Fate of Chloroneb in Animals

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2,5-Dichloro-4-methoxyphenol was isolated as the only metabolite of chloroneb in the urine of dogs, rats, and cows which had been maintained on diets containing chloroneb. This metabolite was found to be present both in the free form and as conjugates, presumably as glucuronides and sulfonates. The urine samples were checked specifically for the possible presence of 2,5-dichlorohydroquinone and 2,5-dichloroquinone and none was found (<0.02 ppm). Neither chloroneb nor any of the three possible metabolites noted above (<0.02 ppm) was found in the milk of a cow which was fed for 30 days on a diet containing 2 ppm chloroneb, a level which corresponds to the existing federal tolerance for total residues of chloroneb and its metabolite in forage crops.

Chloroneb, 1,4-dichloro-2,5-dimethoxybenzene, the active ingredient of Du Pont's Demosan 65W chloroneb fungicide, is useful as a supplemental seed treatment or as an in-furrow soil treatment at planting time for the control of seedling diseases of cotton, beans, and soybeans, and as a supplemental seed treatment for sugar beets. This paper reports on the metabolic fate of chloroneb when ingested by dogs, rats, and cows. The fate of this compound in plants and soils is reported in a companion paper (Rhodes *et al.*, 1971). 2,5-Dichloro-4-methoxyphenol (DCMP) was found to be the principal metabolite of chloroneb in cotton and bean plants. Trace amounts of 2,5-dichlorohydroquinone (DCHQ) and 2,5-dichloroquinone (DCQ) were detected as minor metabolites of chloroneb in these plants.

EXPERIMENTAL

Feeding. A female beagle dog was maintained on a diet containing 500 ppm of chloroneb in its rations, which consisted of Purina dog chow. The dog was maintained on the diet for 1 year, and then was sacrificed.

Male rats (Charles River, CD stock) were maintained on diets containing 2500 ppm of chloroneb in their rations, which consisted of 1% corn oil in ground Purina Lab Chow, for a period of 2 years, and then the rats were sacrificed. Urine and tissues from 10 rats were composited and each composite was analyzed as a single sample.

Two Guernsey dairy cows were maintained on diets containing 2 and 50 ppm of chloroneb in their daily rations, respectively, which consisted of 20 lb of dried alfalfa hay plus 10 lb of grain concentrate. The chloroneb was admixed with the grain concentrate portion of the ration. The cow receiving 2 ppm of chloroneb was maintained on the treated diet for 30 days, and then was sacrificed. The cow receiving 50 ppm of chloroneb was maintained on the treated diet for 30 days, followed by a 7-day period of feeding with untreated rations. Then the cow was sacrificed.

Extraction of Chloroneb and Metabolites from Dog and Rat Urine. NONHYDROLYZED. Urine (25 ml) was adjusted to pH 1 with hydrochloric acid and was extracted continuously for 48 hr with diethyl ether in a liquid–liquid extractor. Nitromethane (25 ml) was added to the ether phase and the volume of the resulting solution was reduced to about 20 ml in a hood. The solution was quantitatively transferred to a 25-ml volumetric flask and made to volume with nitromethane. Recovery studies for chloroneb and its three suspected metabolites from fortified urine samples demonstrated that greater than 90% of chloroneb, 95% of DCMP, and 80% of DCHQ plus DCQ are recovered using the described procedure.

HYDROLYZED. Urine (25 ml) and 10 ml of concentrated hydrochloric acid were placed in a 100-ml round-bottomed flask and the solution was heated under reflux for 4 hr. The solution was cooled to room temperature, adjusted to pH 1, and extracted according to the procedure described above.

METHYLATION. Urine (25-ml portions) was extracted according to each of the procedures described above. The volumes of the etherial solutions were reduced to 50 ml in a hood. Twenty grams of anhydrous sodium sulfate was added and the solution was allowed to stand (covered) overnight. The solution was filtered and the sodium sulfate was washed with three 10-ml portions of ether. Diazomethane (1.5 g), prepared from "Diazald" (Aldrich Chemical Co., Inc.), in 150 ml of ether was added to the extract and allowed to stand for 4 hr. The volume of the solution was reduced to about 20 ml under a hood. Nitromethane (25 ml) was added and the volume of the resulting solution was reduced to 20 ml. The solution was quantitatively transferred to a 25-ml volumetric flask and made to volume with nitromethane. This methylation procedure quantitatively converts phenolic metabolites to chloroneb. Analysis of the metabolite, before and after methylation, provides additional evidence for structural assignment, a method for cross-checking the analyses, and a method to ensure that no other phenolic metabolites are present.

Thin-Layer Chromatography and Infrared Qualitative Analysis. Aliquots of the urine extracts were applied as narrow streaks to thin-layer plates coated with 250-μ layers of Kieselgel with an incorporated phosphor and 5% Baymal as a binder. One microliter each of solutions containing 10 μg/μl of chloroneb, DCMP, DCHQ, and DCQ were spotted next to each streak for use as references and the plates were developed to 10 cm in chloroform.

Only one streak was observed, at an R_f of 0.28, which was not detected in an extract of control urine. For comparison, the R_f values for chloroneb, DCMP, DCHQ, and DCQ are 0.62, 0.28, 0.12, and 0.50, respectively, under these conditions. The area of adsorbent containing the metabolite streak was removed from the plate and eluted with about 10 ml of acetone. The volume of the acetone was reduced to about 0.5 ml under a gentle stream of nitrogen and the ir spectrum was obtained according to the procedure of Kirkland (1955). The ir spectra of the isolated metabolite from dog and rat

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Table I. Gas Chromatographic Analysis of Urine for Chloroneb and Metabolite

Urine	Residue (ppm) Chloroneb Original	Chloroneb Methylated	2,5-Dichloro-4-methoxyphenol Original	2,5-Dichloro-4-methoxyphenol Methylated
Dog				
Unhydrolyzed	N.D.[a]	136	154	N.D.
Hydrolyzed	N.D.	263	261	N.D.
100 ppm spike[b]	N.D.	105	105	N.D.
300 ppm spike[b]	N.D.	288	288	N.D.
Rat				
Unhydrolyzed	N.D.	131	131	N.D.

[a] N.D.—None detected. [b] Urine spiked with 2,5-dichloro-4-methoxyphenol.

urines are shown in Figure 1 in comparison to the spectrum of a standard sample of DCMP. These spectra confirm the identity of the metabolite as DCMP.

Gas Chromatographic Analysis of Dog and Rat Urine for Chloroneb and 2,5-Dichloro-4-methoxyphenol. The methylated and untreated extracts of the hydrolyzed and nonhydrolyzed urine samples were analyzed by gas chromatography on a Beckman GC-5 gas chromatograph equipped with a flame ionization detector. The following conditions were employed. Column: A 1-m, 0.25-in. (o.d.) stainless steel column packed with 20% Dow Corning DC-200 plus 0.2% Epon 1001 on 80-100 mesh "Diatoport S." Inlet temperature: 240° C. Column temperature: 175° C. Detector temperature: 245° C. He carrier flow: 75 cm^3/min. H_2 flow: 55 cm^3/min. Air flow: 300 cm^3/min.

Calibration curves for each compound, DCMP and chloroneb, were obtained by chromatographing a series of standard solutions in nitromethane over the range of 25–500 μg/ml. The column temperature was set at 175° C and 2 μl of the standard solution were injected using a Hamilton syringe. The retention time is about 7.8 min for DCMP and about 9.9 min for chloroneb. The peak height (in millimeters) was measured for each standard solution and the calibration curve was obtained by plotting the peak height (mm) *vs.* concentration (μg/ml).

The concentration of DCMP in the urine extracts and of chloroneb in the solutions of methylated urine extracts were obtained by injecting 2-μl samples of the extracts into the gas chromatograph and measuring the peak height of the appropriate peak.

Recovery studies at the 100 ppm and 300 ppm level showed that essentially 100% of added DCMP is recovered by this procedure. The results of these analyses are listed in Table I.

Analysis of Dog Tissues for Chloroneb and 2,5-Dichloro-4-methoxyphenol. Samples of tissues from the sacrificed dog were analyzed for chloroneb and DCMP by programed temperature gas chromatography according to the procedure described by Pease (1967). The results of these analyses are listed in Table II. The procedure contains a mild acid hydrolysis step; therefore, conjugates of the phenolic metabolite are hydrolyzed to the free phenol and are analyzed as the phenol. Recovery studies for both compounds in each of the tissues showed that greater than 90% of the added material is recovered in all cases except one. The recovery of the metabolite from fat was about 50%.

Table II. Gas Chromatographic Analyses of Dog Tissues for Chloroneb and the 2,5-Dichloro-4-methoxyphenol Metabolite

Sample	Residue (ppm) Metabolite	Chloroneb
Urine	248	8
Feces	39	4
Liver	0.15	0.26
Kidney	<0.10	0.15
Muscle	<0.04	0.04
Fat	<0.08	<0.08
Spleen	<0.04	<0.04
Brain	<0.04	<0.04

Analyses of Cow Tissue and Urine for Chloroneb and Possible Metabolites. Samples of cow tissues and urine were analyzed for chloroneb and its metabolite (DCMP) according to the procedure described by Pease (1967). Urine samples were collected and analyzed at four different times during the feeding period. Recovery studies showed that an average of 95% and 90% of the added chloroneb and metabolite, respectively, were recovered from urine and 96% and 83% from the tissues. No residues (<0.02 ppm) of either compound were detected in lean muscle, subcutaneous fat, liver, or kidney of either animal, except in the kidney of the 2 ppm cow, which showed an apparent residue of 0.05 ppm of the metabolite. No chloroneb (<1 ppm) was detected in the urine of the cow fed 50 ppm of chloroneb, and 6.3, 14, and 17 ppm of DCMP were found in the urine collected 1, 7, and 29 days after treatment, respectively.

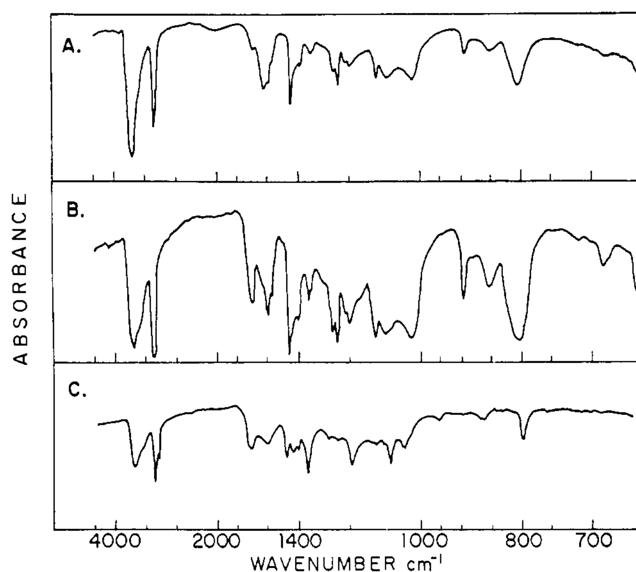

Figure 1. A. Ir spectrum of 2,5-dichloro-4-methoxyphenol. B. Ir spectrum of metabolite isolated from dog urine. C. Ir spectrum of metabolite isolated from rat urine

Table III. Recoveries of Dichlorohydroquinone (DCHQ) and Dichloroquinone (DCQ) from Fortified Cow Urine and Milk[a]

Sample	µg Added		µg DCHQ Found	% Recovery	
	DCHQ	DCQ		DCHQ	DCHQ + DCQ
Urine	2.5	. . .	1.51	60	
	2.5	2.5	3.61		72
	5.0	. . .	3.50	70	
	5.0	5.0	8.79		88
	10.0	. . .	8.52	85	
	10.0	10.0	16.6		
				72	81
Milk[b]	2.0	2.0	3.80		95
	5.0	5.0	10.6		106
	10.0	10.0	19.2		96
					99
Milk[c]	2.0	. . .	1.76		88
	2.0	2.0	3.60		90
	5.0	. . .	3.90		78
	5.0	5.0	6.61		66
	10.0	. . .	8.35		83
					81

[a] Sample size, 100 ml. [b] No hydrolysis. [c] After hydrolysis.

Analysis of Cow Urine for 2,5-Dichlorohydroquinone and 2,5-Dichloroquinone. The residual 6 *N* phosphoric acid solution from the analyses for chloroneb and the metabolite was checked for the possible presence of 2,5-dichlorohydroquinone (DCHQ) and 2,5-dichloroquinone (DCQ). To extract DCHQ, a portion of the acidic solution was adjusted to pH 7 with 50% sodium hydroxide, and the resulting solution was extracted three times with 500 ml of ethyl acetate. The volume of the combined ethyl acetate extracts was reduced to about 2 ml by evaporation under a hood. The resulting solution was chromatographed over a 12-mm column containing 5 g of 60–80 mesh Florisil using ethyl acetate as the eluting solvent. The volume of the solvent was reduced to 2 ml and a 100-µl aliquot of the resulting solution was analyzed for DCHQ using programed temperature microcoulometric gas chromatography with a 4-ft glass column of 10% DC-560 + 0.2% Epon 1001 on 80–100 mesh Chromosorb W. The chromatographic conditions and procedures are those described by Pease (1967). The retention time from the start of programming is *ca.* 7 min. As shown in Table III, an average recovery of 72% has been demonstrated for urine samples fortified with DCHQ at levels down to 0.02 ppm.

To check for the presence of DCQ, a second portion of the phosphoric acid solution was neutralized and extracted with ethyl acetate as described above. Water (20 ml) was added to the ethyl acetate and the volume of the solution was reduced to 20 ml by evaporation in a hood. The aqueous solution was saturated with sodium hydrosulfite to reduce DCQ to DCHQ and allowed to stand covered for 18 hr. The solution was filtered and extracted three times with 50-ml portions of ethyl acetate. The volume of the solution was reduced to 2 ml and a 100-µl aliquot of the solution was analyzed for DCHQ plus DCQ by microcoulometric gas chromatography. An average recovery of 81% has been demonstrated for urine samples fortified with DCHQ plus DCQ at levels down to 0.02 ppm. The results of these recovery studies are listed in Table III.

Using these procedures, no DCQ or DCHQ (<0.02 ppm) was detected in the urine of the cow fed at the dietary level of 50 ppm, even when the hydrolysis step was utilized to try to free possible conjugates.

Analysis of Milk for Chloroneb and Possible Metabolites. CHLORONEB AND 2,5-DICHLORO-4-METHOXYPHENOL. Samples (100 g) of whole milk from the cows fed 2 ppm and 50 ppm of chloroneb in their diets were analyzed for chloroneb and DCMP according to the procedure described by Pease (1967). The results of these analyses are listed in Table IV.

FREE 2,5-DICHLOROHYDROQUINONE (DCHQ) AND 2,5-DICHLOROQUINONE (DCQ). A 100-ml sample of milk was extracted three times with 100 ml of a 1:1 (v/v) mixture of diethyl ether and petroleum ether. This solvent system was found to reduce emulsion problems encountered when diethyl ether alone was used as the extraction solvent. The volume of the combined extracts was reduced to about 10 ml under a hood and 25 ml of water was added. The volume of the resulting mixture was reduced to about 25 ml under a hood and 5 g of sodium hydrosulfite was added to reduce

Table IV. Analysis of Milk for Chloroneb and 2,5-Dichloro-4-methoxyphenol Metabolite

Treated Ration	Residue (ppm) 50 ppm Treatment[a]	
	Chloroneb	Metabolite
1	<0.02	0.19
3	<0.02	0.41
5	<0.02	0.36
8	<0.02	0.32
10	<0.02	0.39
12	<0.02	0.37
15	<0.02	0.35
17	<0.02	0.37
19	<0.02	0.36
22	<0.02	0.35
24	<0.02	0.30
26	<0.02	0.31
29	<0.02	0.33
Days After Withdrawal of Treated Rations		
1	<0.02	0.06
2	<0.02	<0.02
3	<0.02	<0.02
4	<0.02	<0.02
7	<0.02	<0.02

[a] <0.02 ppm chloroneb or metabolite found in milk from 2 ppm treatment cow during study.

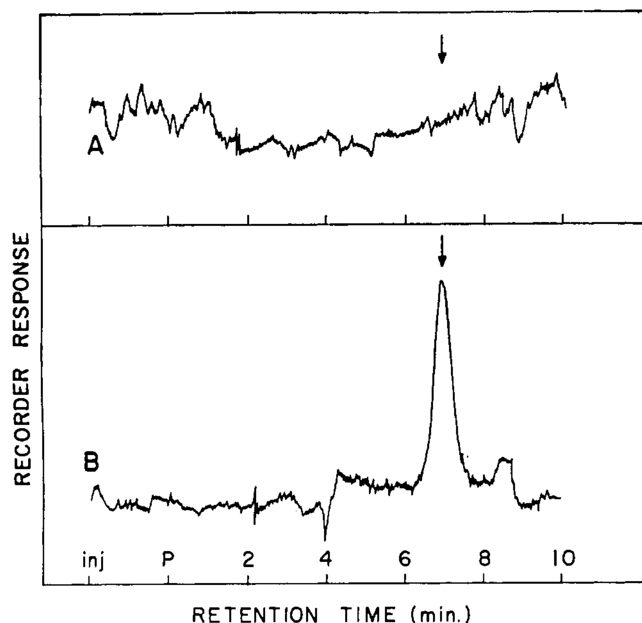

Figure 2. A. Gas chromatogram of control milk. B. Gas chromatogram of control milk fortified with 0.02 ppm of 2,5-dichlorohydroquinone plus 0.02 ppm of 2,5-dichloroquinone

DCQ to DCHQ. The solution was allowed to stand at room temperature, covered with aluminum foil, for 18 hr. The aqueous solution was extracted three times with 25-ml portions of ethyl acetate. The volume of the combined ethyl acetate extracts was reduced to about 2–3 ml under a hood and 25 ml of acetonitrile was added. The volume of the resulting solution was reduced to 20 ml and was extracted two times with equal volumes of petroleum ether. The petroleum ether was discarded. The volume of the acetonitrile was reduced to 1.5 ml under a hood (*do not* reduce volume below 1 ml) and was made to 2 ml with acetonitrile. The resulting solution was analyzed for DCHQ by the procedure previously described. Average recoveries of 99% were demonstrated with milk samples fortified at the 0.02 to 0.10 level (Table III). No DCQ or DCHQ (<0.02 ppm) was found in the milk of the cow which was fed chloroneb at the dietary level of 50 ppm.

TOTAL (FREE PLUS CONJUGATED) 2,5-DICHLOROHYDROQUINONE AND 2,5-DICHLOROQUINONE. A 100-ml sample of milk was acidified by addition of 4 ml of concentrated hydrochloric acid and the solution was heated under reflux for 30 min. The acidic solution was cooled to room temperature and extracted and prepared for gas chromatographic analysis as previously described. Average recoveries of 81% were demonstrated for milk samples fortified with DCHQ and DCQ at the 0.02 to 0.1 ppm level (Table III). No DCQ or DCHQ (<0.02 ppm) was found in the milk of the cow which was fed chloroneb at the dietary level of 50 ppm. The milk samples from the 26th and 29th day collections were analyzed both with no hydrolysis and after acid hydrolysis. Typical chromatograms are shown in Figures 2 and 3. Figure 2 shows a typical control milk plus milk fortified with DCQ and DCHQ at 0.02 ppm each. Figure 3 shows the absence of DCQ and/or DCHQ in milk and urine from treated animals.

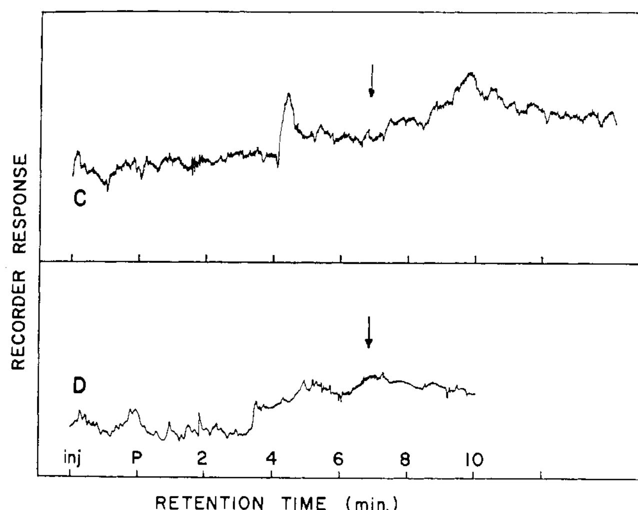

Figure 3. C. Gas chromatogram of milk from 50-ppm cow (26 day). D. Gas chromatogram of urine from 50-ppm cow (26 day)

RESULTS AND DISCUSSION

DCMP was found to be the only metabolite of chloroneb in the urine of dogs, rats, and cows, in contrast to metabolism in plants where DCQ and DCHQ were found as minor metabolites (<1% each) in cotton and bean plants (Rhodes *et al.*, 1971). Extended acid hydrolysis of the urine led to a large increase in the concentration of the metabolite in the extracts, indicating that the metabolite is present in the urine as a conjugate, presumably as a glucuronide or sulfonate. These results are consistent with the findings of Bray *et al.* (1955). Bray studied the fate of aryl ethers in the rabbit and found that 1,4-dimethoxybenzene was converted almost quantitatively to 4-methoxyphenol in rabbit urine and that the phenol was present as a mixture of the sulfonate (27%), the glucuronide (63%), and the free phenol (4%). Gutenmann and Lisk (1969) have also shown that 2,5-dichloro-4-methoxyphenol is a metabolite of chloroneb in cow urine and cow liver homogenate.

No chloroneb, DCMP, DCQ, or DCHQ (<0.02 ppm each) was found in the milk of a cow which was fed chloroneb at a dietary level of 2 ppm. Federal tolerances of 2 ppm for total residues of chloroneb plus DCMP, calculated as chloroneb, have been established for certain forage crops. At the exaggerated dietary level of 50 ppm, residues of DCMP were found in milk at the 0.3 to 0.4 ppm level. The metabolite concentration in the milk remained constant throughout the feeding period but disappeared completely within 2 days of withdrawal of the treated rations. Gutenmann and Lisk (1969) found no chloroneb or metabolite in the milk of a cow which was fed chloroneb at the dietary level of 5 ppm. Federal tolerances for chloroneb and its metabolite, DCMP, calculated as chloroneb, are set at 0.05 ppm in milk and 0.2 ppm in meat, fat, and meat byproducts of cattle, goats, hogs, horses, and sheep.

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