethyl *S*-Isopropyl Phosphorothioate

dose, mg/g	rat	symptoms of poisoning A,[a] B,[b] and C[c] after treatment, days: 1 A	1 B	1 C	2 A	2 B	2 C	3 A	3 B	3 C	4 A	4 B	4 C	5 A	5 B	5 C	6 A	6 B	6 C	7 A	7 B	7 C	8-25 A
90	I	10	−	+	20	−	+	23	+	+	24	+	+	17	+	+	13	−	+	9	+	+	(+31)[d]
	II	7	+	+	17	−	+	19	+	+	24	+	+	21	+	+	19	+	−	16	+	−	(+42)
	III	13	−	−	21	−	+	24	+	+	26	++	++	25	++	++	23	+	+	18	+	+	(+28)
	IV	5	+	−	13	−	−	13	+	+	13	+	+	10	+	−	7	−	−	6	−	−	(+39)
	V	11	−	−	20	−	−	22	+	+	19	+	+	18	+	+	17	+	+	17	+	+	(+29)
100	I	8	+	+	16	+	+	19	++	++	26	++	++	32[f]									
	II	4	−	−	15	−	+	20	+	+	19	+	−	12	+	−	8	−	−	4	−	−	(+4)[e]
	III	10	−	−	15	−	−	18	+	+	15	+	+	13	+	−	11	−	−	8	−	−	(+2)
	IV	7	−	+	15	+	+	18	+	+	15	+	−	15	+	−	13	−	−	9	−	−	(+1)
	V	7	−	−	14	−	+	11	−	+	8	+	+	8	+	+	5	−	−	3	−	−	(+3)
	VI	4	−	−	4	−	+	10	−	+	4	−	−	2	−	−	(+4)	−	−				
	VII	4	−	+	9	−	+	19	+	+	20	+	+	19	+	+	15	+	+	11	+	+	(+1)
110	I	3[f]																					
	II	15	+	+	20	++	++	26[f]															
	III	9	−	−	11	−	+	14	−	+	7	−	+	7	−	−	2	−	−	(+4)	−	−	
	IV	12	+	+	12	+	+	17	+	+	22	++	++	29	++	++	32[f]						
	V	8	+	+	12	+	++	18	++	++	24[f]												
	VI	11	−	+	16	+	+	25	++	++	31	++	++	33[f]									
	VII	10	−	+	12	−	+	13	+	+	16	+	+	17	+	+	13	−	+	9	−	+	(+11)[e]
120	I	5	−	+	13	−	+	17	+	+	14	+	+	15	+	+	8	+	+	5	−	+	(+9)[e]
	II	14	+	+	20	+	+	23	+	+	27	++	++	35	++	++	38[f]						
	III	12	+	+	16	+	+	19	+	+	25	++	++	31	++	++	32	++	++	38[f]			
	IV	3[f]																					
	V	4[f]																					
130	I	3[f]																					
	II	12	+	+	16	+	+	21	++	++	27	++	++	35	++	++	35	++	++	37[f]			
	III	5[f]																					
	IV	3[f]																					
	V	13	+	+	18	+	+	20	++	+	23	++	++	31	++	++	36	++	++	40[f]			
140	I	4[f]																					
	II	16	+	+	18	+	+	23	++	++	28	++	++	28	++	++	34[f]						
	III	2[f]																					
	IV	4[f]																					
	V	13	++	++	13	++	++	14[f]															

[a] Percent loss weight from initial weight; parenthetical numbers which are preceded by a (+) sign indicate percent weight gain. [b] Bleeding from mouth, nose, and ear: (+) observed; (++) heavy; (−) not observed. [c] Diarrhea: (+) observed; (++) heavy; (−) not observed. [d] Rats were weighed on the 18th day after treatment. [e] Rats were weighed on the 11th day after treatment. [f] Died.

Table V. Time of Death of Rats Treated Orally with Different Amounts of *O,O*-Dimethyl *S*-Propyl Phosphorothioate

dose, mg/g	no. of rats treated	time, days, of occurrence of death 1	2	3	4–25	total rats killed	% killed
7.5	5					0	0
10	4		1	2		3	75
15	5	2	1			3	60
18	4	4				4	100
20	5	4	1			5	100

and Järv (1978). A cholinergic mechanism for poisoning by *O,O*-diethyl *S*-alkyl phosphorothioates cannot be ruled out, and it is possible that both cholinergic and noncholinergic mechanisms contribute to intoxication by these esters.

Further work with trialkyl phosphorothioates is in progress.

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Received for review June 26, 1981. Accepted October 5, 1981. This investigation was supported by federal funds from the Environmental Protection Agency under Grant No. R804345 and U.S. Public Health Service Grant No. ES002225. The contents do not necessarily reflect the views and policies of the Environmental Protection Agency nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

Methoxychlor Metabolism in Goats

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Methoxychlor [1,1,1-trichloro-2,2-bis(4-methoxyphenyl)ethane] was given orally to two lactating goats. Goat 66 received 1 g (582 μCi) of methoxychlor, and goat 69 received 200 mg (545 μCi). At the end of 3 days, recovery of ^{14}C in feces, urine, and milk was 68%, 27%, and 0.065%, respectively, for goat 66 and 40%, 58%, and a trace, respectively, for goat 69. Seventeen metabolites plus methoxychlor were isolated from urine and feces and identified by GC–MS. Fecal and some urinary metabolites were demethylated, dechlorinated, or dehydrochlorinated products. Most urinary metabolites were completely demethylated and conjugated with glucuronic acid. Ring hydroxylation occurred in one urinary metabolite. The predominant metabolites were 4,4′-substituted dichloroethanes, and no completely dechlorinated products or acids were identified.

Methoxychlor [1,1,1-trichloro-2,2-bis(4-methoxyphenyl)ethane] is an insecticide used to control flies on livestock and in farm buildings. It has other insecticidal uses, but for this paper we are concerned with its direct contact by farm animals.

The metabolism or distribution of [^{14}C]methoxychlor has not been studied in farm animals. Kapoor et al. (1970) reported five metabolites of [^{14}C]methoxychlor in urine and feces of mice. Woodward et al. (1948) observed no methoxychlor or bis(4-methoxyphenyl)acetic acid in urine of rats fed methoxychlor, and Weikel (1957) observed that ^{14}C was eliminated predominantly in feces of rats given [^{14}C]methoxychlor intravenously.

We report the metabolism of [^{14}C]methoxychlor by lactating goats.

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MATERIALS AND METHODS

Animals. Goat 66 (weight 86 kg, 5 years old, and in first month of lactation) was given 1 g of 4,4′-methoxychlor and 582 μCi of [^{14}C]methoxychlor. Goat 69 (weight 56 kg, 4 years old, and in sixth month of lactation) was given 200 mg of 4,4′-methoxychlor and 545 μCi of [^{14}C]methoxychlor. The goats were placed in metabolism stalls (Robbins and Bakke, 1967) and given the methoxychlor orally in gelatin capsules. Milk, urine, and feces were collected for 3 days. Urine was collected directly from the bladder through a latex catheter, which emptied into a covered stainless steel pan. The goats were killed after 3 days, various tissues were sampled, and then their carcasses were ground and sampled. Tissues, milk, and feces were lyophilized and stored in glass jars. Urine was stored in glass jars at 3 °C.

[^{14}C]Methoxychlor. [*ring*-U-^{14}C]Methoxychlor was obtained from New England Nuclear, Boston, MA 02118. The specific activity was 9.03 mCi/mM. Radiochemical purity was determined by isotope dilution in ether–hexane and ethanol (four recrystallizations in each solvent system); purity was >98%. Purity was also determined by GC–