Metabolism Studies with Torak Insecticide in a Dairy Cow

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Excretion and metabolism of the insecticide Torak [*S*-(2-chloro-1-phthalimidoethyl) *O,O*-diethyl phosphorodithioate] were studied in a dairy cow. At an insecticide level of 5 ppm in the daily ration for 4 days, no residues of Torak or its oxygen analog were found in milk. Torak was also absent in urine. In feces 3.07% of the total Torak fed was excreted as the intact insecticide. A gas chromatographic peak with the retention time of diethyl thiophosphate was found in urine representing 33.14% of the total Torak dose. Hydrolysis of urine prior to extraction and analysis did not release additional amounts of this metabolite. Another peak was observed in urine corresponding in retention time to traces of diethyl phosphate. Torak rapidly decomposed in the presence of beef liver 10,000 × *g* supernate with the production of diethyl thiophosphate. Phosphorus-containing hydrolytic products were not found in unhydrolyzed or hydrolyzed milk or unhydrolyzed feces. Torak was not metabolized in the presence of rumen fluid.

Torak [*S*-(2-chloro-1-phthalimidoethyl) *O,O*-diethyl phosphorodithioate] (Hercules 14503) is a new broad spectrum compound for control of crop insects and mites and other pests such as lice and ticks which affect animals and man. It is particularly effective for controlling the alfalfa weevil and European corn borer and certain insect pests of citrus (Hercules Bulletin AD-8, 1970).

Although studies of the metabolism of Torak in animals have not been published the fate of a similar molecule, Imidan (*O,O*-dimethyl phthalimidomethyl phosphorodithioate) has been investigated in rats (Ford *et al.*, 1966; McBain *et al.*, 1968) and cows (Johnson and Bowman, 1968). In rats orally fed ^{14}C-labeled Imidan, the major metabolites were phthalamic and phthalic acids with smaller amounts of phthaloyl metabolites excreted in the urine. Excretion of unidentified fecal metabolite(s) also occurred. Very little of the intact compound or its oxygen analog was found in urine. Small amounts of unidentified residues were detected unselectively deposited in many tissues. Very little radioactivity was detected in expired carbon dioxide. In lactating Jersey cows receiving 84 mg of Imidan, residues of the insecticide and its oxygen analog were not detected in milk or urine. When ^{14}C-labeled Torak was fed at several levels to rats, total radioactivity measurements indicated major elimination in urine (47.6 to 54.8%) and feces (26.4 to 38.9%), some tissue storage (13.5 to 18.9%), and none in expired carbon dioxide (Bourke *et al.*, 1970). In the work reported, Torak was fed to a dairy cow to study routes of excretion and metabolism of the compound.

EXPERIMENTAL

A Holstein cow weighing 681 kg and with a daily milk production of about 20.5 kg (3.7% butterfat) was catheterized and fed Torak at the 5-ppm level (based on a daily ration of 22.7 kg) for 4 days. The 5-ppm feeding level was chosen as the maximum concentration that one would expect to find in a dairy cattle ration. The pure compound in acetone was thoroughly mixed with the evening grain. Morning and evening subsamples of the total mixed milk were taken 1 day prior to feeding (control sample) daily throughout the feeding period, and for 6 days thereafter. The total daily urine and manure samples were similarly collected, weighed, mixed, and subsampled during the same test period. The manure samples were collected in specially constructed trays. All samples were immediately frozen prior to analysis.

IN VITRO STUDIES

Rumen Fluid. The stability of Torak in the presence of fresh rumen fluid was studied. One milliliter of a solution of Torak in acetone (500 μg/ml) was thoroughly mixed with 100 ml of fresh filtered rumen fluid and held at 38° C. At measured intervals 5 ml of fluid were removed and 5 ml of acetone were added. The mixture was filtered and the filter was rinsed with acetone to a total volume of 25 ml. One milliliter of the acetone filtrate was partitioned with 5 ml of hexane and 44 ml of 2% sodium sulfate solution. Up to 8 μl of the upper hexane layer were analyzed for Torak by electron affinity gas chromatography.

Liver. Possible metabolism of Torak was studied in the presence of the 10,000 × *g* supernatant fraction of fresh beef liver which contains microsomes and soluble enzymes. An Angus steer was slaughtered and the liver was immediately removed. A portion was immersed in 0.25 *M* sucrose solution at 0° C and all further processing for enzyme preparation was conducted in the cold (0–4° C). A 20% liver homogenate in the sucrose solution was prepared using a Dounce homogenizer. The homogenate was centrifuged at 10,000 × *g* max for 30 min. Incubation mixtures contained 5 μg of Torak (100 μl of a 50 μg/ml solution in acetone), 25 μmol of magnesium chloride, 95 μmol of Tris buffer, pH 7.4, 20 μmol of glucose 6-phosphate, 1.5 μmol of TPN (NADP), and 1 ml of the enzyme (10,000 × *g* supernate) preparation in a total volume of 5 ml. Incubations were carried out in a 25-ml Erlenmeyer flask at 37° C in an atmosphere of air for 30 min. The flasks contained a borosilicate marble 0.5 in. in diameter

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and were mechanically shaken 100 times per minute on a reciprocating shaker during incubation. (These samples as well as the controls, which included either no enzyme or no substrate, were carried through the procedure in triplicate.) After 30 min the reactions were terminated by the addition of 3 ml of acetone and each incubation mixture was transferred to a 100-ml volumetric flask using 2 ml of acetone for rinsing. Hexane (10 ml) was added, the flask was made to volume with 2% sodium sulfate solution, and it was shaken vigorously for 1 min. The upper hexane layer (5 μl) was analyzed for Torak by electron affinity gas chromatography.

EXTRACTION, ISOLATION, AND ANALYSIS OF TORAK IN OTHER BODY FLUIDS

Milk and Urine. Milk or urine (25 g) was blended with 60 ml of acetone in an ice bath for 2 min. The mixture was filtered and the filter was rinsed with acetone until the total filtrate volume was 100 ml. The filtrate was transferred to a 250-ml separatory funnel and partitioned with 100 ml and two 25-ml portions of chloroform. After each partitioning the lower organic layer was filtered through anhydrous sodium sulfate and the filtrates were combined in a 500-ml round-bottomed flask and evaporated to dryness using rotary evaporation in a 45° C water bath. The residue was dissolved in 5 ml of benzene and up to 10 μl of the benzene solution was analyzed by electron affinity gas chromatography. The above procedure was also applicable to analysis of Torak oxygen analog in milk.

Feces. For the determination of Torak in feces, 25 g of the well-mixed sample was blended for 2 min with 60 ml of acetone using an ice bath. The mixture was filtered and the filter was rinsed with acetone until 100 ml of filtrate was collected. The filtrate was evaporated in a 100-ml volumetric flask using air. Benzene (10 ml) was added, the flask was made to volume with 2% sodium sulfate solution, and the contents were shaken vigorously for 1 min. Up to 5 μl were analyzed by alkali thermionic gas chromatography.

EXTRACTION, ISOLATION, AND ANALYSIS OF HYDROLYTIC PHOSPHORUS METABOLITES

Milk. Analysis of milk for possible water-soluble, phosphorus-containing hydrolytic products of Torak was performed. Well-mixed whole milk was centrifuged at 1000 rpm for 30 min. The skim milk (10 g) was transferred to a 50-ml volumetric flask. One milliliter of 5 *N* hydrochloric acid and 5 ml of 20% ethyl acetate in diethyl ether (v/v) was added, the flask was made to volume with saturated sodium chloride solution, and was shaken vigorously. The mixture was centrifuged and a 2-ml aliquot of the upper organic layer was transferred to a calibrated concentration tube, 0.22 ml of methanol was added, and the solution was methylated with diazomethane following the procedure of Schlenk and Gellerman (1960). After removal of excess diazomethane with nitrogen bubbling, the solution was made to a volume of 1 ml with the ethyl acetate–ether solution. Up to 10 μl of this solution was analyzed by alkali thermionic gas chromatography.

Urine. Urine was also analyzed for free phosphorus-containing cleavage products of Torak. The method was identical to that used for these hydrolytic products in milk except that 5 g of urine was taken for analysis and the final organic solution after methylation was adjusted to either 2 or 10 ml (depending on concentration) prior to gas chromatographic analyses.

Feces. Analysis of feces for free phosphorus-containing hydrolytic products was performed. Feces (25 g) was extracted with 100 ml of acetone containing 1 ml of orthophosphoric acid. A 20-ml portion of this solution was placed in a 50-ml volumetric flask and the acetone was evaporated using air. One milliliter of 5 *N* hydrochloric acid and 5 ml of the ethyl acetate–ether solution were added and the flask was made to volume with saturated sodium chloride solution and shaken vigorously. A 2-ml portion of the upper organic layer was then methylated by the procedure described above for these hydrolytic products in liver. Analysis was made by alkali thermionic gas chromatography.

Liver. A second incubation study with Torak in the presence of the 10,000 $\times$ *g* supernatant fraction of Angus steer liver was conducted in which analysis for phosphorus cleavage products was performed. After terminating the reaction at 30 min with 3 ml of acetone the incubate solution was transferred to a 50-ml volumetric flask using about 3 ml of acetone for rinsing. 20% ethyl acetate in ether (5 ml) and 1 ml of 5 *N* hydrochloric acid was added and the flask was diluted to volume with saturated sodium chloride solution and shaken vigorously. A 2-ml aliquot of the upper organic layer was transferred to a calibrated concentration tube and the solution was methylated as described for analysis of milk beginning with the addition of methanol. Up to 5 μl of ethyl acetate–ether solution were analyzed by alkali thermionic gas chromatography.

EXTRACTION, ISOLATION, AND ANALYSIS OF CONJUGATED HYDROLYTIC PHOSPHORUS METABOLITES

Milk. Milk samples were hydrolyzed to release possible hydrolytic phosphorus metabolites which may have been present in bound or conjugated form. Two 10-g portions of skim milk were transferred to 50-ml volumetric flasks. Both an acid and base hydrolysis procedure were compared. 1 *N* hydrochloric acid (5 ml) was added to the milk in one flask and 5 ml of 1 *N* sodium hydroxide was added to that in the other. Both flasks were fitted with a 10/30 ground glass joint to serve as an air condenser and the contents were heated in a boiling water bath for 30 min. The contents were cooled and each condenser was rinsed down with 2 ml of water. 5 *N* hydrochloric acid (2 ml) was added to the flask containing the alkaline hydrolysate. The remainder of the procedure including methylation was identical to that used for analysis of free Torak hydrolytic products in milk, beginning with the addition of 5 ml of the ethyl acetate–ether solution.

Urine. Urine samples were subjected to alkaline hydrolysis. The procedure was identical to that above for alkaline hydrolysis of milk to liberate possible conjugates of hydrolytic phosphorus metabolites except that 5 g of urine were taken for analysis and 1 ml of 5 *N* sodium hydroxide was used during the initial reflux.

DETERMINATION

Final analysis for Torak and possible metabolites was made by gas chromatography. The gas chromatograph was a Barber-Colman Model 10. It was equipped with both an electron affinity and an alkali thermionic detector. The electron affinity detector was a battery-operated No. A-4071 of 6 cm^3 volume and containing 56 μCi of ^{226}Ra. The alkali thermionic detector was described earlier (St. John and Lisk, 1968). The recorder was a Wheelco, 0 to 50 mV, equipped with 10-in. chart paper, running 10 in./hr. The electrometer gain was 10,000 or 30,000. Nitrogen (60 cm^3/min) was the carrier gas. The columns were U-shaped, made of borosili-

Table I. Gas Chromatographic Operating Parameters

Sample	Column substrate and length	Detection mode	Temperature, °C: Column	Flash heater	Detector	Retention time, min
		Torak				
Milk, urine, rumen fluid, liver 10,000 × *g* supernate	A 1:1 (w/w) mixture of 10% DC-200 and 15% QF-1 on 80 to 100 mesh Gas-Chrom Q; 2 ft	Electron affinity	200	265	235	14.1
Feces	A 1:1 (w/w) mixture of 10% DC-200 and 15% QF-1 on 80 to 100 mesh Gas-Chrom Q; 2 ft	Alkali thermionic	200	265	235	14.1
		Torak oxygen analog				
Milk	A 1:1 (w/w) mixture of 10% DC-200 and 15% QF-1 on 80 to 100 mesh Gas-Chrom Q; 2 ft	Electron affinity	200	265	235	14.3
		Phosphorus-containing hydrolytic products				
Milk and urine (both either hydrolyzed or unhydrolyzed); feces unhydrolyzed; liver 10,000 × *g* supernate	10% OV-17 on 80 to 100 mesh Gas-Chrom Q; 6 ft	Alkali thermionic	110	200	300	DETP[a] 10.5

[a] Diethyl thiophosphate.

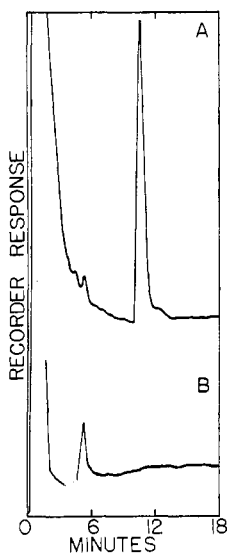

Figure 1. Gas chromatograms showing (A) the peak corresponding in retention time to diethyl thiophosphate in urine collected on the last day Torak was fed and (B) control urine

Table II. Recovery of Torak and Metabolites from Samples

Sample	Added, ppm	Recovery, %	Sensitivity, ppm
	Torak		
Milk	0.05	92, 80, 94, 92	0.01
	0.1	79	
	0.2	82	
Urine	0.1	98	0.02
Feces	0.1	78	0.05
	0.2	88, 99	
	2	70, 92	
Rumen fluid	5	102	
Liver 10,000 × *g* supernate	5	82, 86, 74	
	Torak oxygen analog		
Milk	0.05	78, 62, 72	0.01
	0.1	73	
	Diethyl thiophosphate		
Milk	1	70	0.1
Milk			
acid hydrolyzed	1	144	0.1
base hydrolyzed	1	115	0.1
Urine	0.08	72	0.01
	0.2	79	
	0.4	92	
Feces	0.2	110	

cate glass, and of 6 mm i.d. Table I lists the other operating parameters. Table II lists the percent recoveries and estimated sensitivities obtained using the method described for Torak and metabolites in various samples.

RESULTS AND DISCUSSION

No residues of intact Torak or its oxygen analog were detected in milk. Torak was not detected in urine. About 3.05% of the total Torak fed (454 mg) was found intact in the feces. Torak was stable in the presence of rumen fluid for 8 hr. The compound decomposed in the presence of the beef liver 10,000 × *g* supernate for 30 min. The percent decomposition of Torak in the triply replicated incubates of the liver was 76, 77, and 77%, respectively. In the second incubation, study of Torak with the liver fraction analysis of each liver incubate for phosphorus-containing hydrolytic products showed a peak with the retention time of diethyl thiophosphate equivalent to 37, 35, and 33% of the total Torak (5 μg) added to the triple replicates. Analysis of the liver incubates for Torak oxygen analog, a possible metabolite, was not done because attempts to recover this compound when added as a pure standard to liver incubates were unsuccessful.

No residues of phosphorus-containing hydrolytic products of Torak were found in unhydrolyzed milk or milk which was subjected to acid or alkaline hydrolysis. Analysis of unhydrolyzed urine for phosphorus-containing metabolites again showed a peak with the retention time of diethyl thiophosphate. It represented 33.14% of the total Torak fed

Table III. Daily Excretion of Diethyl Thiophosphate in Cow Urine

Day	% of total Torak excreted as diethyl thiophosphate
1[a]	Not detectable
2	3.77
3	7.25
4[b]	8.96
5	8.59
6	2.78
7	1.56
8	0.19
9	0.04
10	0.02
Total	33.14

[a] First day of feeding Torak. [b] Last day of feeding Torak.

(454 mg). Table III lists the daily amounts of diethyl thiophosphate excreted in unhydrolyzed urine in terms of the percent of total Torak fed. Figure 1 shows chromatograms of this metabolite in unhydrolyzed urine on the last day Torak was fed, and also shows control urine. Hydrolysis of urine with alkali did not release additional amounts of the diethyl thiophosphate metabolite. Traces of another hydrolytic phosphorus metabolite were excreted in urine. This metabolic peak had a retention time (5.2 min) corresponding to diethyl phosphate. This earlier eluting metabolite, if present in minute amounts, would have been particularly difficult to detect in urine samples because the urine extracts had to be concentrated prior to analysis and many background peaks appeared which would have interfered with those of this metabolite. No residues of phosphorus-containing hydrolytic products were found in feces samples. Residues of Torak may have been deposited in tissue samples. Since the cow used was part of the University's milking herd it could not be slaughtered to perform tissue analysis. Possible nitrogen-containing hydrolytic products of Torak were not determined.

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