Metabolism of Four Thiobis(formamidine) Insecticides in the Mouse and the Rat

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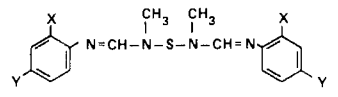
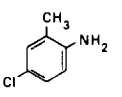
Four ^{14}C-labeled thiobis(formamidine) insecticides, derived from a series of 2,4-disubstituted anilines (2,4-dichloro-, 2-chloro-4-methyl-, 4-chloro-2-methyl-, and 2,4-dimethyl-), were orally administered to female mice and rats in single dose studies. In all cases, the mice excreted 93–97% of the dose and the rats excreted 96–100% of the dose within 48 h posttreatment. The primary mode of excretion in all cases was via the urinary tract (70–90%). The 12- and 24-h urine samples were analyzed for various metabolites. No parent thiobis(formamidine) was detected in any sample: Hydrolysis to the simple formamidines and to the *N*-formylanilines was the likely initial fate. Subsequent metabolism was then highly efficient. Only the dichloro analogue produced an amount of free aniline greater than 1% of the dose. Oxidation of the methyl group(s) in the other compounds was a major fate, especially when the methyl was in the 4-position. No apparent differences in the metabolism of these compounds between mice and rats were detected. Further, the general metabolism schemes for all four compounds were similar and followed much the same pattern for the metabolism of chlordimeform in the rat.

The thiobis(formamidine) series of compounds (I) are

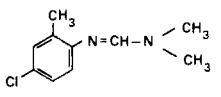


I

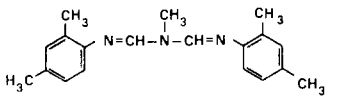
I-A: X = Y = Cl I-C: X = CH_3, Y = Cl
I-B: X = Cl, Y = CH_3 I-D: X = Y = CH_3



IV-C



VII



VIII

of interest because of their exceptional insecticidal activity toward various lepidopteran pests of cole and cotton crops, as well as their acaracidal activity toward various mites and ticks. These compounds are an extension of the formamidine family of pesticides of which chlordimeform (VII) and amitraz (VIII) are perhaps the most familiar.

There are two structural features of I to be noted. First, there are two identical formamidine subunits joined through a sulfur bridge. Second, and perhaps the most prominant feature, is the 2,4-disubstituted aniline moiety in each subunit from which these compounds are derived. Structure–activity studies have indicated that this structural feature and substitution pattern produce optimal pesticidal activity (Gemrich et al., 1976).

The aniline moieties of all such compounds are the focal point of toxicological concern since many anilines have been found to be mutagenic and/or weakly carcinogenic. Chlordimeform (VII) is a case in point. The 4-chloro-2-methylaniline (IV-C), which serves as the structural foundation, has been shown to be a weak carcinogen (IRAC, 1978) and a strong mutagen (Zimmer et al., 1980). Several studies of the metabolic fate of VII in plants (Knowles and Sen Gupta, 1969; Knowles and Ehrhardt, 1970; Withonton and Erregovich, 1972) and animals (Knowles and Sen Gupta, 1970a,b; Knowles and Johnson, 1970; Knowles, 1970; Knowles and Benezet, 1977) have indicated the generation of small but measurable levels of IV.

These considerations dictated the primary objective of the metabolism studies which are the subject of this paper. This objective was to develop the metabolism scheme of each of the four compounds (I) with emphasis on determining the quantities of the corresponding aniline generated by the animal systems. The four compounds under consideration result from the four combinations of chlorine and methyl substituents in the 2- and 4-positions of aniline. The metabolism of these thiobis(formamidines) was anticipated to closely parallel that of chlordimeform, with perhaps some variations due to the substituents X and Y.

The metabolism studies were done in the mouse and the rat since most of the toxicology data on these compounds have been accumulated in these species. In this initial preliminary study, only female mice and rats were studied since the toxicology data on a few of these compounds or intermediates showed a low, but statistically significant, incidence of mammary tumors. A single oral dose of ^{14}C-radiolabeled I, at a dose rate of 10–13 mg/kg, was administered as a corn oil solution by gavage. Urine and feces were collected at regular intervals and the excretion patterns established. Most (80 ± 6%) of the radiolabel was excreted in the urine, and the metabolite identification efforts were restricted to this fraction.

EXPERIMENTAL SECTION

Synthesis of [^{14}C]I. In a typical experiment, ^{14}C-radiolabeled aniline hydrochloride (uniformly ring labeled, New England Nuclear, Boston, MA) and nonlabeled aniline hydrochloride were mixed to adjust the specific activity by weighing into a 50-mL glass-stoppered centrifuge tube that contained a $^5/_8$-in. Teflon-coated magnetic stir bar. Then 1.5 mL of freshly distilled *N*-methylformamide (Aldrich Chemical Co., Milwaukee, WI) was added, the mixture stirred until the solids were dissolved, and then the solution cooled to 0 °C. Methanesulfonyl chloride (Aldrich), 0.5 mL, was added all at once and the mixture allowed to slowly warm to room temperature over 20–30 min and stirred an additional 3 h. Water, 10 mL, was added and the pH adjusted to 10–11 by the dropwise addition of 1.0 N NaOH. The basic solution was then extracted 3 times with 10-mL volumes of methylene chloride. The extracts were combined, dried over anhydrous Na_2SO_4 for 3 h, filtered, and transferred to a clean 50-mL tube. The tube was partially immersed in a water bath at 40 °C and the methylene chloride evaporated to dryness with a stream of nitrogen. The oily residue was dissolved in 2 mL of fresh CH_2Cl_2, and 265 μL (190 mg) of freshly distilled triethylamine and a stir bar were added. The air above the solution was displaced by nitrogen and the solution then cooled to 0 °C. Then 92 mg (0.9 mmol) of sulfur dichloride in 0.50 mL of methylene chloride was added dropwise. The solution was then allowed to warm to room

The Upjohn Company, Agricultural Division, Kalamazoo, Michigan.

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temperature and stirred an additional hour.

The reaction mixture was diluted with 10 mL of methylene chloride and then washed successively with 10-mL volumes of water, 4% citric acid, and water. The washings were removed by pipet and the organic solution was dried over 3 g of anhydrous Na_2SO_4. The dried solution was transferred in 5-mL portions to a 10-mL Erlenmeyer flask, and each portion was evaporated to dryness with a stream of nitrogen before introduction of the second portion. The total residue was then dissolved in 1 mL of CH_2Cl_2 and 2 mL of 2-propanol. The solution was gently warmed to reduce the CH_2Cl_2 content. On cooling of the solution, crystallization was rapid. The off-white crystalline product was collected and washed with 2–3 mL of ice-cold 2-propanol. The crystallization from 2-propanol was effective for all but the dimethyl analogue (I-D), which was crystallized from pentane. The quantities in each case were

compound	amt of [^{14}C]-IV·HCl, mmol	amt of [^{12}C]-IV·HCl, mmol	yield, mmol	sp act., mCi/mmol
I-A	0.69	1.31	0.57	3.88
I-B	0.86	1.14	0.48	3.94
I-C	1.01	1.01	0.59	4.62
I-D	1.06	0.96	0.53	3.09

Reference Standards. The nonlabeled standards and intermediates were generously provided by S. J. Nelson and V. L. Rizzo of the Upjohn Co.

Animal Treatment. For each compound, two female Sprague-Dawley rats and one group of four female ICR-Upjohn mice were housed in separate all-metal metabolism cages (Acme Metal Products, Chicago), which allowed for the separation and collection of urine and feces. Feed (Purina-Upjohn rat/mouse chow) and water were provided ad libitum throughout the study, except for the 12-h period just prior to dosing when feed was withdrawn.

The radiolabeled compounds were dissolved in USP-grade corn oil at 2.45–4.42 mg/mL for the rats and 1.20–2.95 mg/mL for the mice. The respective solutions were administered by gavage, 0.5 mL to each rat and 0.25 mL to each mouse.

Excretion Patterns. Excreta collections were made at 12, 24, 48, 72, and 96 h posttreatment. The urine was collected into 25-mL glass-stoppered volumetric mixing cylinders, which were packed in ice during the collection period, and diluted with ice-cold water to the 25-mL mark at the end of the period. Three 0.100-mL aliquots were placed in separate scintillation vials followed by 15 mL of Diotol scintillation cocktail (Burdick and Jackson Laboratories, Muskegon, MI) and counted as described below. The bulk urine sample was then stored frozen (−20 °C) until assayed.

The feces samples were weighed into tared 2-oz wide-mouthed jars and homogenized with a 3–4-fold weight of water for 3–5 min with a Tissuemizer (Tekmar, Cincinnati, OH) equipped with an S10N generator. Three 300–400-mg aliquots of the homogenate slurry were weighed into quartz combustion boats for combustion analysis with a biological material oxidizer (Beckman Instruments, Fullerton, CA). The evolved [^{14}C]CO_2 was trapped in 15 mL of CO_2 absorbent scintillator solvent (Burdick and Jackson) contained in a glass bubbler trap, and the solution was then drained into a scintillation vial.

A Model 3375 Packard Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., Downers Grove, IL) was used for all radioactive assays. All vials were counted 3 times for either 10.0 min or 20 000 counts each, whichever occurred first. The counting efficiency was determined by the internal standard technique using [^{14}C]-toluene. Data computations were made with computer programs that apply the appropriate corrections for background, counting efficiency, combustion efficiency, and sample aliquot size.

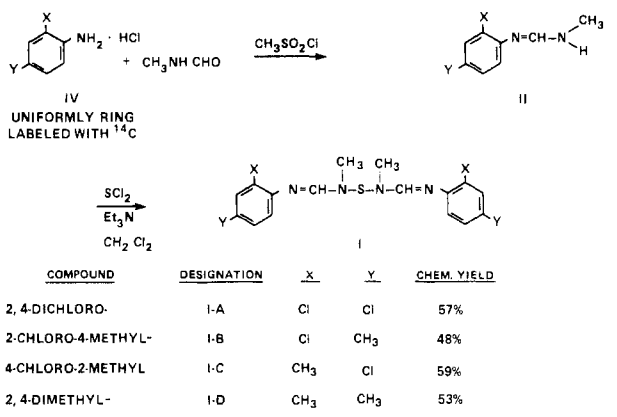
Extraction of Urinary Metabolites. A 10.0-mL aliquot of the thawed urine sample was transferred to a 50-mL glass-stoppered centrifuge tube and the pH adjusted to 10–11 with dropwise addition of 1 N NaOH. The tube was partially immersed in an ice bath throughout. The sample was then extracted 4 times with an equivalent volumn of ice-cold CH_2Cl_2. An emulsion was generally produced but was easily broken by centrifugation. The extracts were combined and dried over anhydrous Na_2SO_4. The pH of the aqueous solution was then adjusted to 1–2 with 2 N HCl and this acidic solution extracted again 4 times with CH_2Cl_2 as above. Both extracts were sampled in triplicate for radioactive assay.

Stability and Extraction of Standards. In four separate trials, 10 mL of fresh blank rat urine, containing no residues of any of these compounds, was spiked with ^{14}C-radiolabeled I-A, I-D, II-D, or III-D. At each of five sampling times, 2.0-mL aliquots were taken for extraction and TLC assay for compound integrity. The sampling times were 0, 6, 24, 48, and 120 h. The spiked urine samples were kept at 0 °C through the first 6 h and then frozen at −20 °C for the duration.

Enzyme Hydrolysis of Urine. A 10.0-mL aliquot of the urine sample was transferred to a 50-mL glass-stoppered centrifuge tube containing a $^5/_8$-in. Teflon-coated magnetic stir bar and the pH adjusted to 5.2 with 0.5 mL of 2.0 M acetate buffer. Chloroform (approximately 50 μL) was added followed by 0.30 mL of Glusulase (Endo Laboratories, Inc., Garden City, NY), which contained approximately 4500 units of aryl sulfatase and approximately 50 000 units of β-glucuronidase. The stirred mixture was then incubated at 37 °C for 42–48 h. Base and acid extracts were then obtained as described above.

TLC Analysis. The extracts were flash evaporated to dryness and the residues taken up in 1.0 mL of acetone and transferred to screw-cap vials. A 5–25-μL aliquot was applied as a single spot, 20 mm from the bottom edge and to the left of center, to a 5 cm × 20 cm TLC plate, 250 μm of silica gel GF (Analtech, Newark, DE). A mixture of nonradiolabeled reference standards, II–VI in each series as appropriate, was applied to the right of the sample spot. Duplicate plates were prepared for one-dimensional development in two solvent systems: system 1 was 95:5 benzene–diethylamine and system 2 was 90:25:4 benzene–dioxane–acetic acid. The plates were developed to a height of 10 cm above the spot origin. The plates were air-dried and photographed under UV light (254 nm) to determine the R_f of the reference standards, and then the silica gel absorbent was segmented into 5-mm horizontal bands with a Camag zonal plate scraper (Analabs, North Haven, CT). Each band was channeled into a scintillation vial, and the silica gel was wetted with 200 μL of 1:1 MeOH–H_2O and then slurried with 15 mL of Diotol scintillator. Computer analysis of the data generated histograms of the chromatographically dispersed radiolabeled metabolites for comparison to R_f values for the reference standards. A second set of TLC plates was prepared for autoradiography (Industrex Type 5-AA X-ray film, Kodak): Exposure times varied from 1 to 5 weeks depending on the total activity applied to the TLC plate.

GC Analysis. *Sample Preparation.* The metabolites were separated by preparative TLC on 500-μm silica gel plates (Analtech) with the appropriate solvent system. The



COMPOUND	DESIGNATION	X	Y	CHEM. YIELD
2,4-DICHLORO-	I-A	Cl	Cl	57%
2-CHLORO-4-METHYL-	I-B	Cl	CH_3	48%
4-CHLORO-2-METHYL	I-C	CH_3	Cl	59%
2,4-DIMETHYL-	I-D	CH_3	CH_3	53%

Figure 1. Synthetic scheme for the thiobis([^{14}C]formamidines), their substitution patterns, and respective designations.

Table I. Oral Dosage Data of the Thiobis(formamidines) in Mice and Rats

compound	species	animal weight, g	dose,[a] mg	dose rate, mg/kg
2,4-diCl	mouse[b]	99.0	1.20	12.1
(I-A)	rat 1	190.5	2.18	11.4
	rat 2	203.5	2.26	11.1
2-Cl-4-Me	mouse[b]	94.5	1.24	13.1
(I-B)	rat 1	206.5	2.20	10.6
	rat 2	198.2	2.15	10.8
4-Cl-2-Me	mouse[b]	96.0	0.98	10.2
(I-C)	rat 1	194.0	2.45	12.6
	rat 2	205.5	2.21	10.8
2,4-diMe	mouse[c]	64.5	2.21	34.3
(I-D)	rat 1	200.1	2.21	11.1
	rat 2	208.7	2.16	10.3

[a] Dissolved in corn oil, administered by intubation. [b] Four animals per trial. [c] Only three animals in this trial.

developed and dried plates were exposed to X-ray film (15–24 h) to establish the precise locations of the radioactive components. The areas of interest were carefully removed with a razor blade into 3-mL screw-cap vials and the silica gel was slurried in 2 mL of acetone for 30–60 min. The slurry was filtered through a medium-porosity (10–15 μm) 2-mL glass-scintered funnel. The filtrate was evaporated to dryness with nitrogen and the residue redissolved in 50 or 100 μL of acetone.

GC/RAM. The TLC-purified sample fractions were analyzed on a gas chromatograph (Model 402, Hewlett-Packard) equipped with a FID and a gas proportional counter, or radioactive monitor, RAM (Nuclear Chicago), and fitted with a 0.25 in. × 2 ft glass column packed with 3% Versamid 900 on GC-Q (Applied Science, State College, PA). A temperature program from 130 to 230 °C at 10 °C/min and a helium carrier gas flow rate of 60 mL/min were generally used. Acids V and VI were derivatized to the methyl esters with diazomethane prior to GC. Many of the metabolite samples were analyzed both before and after methylation.

GC/MS. The TLC-purified sample fractions were analyzed on an LKB-9000 GC/MS interfaced with an IBM 1800 data system. The GC conditions were as described above except that the helium carrier flow rate was reduced to 40 mL/min. Mass spectra were generated by electron impact ionization operated at 70 eV.

RESULTS AND DISCUSSION

Dose and Excretion Patterns. The ^{14}C-radiolabeled thiobis(formamidines) (I) were readily prepared as outlined in Figure 1 beginning with the uniformly ring-labeled [^{14}C]-aniline hydrochlorides. The compounds were administered as corn oil solutions to mice and rats by gavage. The dose rates, tabulated in Table I, were in the range 10–13 mg/kg, with the exception of the dimethyl analogue (I-D) where the dose preparation for the rats was inadvertently administered to the mice, resulting in an elevated dose rate of 34.4 mg/kg. The 10–13 mg/kg dose rate was well below the physiological effect level of 30–40 mg/kg for these compounds (Kakuk, 1978). The excretion data, summarized in Table II, showed that 94–100% of the dose was excreted within 96 h in every case. The overall high yield of excreted ^{14}C residue demonstrated a rapid and thorough passage of the thiobis(formamidines) through the animals with little or no apparent retention of metabolites.

Table II. Excretion of Thiobis([^{14}C]formamidine) Residues after Oral Dosage

compound	species	duration, h	amount excreted, % of dose: in urine	in feces	total
2,4-diCl (I-A)	mouse	96	73.8	22.3	96.1
	rat 1	96	88.9	11.2	100.1
	rat 2	96	83.4	15.7	99.1
2-Cl-4-Me (I-B)	mouse	96	81.4	13.8	95.2
	rat 1	96	78.2	22.6	100.8
	rat 2	96	82.1	16.5	98.6
4-Cl-2-Me (I-C)	mouse	72	70.6	22.9	93.5
	rat 1	72	76.7	19.5	96.2
	rat 2	96	78.9	20.2	99.1
2,4-diMe (I-D)	mouse	72	80.5	13.9	94.4
	rat 1	72	90.0	9.3	99.3
	rat 2	96	83.8	16.7	100.5

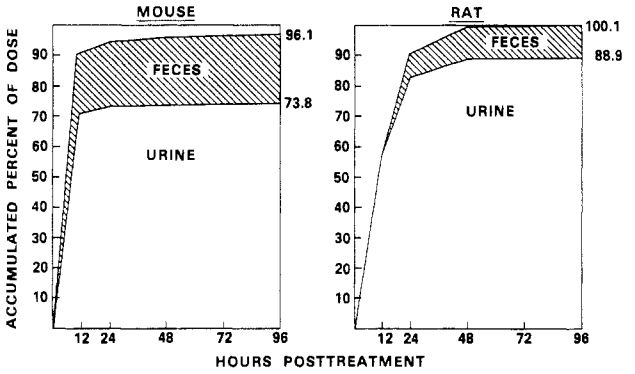


Figure 2. Excretion pattern of thiobis(formamidine) I-A following a single oral dose. These patterns were nearly identical for all four thiobis(formamidines).

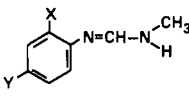
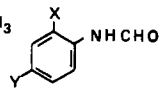
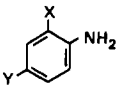
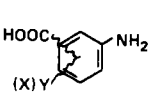
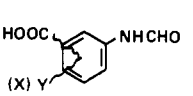
A typical graphical representation of the excretion patterns found in the mouse and rat is given in Figure 2, here for the 2,4-dichloro analogue (I-A). All thiobis(formamidines) generated vitually the same pattern. In all cases, the primary mode of excretion was via the urinary tract. For this reason, metabolite identification work was restricted to the urine samples.

Metabolite Identification. The isolation and identification of the urinary metabolites were restricted to those urine samples that contained the highest level of total ^{14}C activity. These usually included the 12-h mouse sample and the 12- and 24- h rat samples; urine samples were not pooled since the metabolite composition was expected to change with time. Extraction of the raw urine (but pH adjusted) and of the enzyme (β-glucuronidase and arylsulfatase) hydrolyzed urine, following the extraction scheme outlined in Figure 3, was carried out to establish the extent of glucuronide and sulfate conjugation. There were no significant differences in the extractability of the urine samples as a function of time, and the combined

Table III. Methylene Chloride Extraction of Urinary Thiobis([^{14}C]formamidine) Residues before and after Enzyme Hydrolysis, Pooled Values

			% of urine sample radioactivity					
			unhydrolyzed			Glusulase hydrolyzed		
compound	species	(sample)	base extr	acid extr	unextr	base extr	acid extr	unextr
2,4-diCl (I-A)	M	(12 h)	7.8	16.9	75.3	54.2	13.9	31.9
	R	(12 + 24 h)	6.8	13.1	80.0	36.1	29.8	34.1
2-Cl-4-Me (I-B)	M	(12 h)	3.3	40.4	56.3	10.4	47.3	42.3
	R	(12 + 24 h)	4.2	36.1	59.6	11.0	37.1	51.9
4-Cl-2-Me (I-C)	M	(12 + 24 h)	15.3	36.5	48.2	38.9	45.3	15.9
	R	(12 + 24 h)	19.2	42.1	38.7	21.6	66.3	12.1
2,4-diMe (I-D)	M	(12 + 48 h)	13.1	18.3	68.6	14.8	27.1	58.1
	R	(12 + 24 h)	13.5	15.0	71.5	14.0	23.4	62.6

Table IV. Urinary Thiobis(formamidine) Metabolites following Glusulase Hydrolysis of Urine from Mice and Rats: Pooled Data Expressed as a Percent of the Dose

						
compound	species	II	III	IV	V	VI
I-A	M	0	0.4	12.2 (4.1)		
	R	0	0.5	10.8 (6.0)		
I-B	M	0	0.2	0.7 (0.7)	12.2	19.6
	R	0	0.4	1.1 (0.4)	8.5[a]	16.5[a]
I-C	M	0.3	4.1	1.5 (1.2)	2.9	2.0 (2.0)[b]
	R	0.5	2.0	0.6 (0.6)	5.8	4.1 (1.4)[b]
I-D	M	4.4 (5.0)[d]	1.1	0.2 (0.2)	1.5[c]	14.1 (9.5)[c]
	R	6.7 (8.4)	2.1	0.3 (0.1)	1.2[c]	16.6 (10.8)[c]

[a] Estimated from insufficiently resolved TLC data. [b] Estimated; structural verification was inconclusive. [c] *p*-Methyl oxidation only. [d] Values in parentheses are from unhydrolyzed urine samples.

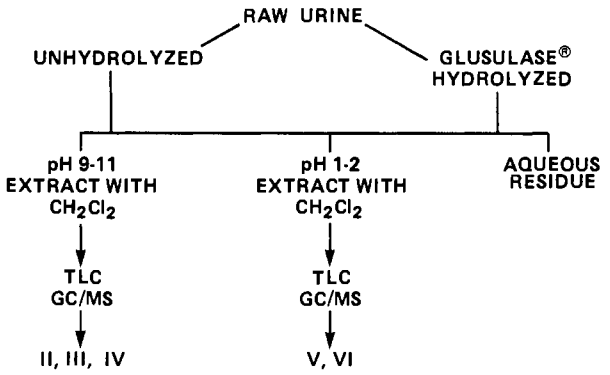


Figure 3. Flow chart for the extraction of the ^{14}C residue from mouse and rat urine.

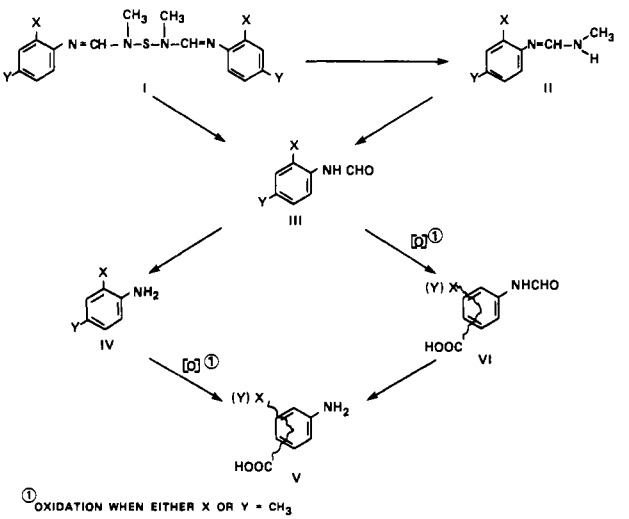


Figure 4. Metabolism scheme for the thiobis(formamidine) compounds orally administered to mice and rats.

extraction data are summarized in Table III. The quantities of organosolubles generally increased following the enzymatic hydrolysis, from a low of 9–10% for compound I-D to a high of 43–46% for I-A. The bulk of the increase in compounds I-A and I-B was found in the base extracts, which contained the neutral and basic metabolites II, III, and IV (see Figure 4). The bulk of the increase in I-C and I-D was found in the acid extracts, which contained the acidic metabolites V and VI. However, much of the activity still remained unextracted, particularly for I-B and I-D. The nature of these water-soluble residues was not investigated.

Preliminary identification of the organosoluble metabolites were carried out by comparison of the relative migrations of radioactive components to reference standards in two TLC solvent systems. The quantities of these metabolites were determined from the histogram data. Structural verification was carried out by gas chromatography using GC/RAM and GC/MS techniques. Quantitative estimates of the identified metabolites are presented in Table IV. The data are pooled values since there were no significant differences in the metabolite composition as a function of time for the samples analyzed here. The major entries are from the enzyme-hydrolyzed samples and thus represent both unconjugated or "free" metabolites plus the proportion derived from glucuronide and sulfate conjugation. The minor entries (in parentheses) represent the quantities of free metabolites obtained from raw nonhydrolyzed urine samples. These data suggested that the conjugation of at least a portion of the metabolites was an incomplete process. Reasons for this are not entirely clear.

Parent compounds (I) were not found to a minimum detectability of 0.01% of the dose in any urine sample examined. The extractability and urine stability of compounds I-A, I-D, II-D, and III-D were examined to ensure that the methods used in this study would preserve and

Table V. Extractability and Stability of Various Compounds in the Thiobis(formamidine) Series: Fresh Control (Blank) Rat Urine Fortified with ^{14}C Compound, Stored at 0 °C for 6 h, and Then Stored Frozen for the Duration

compound	time, h	% extracted	% pure
I-A	0	98	100
	6	88	97
	24	91	98
	48	85	93
	120	87	91
I-D	0	88	98
	6	86	96
	24	78	95
	48	85	93
	120	82	90
II-D	0	100	100
	6	89	97
	24	91	98
	48	85	93
	120	86	91
III-D	0	98	100
	6	82	98
	24	94	96
	48	103	96
	120	95	95

detect these compounds if excreted "free" in the urine. These data are summarized in Table V. There appears to be slight degradation of compounds I-D and II-D out to 5 days in frozen urine, but there is clearly not a rapid loss of compound, relative to the time of analysis following sample collection, such that measurable levels of these compounds in treated animals would have avoided detection.

The data taken as a whole suggested either a rapid metabolism of I or, more likely, a rapid hydrolysis of I in the acidic environment of the stomach. The hydrolysis of I was implicated during the synthesis of these compounds, since all were found to be somewhat hydrolytically labile. The relatively high quantity of compound II-D was unexpected since the simple formamidines were generally found to be slightly acid labile as well.

In every case, the corresponding anilines (IV) were detected as the free aniline, although in minor amounts except for the dichloro series. The enzymatic hydrolysis generally produced very small additional amounts of the anilines, except in the case of the dichloro series where significant quantities of additional aniline were produced. The higher levels of the dichloroaniline relative to the anilines that contain a methyl substituent were anticipated because the facile alkyl side chain oxidation, which so readily occurs on benzylic methyl groups, was negated in this series. However, the "total" aniline residue from all four thiobis(formamidines) was generally a very small portion of the total urinary metabolite pool.

The oxidation of benzylic methyl groups is a well-known, sufficiently documented, facile metabolic process (Testa and Jenner, 1976). The major identifiable metabolic components in I-B (2-chloro-4-methyl-) and I-D (2,4-dimethyl-) contained the carboxylic acid function. The relative quantities of the (formylamino)benzoic acids (VI) were greater than the simpler aminobenzoic acids, which suggested that oxidation of the methyl group was a more facile metabolic fate than the *N*-deformylation. The two acids in the rat urine from compound I-B were insufficiently resolved by TLC to yield clear-cut quantitation. However, a 2:1 ratio in favor of the *N*-formyl acid was estimated. In addition, the quantity of the *N*-formyl acid in the I-C series (4-chloro-2-methyl-) was estimated by TLC to be slightly less than that of the deformylated acid: The values were not definitively established since structural verification by GC/MS was inconclusive. The overall results do, however, suggest that benzylic methyl group oxidation was more facile than *N*-deformylation, perhaps even in those compounds where the methyl group is ortho to the nitrogen.

In the dimethyl series (I-D), only *p*-methyl oxidation was observed, despite efforts to locate the corresponding *o*-methyl oxidation products (the 5-methylanthranilic acid analogue); authentic standards were available to aid this search. Steric factors are the likely driving forces for this observed direction of oxidation. Similar results have been observed in other compounds (Testa and Jenner, 1976).

There were no significant differences observed between the metabolism patterns of the mouse and rat for the four thiobis(formamidines) studied. In the case of the rat, when two collection periods were examined, there also were no significant differences in the metabolic content of the urine as a function of time. The relative amounts of total aniline produced from each compound by each species were nearly the same. At the dose level studied, under the single-dose regimen, and to the extent to which the excreted metabolites have been analyzed, there were no apparent trends to suggest differences in metabolism by the two species.

A direct comparison to similar metabolism studies can only be made for I-C. Knowles has studied the metabolism of chlordimeform (VII) in the rat (Knowles, 1970). Both compounds are derived from 4-chloro-2-methylaniline. Both compounds gave quantitatively similar metabolites. The formation of compounds II–VI represented the minor but identifiable metabolite fraction. The metabolism of chlordimeform apparently generated a larger proportion of the free aniline than I-C. And larger quantities of the *N*-formyl acid relative to the deformylated acid were observed for chlordimeform than for I-C.

In summary, the metabolism scheme which evolved from this initial work is presented in Figure 4. There was an apparent rapid hydrolysis of parent compound in the stomach to the simple formamidine II, most of which did not survive the hydrolytic or metabolic conditions in the body. The formamidine was then converted to the *N*-formylaniline III, which subsequently could have suffered more than one fate. When side chain oxidation was possible, as in all compounds except the dichloro, I-A, generation of the acid VI occurred. To a lesser degree, N-deformylation of III gave the simple aniline IV. This clearly became the dominant pathway in the absence of side chain oxidation as was found in I-A. The formation of the aminobenzoic acid V could clearly result from either IV or VI. When the molecule provided two potentially oxidizable side chains, or methyl groups, as in I-D, *p*-methyl oxidation preferentially occurred over *o*-methyl oxidation. In all four cases, additional metabolism no doubt occurred, including glucuronide and/or sulfate conjugation. Other oxidation products were likely produced, but no attempts were made to isolate these products such as the intermediate oxidative compounds between IV and V or between III and VI, for example: Such products have been documented in other studies involving compounds with similar structural features (Testa and Jenner, 1976).

Registry No. I-A, 80029-35-2; I-A (^{14}C-labeled), 87923-96-4; I-B, 80029-36-3; I-B (^{14}C-labeled), 87923-97-5; I-C, 59295-57-7; I-C (^{14}C-labeled), 87923-98-6; I-D, 59263-66-0; I-D (^{14}C-labeled), 87923-99-7; II-C, 21787-80-4; II-D, 33089-74-6; III-A, 22923-00-8; III-B, 18931-77-6; III-C, 21787-81-5; III-D, 60397-77-5; IV-A, 554-00-7; IV-A·HCl (^{14}C-labeled), 65799-64-6; IV-B, 615-65-6; IV-B·HCl (^{14}C-labeled), 87923-94-2; IV-C, 95-69-2; IV-C·HCl (^{14}C-labeled), 87999-30-2; IV-D, 95-68-1; IV-D·HCl (^{14}C-labeled),

87923-95-3; V-B, 2486-71-7; V-C, 635-21-2; V-D, 2486-70-6; VI-B, 87923-93-1; VI-C, 26208-56-0; VI-D, 80029-31-8; CH_3NHCHO, 123-39-7; SCl_2, 10545-99-0.

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Synthesis and Biological Activity Studies of Selected Organophosphorus Esters

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Thirty organophosphorus esters were synthesized and evaluated as possible insecticide synergists against boll weevils, *Anthonomous grandis*. B-Esterase and acetylcholinesterase activity from organophosphorus-susceptible weevils were measured spectrophotometrically with *S*-phenyl thiobenzoate and acetylthiocholine as substrates. The structure–biological activity relation may be divided into three major effects—a lipophilic effect, an electronic effect, and a steric effect. In vitro and in vivo inhibition and toxicity data support the hypothesis that synergism of methyl paraoxon results from the inhibition of *S*-phenyl thiobenzoate hydrolyzing esterase by selected organophosphorus esters.

The most thoroughly studied and best known examples of organophosphorus synergism are those involving carboxylesterase inhibitions with malathion (Frawley et al., 1957; Murphy et al., 1959; Eto and Casida, 1962; Eto et al., 1965; Casida, 1961). These B-esterase enzymes hydrolyze the α-carboxyl ester group of malathion to yield the α-monoacid and/or the β-ester group to the β-monoacid, both very weak cholinesterase inhibitors (Cook et al., 1958). Because the low mammalian toxicity of malathion is the result of its degradation by carboxylesterase, the inhibition of this enzyme brings about a subsequent increase in toxicity.

DEF (*S,S,S*-tributyl phosphorotrithioate) was found to synergize methyl parathion and methyl paraoxon against boll weevils, *Anthonomous grandis* (Chambers, 1979a). Additionally, DEF has been found to synergize the carbamate and organochlorine insecticides when applied to certain resistant insect strains (Sun et al., 1967). Striking differences in the patterns of synergisms of organophosphorus, carbamate, and pyrethroid insecticides in boll weevils by DEF, and by piperonyl butoxide and sesamex, suggest that the DEF-induced synergism is not via mixed-function oxidase inhibition (Wilkinson, 1976). Rather, inhibition of β-esterases appears to be the mechanism involved, though the role of these enzymes in limiting insecticide toxicity has not been fully explained (chambers, unpublished data). Recently, mixtures of carbamate–organophosphorus derivatives have been shown to have synergistic possibilities (Bakunick, 1979).

In this paper, the preparation of organophosphorus esters (structurally similar analogues of DEF) and their synergistic activity toward methyl paraoxon are reported.

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EXPERIMENTAL SECTION

Methods and Materials. Column adsorption chromatography (Brinkman silica gel 60) was used to purify all ester compounds except the *o*-phenylene phosphorothioates (distill). An ether–hexane (1:1) solvent system was used. Compound purity was checked by thin-layer chromatography using Eastman Chromagram precoated sheets (13181 silica gel).

All nuclear magnetic resonance spectra (^1H NMR) were obtained at 60 MHz by using a Varian A-60 nuclear magnetic resonance spectrometer. Tetramethylsilane was used as the internal standard and 99.8% chloroform-*d* was used as the solvent. All mass spectroscopy data were obtained by using a Hewlett-Packard Model 5930 quadrupole electron impact mass spectrometer. All infrared spectra were obtained by using a Perkin-Elmer Model 283 grating infrared spectrophotometer. The spectra of liquids were taken as films formed between two cesium iodide plates; potassium bromide was used in preparing pellets of solid samples.

Synthesis. The analytical data are given in Table I. The IR, ^1H NMR, and MS spectra were consistent with the proposed structure (see supplementary material for a listing of the data; see paragraph at end of paper regarding supplementary material).

S,S,S-Trialkyl Phosphorotrithioates (**1–5**). One equivalent of phosphorus trichloride was alkylated with a mixture of 3 equiv of the appropriate mercaptan and 3 equiv of triethylamine in benzene at 0 °C (Ford-Moore and Perry, 1963). After the reaction mixture was warmed to room temperature and stirred for 12 h, triethylammonium hydrochloride was removed by filtration. Unreacted phosphorus trichloride and benzene were removed by using a rotary evaporator. One equivalent of the *S,S,S*-trialkyl

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