N'-(4-Chloro-*o*-tolyl)-N-methylformamidine (Demethylchlordimeform) Metabolism in the Rat

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N'-(4-Chloro-*o*-tolyl)-N-methylformamidine-^{14}C was rapidly metabolized and eliminated when administered orally to rats. Excretion balance data indicated that during the 72-hr experimental period the majority of the radioactive materials were eliminated in the feces (64%) but significant amounts also were eliminated in the urine (35%). Radioactive compounds identified in the urine and feces included demethylchlordimeform, 4'-chloro-*o*-formotoluidide, 4-chloro-*o*-toluidine, 5-chloroanthranilic acid, and N-formyl-5-chloroanthranilic acid. Several unidentified polar metabolites, presumably conjugates, also were present. A new formamidine metabolite, N'-(4-chloro-*o*-tolyl)formamidine, was tentatively identified from rat urine and feces. Levels of radioactivity in tissues examined were in the parts per billion range.

N'-(4-Chloro-*o*-tolyl)-N-methylformamidine or demethylchlordimeform is a toxic metabolite of the important formamidine acaricide–insecticide N'-(4-chloro-*o*-tolyl)-N,N-dimethylformamidine (chlordimeform) (Knowles, 1970). It has been identified during studies of chlordimeform-^{14}C metabolism in mammals (Knowles and Sen Gupta, 1970; Sen Gupta and Knowles, 1970; Ahmad and Knowles, 1971), insects (Knowles and Shrivastava, 1973; Crecelius and Knowles, 1975), acarines (Knowles and Schuntner, 1974), plants (Sen Gupta and Knowles, 1969; Ehrhardt and Knowles, 1970; Bull, 1973), and microorganisms (Johnson and Knowles, 1970). Since it is imperative to understand the fate of toxic pesticide metabolites, we investigated the metabolism of demethylchlordimeform-^{14}C in the white rat.

MATERIALS AND METHODS

Compounds. Radioactive demethylchlordimeform-^{14}C was synthesized as described by Arndt and Steinhausen (1968) but on a reduced scale according to the following procedure. To 0.02 ml of N-methylformamide dissolved in 1 ml of ethylene chloride was added 0.01 ml of phosphorus oxychloride. This solution was added to 4.91 mg of 4-chloro-*o*-toluidine-*tolyl*-^{14}C (sp act., 10 mCi/mmol), and the mixture was heated in a 65°C oil bath for 3 hr. The mixture was allowed to cool and was made alkaline with 2 N sodium hydroxide. The two phases were separated. The organic phase was washed twice with water and concentrated to about 0.5 ml with nitrogen. The concentrate was streaked on a thin-layer plate coated with a 500-μm layer of silica gel GF_{254}, and the chromatogram was developed with benzene–diethylamine (95:5) (Sen Gupta and Knowles, 1969). The zone corresponding to authentic demethylchlordimeform (provided by NOR-AM Agricultural Products, Inc., Woodstock, Ill.) was removed from the plate and extracted with acetone. After removal of the acetone with nitrogen 5.36 mg of demethylchlordimeform-*tolyl*-^{14}C (yellow liquid; sp act., 10 mCi/mmol) remained. The yield was 85% of theory.

4-Chloro-*o*-toluidine-^{14}C, a starting material for the radiosynthesis of demethylchlordimeform, was prepared as follows. To a mixture of 4'-chloro-*o*-formotoluidide-*tolyl*-^{14}C (3.53 mg; sp act., 20.7 mCi/mmol) and nonradioactive 4'-chloro-*o*-formotoluidide (3.57 mg) was added 0.5 ml of 2 N sodium hydroxide, and the suspension was heated in an oil bath at 65°C for 1.5 hr. The mixture was allowed to cool and was extracted with 0.5 ml of ethylene chloride. The ethylene chloride phase was washed twice with water and was concentrated to about 0.2 ml with nitrogen. The concentrate was subjected to TLC as described above, and the zone corresponding to authentic 4-chloro-*o*-toluidine was isolated and extracted from the silica gel with acetone. After removal of the acetone with nitrogen 4.91 mg of 4-chloro-*o*-toluidine-*tolyl*-^{14}C remained (82.4% of theory).

The following compounds were examined by cochromatography as potential demethylchlordimeform metabolites: 4'-chloro-*o*-formotoluidide (CIBA-GEIGY Corp., Greensboro, N.C.), N'-(4-chloro-*o*-tolyl)formamidine hydrochloride (NOR-AM Agricultural Products, Inc.), N'-methyl-N-4-chloro-*o*-tolyl-N-(N-4-chloro-*o*-tolylformamidoyl)formamidine (BTS-23376, Boots Pure Drug Co. Ltd., Nottingham, England), 4-chloro-*o*-toluidine (Aldrich Chemical Co., Inc., Milwaukee, Wis.), and 5-chloroanthranilic acid (K and K Laboratories, Inc., Plainview, N.Y.). 4'-Chloro-2'-methylacetanilide (mp 141–142°C) was prepared by acetylation of 4-chloro-*o*-toluidine, and N-formyl-5-chloroanthranilic acid (mp 289°C dec) was prepared by formylation of 5-chloroanthranilic acid (Knowles and Sen Gupta, 1970).

Treatment and Handling of Rats. Eight male white rats (Sprague-Dawley) weighing about 150 g each were treated by oral intubation with 1.5 μCi of demethylchlordimeform-^{14}C dissolved in corn oil and acetone (2:1). The demethylchlordimeform was purified to greater than 95% by TLC immediately prior to use. After treatment each rat was placed in a modified Roth metabolism cage for 72 hr; food and water were provided ad libitum.

Analysis of Urine. Urine samples were collected at posttreatment intervals of 3, 8, 12, 18, 24, 48, and 72 hr. The total radioactivity in each urine sample was determined by radioassay of duplicate 50-μl aliquots in a liquid scintillation spectrometer (Knowles and Sen Gupta, 1970).

The urine remaining after determination of total radioactivity was extracted twice with ethyl acetate. The ethyl acetate extracts were combined, dried over anhydrous sodium sulfate, and concentrated to about 0.1 ml under a stream of air. The concentrate was spotted, along with samples of potential metabolite standards, on TLC plates coated with silica gel GF_{254}. The chromatogram was developed in benzene–diethylamine (95:5) in most instances, and a radioautograph was prepared. The chromatographic behavior of demethylchlordimeform and potential metabolites in three solvent systems is given in Table I. The silica gel corresponding to darkened images

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Table I. Chromatographic Behavior of Demethylchlordimeform and Potential Metabolites

Compound	R_f value in solvent system[a] A	B	C
Demethylchlordimeform	0.25	0.00	0.47
N'-(4-Chloro-*o*-tolyl)formamidine	0.04	0.00	0.43
BTS-23376	0.75		
4'-Chloro-*o*-formotoluidide	0.08	0.44	0.83
4-Chloro-*o*-toluidine	0.43	0.59	0.78
5-Chloroanthranilic acid	0.00	0.47	0.72
N-Formyl-5-chloroanthranilic acid	0.00	0.37	0.43
2'-Methyl-4'-chloroacetanilide	0.15	0.35	0.76

[a] Adsorbent for TLC was silica gel GF_{254}. Solvent systems: A, benzene–diethylamine (95:5); B, benzene–dioxane–glacial acetic acid (90:25:4); C, ethyl acetate–1-propanol–water (64:24:12).

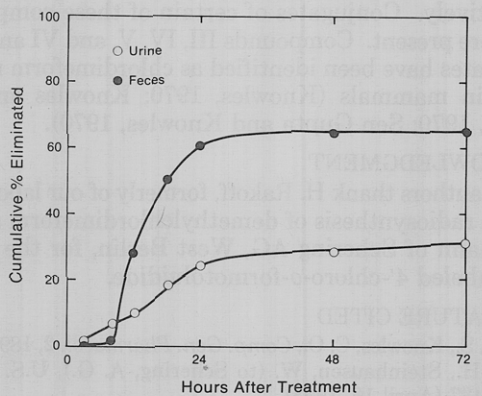

Figure 1. Cumulative percentage of the administered dose eliminated in the urine and feces of a rat treated orally with demethylchlordimeform-^{14}C.

on the film was scraped into scintillation vials, the counting solution was added, and the radioactivity was measured. The radioactivity remaining in the urine after ethyl acetate extraction was measured as described for total urinary radioactivity. From these determinations it was possible to determine the rate of elimination of the demethylchlordimeform-^{14}C equivalents in the urine and the nature and relative concentration of the radioactive metabolites.

Analysis of Feces. Fecal samples were collected from each rat at the same time intervals used for collection of urine. Each sample was thoroughly ground in a mortar, and the total radioactivity was determined by oxygen flask combustion of duplicate 100-mg samples (wet weight) (Knowles and Sen Gupta, 1970). Some fecal samples were extracted with acetone, and the acetone extracts were analyzed as described for ethyl acetate extracts of urine.

Analysis of Tissues. At 72-hr posttreatment each rat was killed and selected tissues were removed. Total radioactivity in the tissue was determined by oxygen flask combustion of duplicate 100-mg samples as described above for feces.

RESULTS AND DISCUSSION

Figure 1 shows that rats eliminated more ^{14}C equivalents in the feces than in the urine following a single oral dose of demethylchlordimeform. For four rats the mean ± standard error cumulative percentage elimination was 35.3 ± 6.4 for the urine and 64.2 ± 4.6 for the feces. The peak levels of radioactive materials occurred in the urine between 12 and 24 hr and in the feces between 18 and 48 hr.

From 16 to 26% of the total radioactive materials in the urine were extractable with ethyl acetate, and the nature and relative amounts of these compounds are given in Table II and Figure 2. Compounds present included demethylchlordimeform, *N'*-(4-chloro-*o*-tolyl)formamidine,

Table II. Nature and Relative Amount of Radioactive Compounds in the Urine of Rats Treated Orally with Demethylchlordimeform-^{14}C[a]

Compound[b]	Relative % at indicated posttreatment interval (hr) 0 to 3	3 to 12	12 to 18	18 to 24	48 to 72
Demethylchlordimeform	3.4	1.7	1.4	0.7	0.1
N'-(4-Chloro-*o*-tolyl)-formamidine	6.2	4.8	5.5	8.3	11.0
4'-Chloro-*o*-formotoluidide	14.4	3.8	3.6	3.3	5.0
4-Chloro-*o*-toluidine	<0.1	4.6	3.5	3.0	<0.1
Unknown 3	<0.1	0.5	0.5	<0.1	<0.1
Unknown 5	<0.1	1.4	1.2	<0.1	<0.1
Unknown 6	<0.1	1.1	1.2	1.4	<0.1
Unknown 8	<0.1	0.8	1.2	1.7	1.8
Origin	76.0	81.3	81.9	81.6	82.2

[a] Benzene–diethylamine (95:5) was the solvent system for TLC. [b] See Figure 2 for corresponding radioautograph.

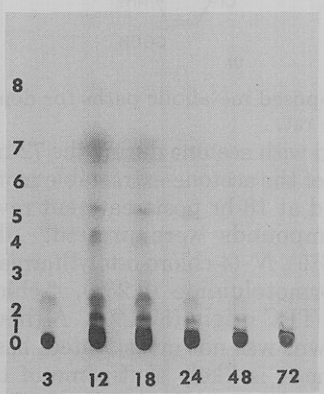

Figure 2. Radioautograph of urinary ethyl acetate extracts at 3, 12, 18, 24, 48, and 72 hr following treatment of rats with demethylchlordimeform-^{14}C: 0 = TLC origin; 1 = *N'*-(4-chloro-*o*-tolyl)formamidine; 2 = 4'-chloro-*o*-formotoluidide; 4 = demethylchlordimeform; 7 = 4-chloro-*o*-toluidine; 3, 5, 6, and 8 are unknowns. Solvent system was benzene–diethylamine (95:5).

4'-chloro-*o*-formotoluidide, 4-chloro-*o*-toluidine, and several unidentified compounds. The vast majority of ethyl acetate extractable radioactive material was recovered at the TLC origin with the benzene–diethylamine solvent system. However, when the ethyl acetate fraction was chromatographed with the benzene–dioxane–acetic acid system substantial amounts of 5-chloroanthranilic acid and *N*-formyl-5-chloroanthranilic acid were found; these two compounds were too polar to migrate in the benzene–diethylamine system. There was no evidence for the formation of BTS-23376 or of 4'-chloro-2'-methylacetanilide.

The aqueous fraction remaining after ethyl acetate extraction of the urine contained from 74 to 85% of the total radioactivity during the 72-hr period. This fraction could be resolved into at least 15 compounds with a solvent system consisting of ethyl acetate–1-propanol–water (64:24:12). Acid hydrolysis of this aqueous fraction prior to TLC in the above system reduced the number of compounds to 5; two of these compounds cochromatographed with 5-chloroanthranilic acid (R_f 0.72) and *N*-formyl-5-chloroanthranilic acid (R_f 0.43). Thus, many of these compounds probably were acid-labile conjugates, possibly glucuronides and ethereal sulfates.

About 25% of the total radioactive materials in the feces

Table III. Demethylchlordimeform-^{14}C Equivalents in Rat Tissues 72 hr after Treatment

Tissue	ppb, mean ± SE
Blood	134.6 ± 7.8
Brain	18.2 ± 3.2
Heart	64.0 ± 26.8
Intestine	59.6 ± 12.2
Kidney	80.8 ± 6.1
Liver	322.2 ± 33.8
Lung	85.4 ± 15.0
Spleen	35.4 ± 9.8
Stomach	73.8 ± 5.0
Testes	38.8 ± 20.4

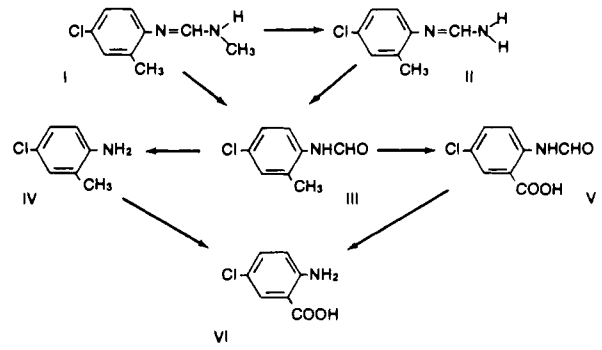

Figure 3. Proposed metabolic paths for demethylchlordimeform in the rat.

were extracted with acetone during the 72-hr experimental period. TLC of the acetone-extractable materials from the feces collected at 18-hr posttreatment revealed that the following compounds were present: demethylchlordimeform (6.8%), *N'*-(4-chloro-*o*-tolyl)formamidine (5.4%), 4'-chloro-*o*-formotoluidide (8.2%), 4-chloro-*o*-toluidine (22.5%), and TLC origin (57.1%). Although the nature of the unknowns was not investigated, based on analogy with the urine it is likely that some of the radioactive material at the TLC origin consisted of the anthranilic acids. No attempts were made to determine the nature of the radioactive material(s) remaining in the feces following acetone extraction.

Table III shows that demethylchlordimeform-^{14}C equivalents in rat tissues at 72-hr posttreatment were in the parts per billion range with only blood and liver containing greater than 100 ppb.

Figure 3 gives the proposed paths for demethylchlordimeform (I) metabolism in the rat. There apparently was N-demethylation of the parent compound to form *N'*-(4-chloro-*o*-tolyl)formamidine (II). However, the identity of compound II was based on cochromatography with the authentic standard in one TLC solvent system; therefore, its identification should be regarded as tentative. There also is some evidence that compound II was formed when chlordimeform-^{14}C was administered orally to mammals (Knowles and Sen Gupta, 1970; Sen Gupta and Knowles, 1970) and when it was incubated with a microsomal preparation from rat liver in the presence of NADPH and oxygen (Ahmad and Knowles, 1971). Demethylchlordimeform (I) and *N'*-(4-chloro-*o*-tolyl)formamidine were converted to 4'-chloro-*o*-formotoluidide (III) which was subsequently deformylated to yield 4-chloro-*o*-toluidine (IV) or oxidized at the phenyl methyl moiety to yield *N*-formyl-5-chloroanthranilic acid (V). Compounds IV and V were converted to 5-chloroanthranilic acid (VI) by oxidation of the phenyl methyl moiety and deformylation, respectively. Conjugates of certain of these compounds also were present. Compounds III, IV, V, and VI and their conjugates have been identified as chlordimeform metabolites in mammals (Knowles, 1970; Knowles and Sen Gupta, 1970; Sen Gupta and Knowles, 1970).

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