Identification of Metabolites of *N*-(1,1-Dimethylpropynyl)-3,5-dichlorobenzamide in Rat and Cow Urine and Rat Feces

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Results of a study of the metabolic products identified in rat and cow urine and rat feces from oral ingestion of the new herbicide *N*-(1,1-dimethylpropynyl)-3,5-dichlorobenzamide (Compound A) are presented. Half the radioactivity in rat feces and less than 1% in rat urine was unchanged Compound A. Cow urine contained no Compound A. In rat feces the metabolites were: 2-(3,5-dichlorophenyl)-4,4-dimethyl-5-methyleneoxazoline (I), *N*-(1,1-dimethylacetonyl)-3,5-dichlorobenzamide (II), 2-(3,5-dichlorophenyl)-4,4-dimethyl-5-hydroxymethyloxazoline (III), *N*-(1,1-dimethyl-3-hydroxyacetonyl)-3,5-dichlorobenzamide (IV), *N*-(1,1-dimethyl-3-hydroxypropyl)-3,5-dichlorobenzamide (V), *N*-(1,1-dimethyl-2,3-dihydroxypropyl)-3,5-dichlorobenzamide (VI), β-(3,5-dichlorobenzamido)-β-methylbutyric acid (VII), α-(3,5-dichlorobenzamido)isobutyric acid (VIII), plus one metabolite which has not been identified. In rat urine the metabolites were III, IV, VI, VII, VIII, plus β-(3,5-dichlorobenzamido)-α-hydroxy-β-methylbutyric acid (XII), and three unidentified metabolites different from that found in the feces. In cow urine the metabolites were VII, VIII, XII, and one of the unidentified metabolites found in rat urine. A tentative pathway by which these metabolites may have been formed is discussed.

This is the continuation of studies to determine the comparative metabolism of *N*-(1,1-dimethylpropynyl)-3,5-dichlorobenzamide (Kerb, Rohm and Haas RH-315, hereafter designated Compound A) in soil, plants, and animals.

The initial transformation of Compound A in soil has been reported (Yih *et al.*, 1970). Nine metabolites were identified in C^{14}-labeled Compound A treated soil and alfalfa (Yih and Swithenbank, 1971). The present study concerns the isolation, identification, and synthesis of metabolites of Compound A in rat and cow urine and rat feces. As a result, sufficient data exist to postulate tentative metabolic pathways for Compound A, and its comparative metabolism in soils, plants, and mammals.

EXPERIMENTAL

Synthesis of Compounds and Metabolites. The syntheses of *N*-(1,1-dimethylpropynyl)-3,5-dichlorobenzamide (Compound A), and the compounds found in soil and alfalfa are described in the preceding paper (Yih and Swithenbank, 1971). Following is the synthesis of the one additional compound found in urine

β-(3,5-DICHLOROBENZAMIDO)-α-HYDROXY-β-METHYLBUTYRIC ACID (COMPOUND XII)

This preparation is a modification of that described by Danilov and Martynov (1952).

3-METHYL-3-AMINO-2-HYDROXYBUTYRAMIDE. A two-phase mixture of methyl 2,3-epoxy-3-methylbutyrate (62.7 g, 0.483 mole) and 28% ammonium hydroxide (313.5 ml, 5.15 moles) was stirred at 25° C for 24 hr until homogeneous to give a solution of 3-methyl-2,3-epoxybutyramide. This solution was transferred to pressure bottles and heated on a steam bath for 8 hr. After cooling, the solution was concentrated under reduced pressure to a syrup which was dissolved in methanol. The solution was treated with anhydrous hydrogen chloride to give 3-methyl-3-amino-2-hydroxybutyramide hydrochloride (39.2 g, 23%): m.p. 233–240° C (dec).

Rohm and Haas Company, Research Laboratories, Spring House, Pa. 19477

Neutralization equivalent. Calcd. for $C_5H_{13}ClN_2O_2$: 168.6. Found: 160.

β-(3,5-DICHLOROBENZAMIDO)-α-(3,5-DICHLOROBENZOXY)-β-METHYLBUTYRAMIDE. A solution of 3,5-dichlorobenzoylchloride (97.2 g, 0.464 mole) in benzene (200 ml) was slowly added to a well stirred solution of 3-methyl-3-amino-2-hydroxybutyramide hydrochloride (39.2 g, 0.232 mole) and sodium hydroxide (27.9 g, 0.696 mole) in water (200 ml) at 20–25° C over 90 min. After stirring a further 90 min, the aqueous phase was neutral and β-(3,5-dichlorobenzamido)-α-(3,5-dichlorobenzoxy)-β-methylbutyramide (80 g, 72%) was filtered off and washed with water, benzene, and methanol, and air dried. A sample was recrystallized from ethanol: m.p. 196–198° C.

ANAL. Calcd. for $C_{19}H_{16}Cl_4N_2O_4$: C, 47.43; H, 3.37; N, 5.86. Found: C, 48.10; H, 3.68; N, 6.03.

β-(3,5-DICHLOROBENZAMIDO)-α-HYDROXY-β-METHYLBUTYRAMIDE. Sodium hydroxide (2 g, 0.05 mole) was dissolved in ethanol (200 ml) and the solution heated under reflux with β-(3,5-dichlorobenzamido)-α-(3,5-dichlorobenzoxy)-β-methylbutyramide (23.9 g, 0.05 mole). After 1 hr the solid had dissolved and the solution was only slightly alkaline. The solvent was removed under reduced pressure and the residue slurried with water, filtered, and the solid washed with water and benzene and air dried to give β-(3,5-dichlorobenzamido)-α-hydroxy-β-methylbutyramide (12 g, 79%) which was recrystallized from ethanol: m.p. 225–227° C.

ANAL. Calcd. for $C_{12}H_{14}Cl_2N_2O_3$: C, 47.22; H, 4.63; N, 9.18. Found: C, 47.42; H, 4.57; N, 9.15.

β-(3,5-DICHLOROBENZAMIDO)-α-HYDROXY-β-METHYLBUTYRIC ACID. Finely powdered sodium nitrite (45.0 g, 0.655 mole) was added to a solution of β-(3,5-dichlorobenzamido)-α-hydroxy-β-methylbutyramide (20 g, 0.065 mole) and concentrated hydrochloric acid (54.6 ml, 0.65 mole) in dioxane (375 ml) and the solution stirred vigorously at 20–25° C for 24 hr until nitrogen evolution ceased. The mixture was diluted with water (1200 ml) and extracted with ether. The extract was dried and the solvent removed to give β-(3,5-dichlorobenzamido)-α-hydroxy-β-methylbutyric acid (17.5 g, 85%) which was recrystallized from 20% acetone in benzene: m.p. 161–163° C.

ANAL. Calcd. for $C_{12}H_{13}Cl_2NO_4$: C, 47.07; H, 4.28; N, 4.57. Found: C, 47.21; H, 4.48; N, 4.50.

METHYL β-(3,5-DICHLOROBENZAMIDO)-α-HYDROXY-β-METHYLBUTYRATE. A solution of β-(3,5-dichlorobenzamido)-α-hydroxy-β-methylbutyric acid (4.0 g, 0.013 mole) and concentrated hydrochloric acid (eight drops) in methanol (100 ml) was warmed at 50–60° C for $2^1/_2$ hr. The solvent was removed under reduced pressure, the residue taken up in ether, and the solution washed with sodium bicarbonate solution and water, and dried. The ether was evaporated and the residue recrystallized from benzene/hexane to give methyl β-(3,5-dichlorobenzamido)-α-hydroxy-β-methylbutyrate (2 g, 48%): m.p. 92–92.5° C.

ANAL. Calcd. for $C_{13}H_{15}Cl_2NO_4$: C, 48.76; H, 4.72; N, 4.38. Found: C, 48.99; H, 4.91; N, 4.34.

Compound A Feeding. Rat urine and feces used for identification were obtained from a material balance study. The detailed experimental procedures of feeding C^{14}-carbonyl labeled Compound A to rats will be described elsewhere (Wargo and Gordon, 1970). One cow was fed C^{14}-carbonyl labeled Compound A (specific activity of 0.11 mc per g) at 0.2, 1.0, and 5.0 ppm daily for a 2 week period at each concentration at Affiliated Medical Enterprises, Inc., Princeton, N.J. Cow urine used for metabolite identification was collected from this cow during the second week of the third period (final test period). The C^{14} radioactive residue from the urine on this particular day was 14.4 ppm.

Extraction of Compound A and Metabolites from Rat and Cow Urine and Rat Feces. Rat urine samples (5 to 10 ml) or cow urine samples (50 to 100 ml) were freeze dried, 5 to 10 ml of methanol was added, and the insoluble materials were removed by centrifugation. Rat feces (2 to 5 g) were extracted in a Soxhlet with methanol. The methanol extracts were evaporated to dryness and made to 2 ml with methanol.

Separation and Identification of Compound A and Its Metabolites. Techniques used for separation and identification have been described (Yih and Swithenbank, 1971).

RESULTS AND DISCUSSION

Identification of Rat Feces Metabolites. Preliminary studies showed that the same metabolites were present in eight daily feces samples; consequently, daily feces samples were pooled for subsequent metabolism studies. The C^{14} radioactivity in the feces (655 ppm) was readily extractable by Soxhlet extraction with methanol (Table I) about 88% of the total radioactivity being present in the extract. This extract was used to determine the number and amount of metabolites and for their identification. The minor amount of radioactivity in the feces residue after extraction was not studied.

A typical set of chromatograms obtained from the analysis of rat feces fed with C^{14} Compound A is shown in Figure 1. In the first solvent system (Figure 1A) it may be seen that most of the radioactivity remained at the origin; five components moved and a major one has the same R_f value as standard Compound A. In the second solvent system (Figure 1B), Compound A and seven metabolites were separated. In the third solvent system (Figure 1C) there were three metabolites that separated, and the other metabolites moved to the solvent front. (There was still a trace amount of radioactivity that remained at the origin.) Thus, it is possible to separate nine metabolites from the feces of Compound A fed rats using tlc. The amount of the individual metabolites found in the methanol soluble fraction of the feces (ref. Figure 1A,B,C) is shown in Table II, where it may be seen that Compound A constitutes 54% of the recovered activity, the major metabolism products being metabolite III (15%) and metabolite IV (4.7%).

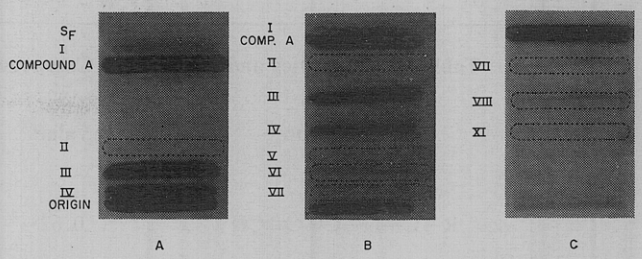


Figure 1. The presence of metabolites in feces of C^{14}-labeled Compound A fed rats. Radioautographs of thin-layer chromatograms (Brinkmann tlc-plates, Silica Gel F-254, thickness 0.5 mm). A. Developed with acetone:benzene = 5:95. B. Developed with acetone:benzene = 25:75. C. Developed with isopropyl alcohol: ethyl acetate:water = 25:65:10

Table I. Percent C^{14} Radioactivity in Various Fractions of Rat Feces

Fraction	% C^{14} Radioactivity
Methanol	88.2
Feces residue (after extraction)	9.4
Total recovered	97.6

The metabolites recovered from feces were cochromatographed with synthetic compounds by tlc, and this led to the assignment of structure for the feces metabolites as shown in Table II. In addition to chromatographic evidence, metabolite III was identified by infrared spectra, gas-liquid chromatography, mass spectra, and reverse radioisotope dilution.

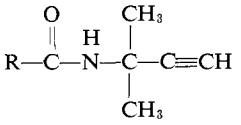
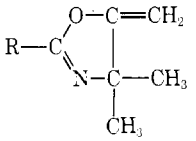
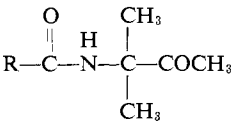
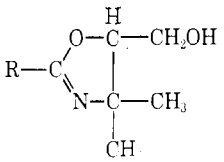
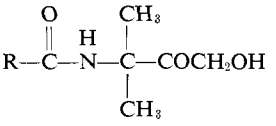
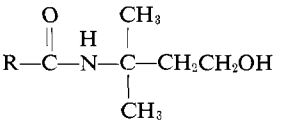
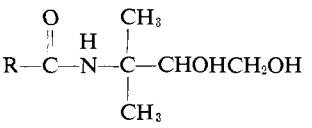
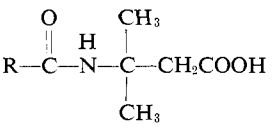
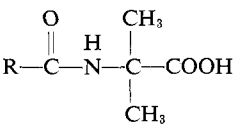
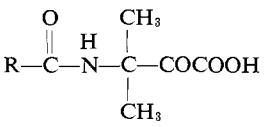
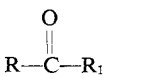
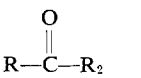
Metabolite XI has not been identified. However, methyl 3,5-dichlorobenzoate was obtained after vigorous treatment with methanol/HCl, showing that metabolite XI is a derivative of 3,5-dichlorobenzoic acid.

As indicated in Table II, metabolites VIII and IX have the same R_f value using isopropyl alcohol/ethyl acetate/water as elution solvent. The presence of metabolite VIII and absence of metabolite IX in rat feces was determined as described in the previous paper (Yih and Swithenbank, 1971) by methylation prior to chromatography.

Identification of Rat Urine Metabolites. Before using a composite urine sample for metabolism studies, eight daily urine samples were chromatographed on three thin-layer chromatograms and separated in three different solvent systems to determine the possibility of variable metabolism from day to day. It was found that the amount of the metabolites varied from day to day, but there was no daily difference in the number of metabolites. Consequently, daily urine samples were pooled for subsequent metabolism studies.

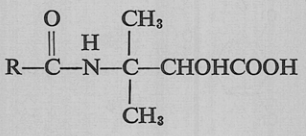
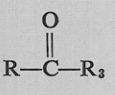
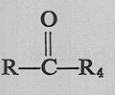
The composite urine sample was freeze dried and the radioactivity was quantitatively recovered into methanol. This methanol extract was chromatographed with three solvent systems, as shown in Figure 2, where it may be seen that a total of nine metabolites were present in the urine from C^{14}-labeled Compound A fed rats. The amount of the individual metabolites found in the urine is shown in Table II, where it may be seen that most of the activity is found in metabolites VIII, XI, XII, and XIV and that they account for approximately 82% of the total C^{14} recovered. Compound A is present in the urine, but constitutes less than 1% of the total radioactivity recovered.

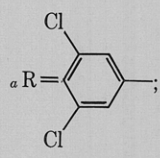
Table II. Identities and Amounts of Compound A Metabolites in Rat and Cow Urine and Rat Feces

Metabolite Number	Structure[a]	R_f Values[b]	Rat Feces		Rat Urine		Cow Urine	
			%	μg/g	%	mg/l.	%	mg/l.
Compound A		0.62[a]	53.7	351.74	0.6	1.36	...	...
I		0.82[a]	0.9	5.90	...	...	...	...
II		0.42[a]	1.5	9.83	...	...	...	...
III		0.23[a]	15.0	98.25	5.9	13.38	...	...
IV		0.61[b]	4.7	30.79	3.6	8.17	...	...
V		0.49[b]	1.2	7.86	...	...	...	...
VI		0.46[b]	2.2	14.41	3.1	7.03	...	...
VII		0.71[c]	1.9	12.45	2.4	5.44	3.3	0.47
VIII		0.49[c]	1.8	11.79	22.4	50.82	4.4	0.63
IX		0.49[c]	...	...	...	...	...	...
X		0.63[c]	...	...	...	...	...	...
XI		0.32[c]	1.1	7.21	24.1	54.67	...	...

(continued)

Table II (*Continued*)

Metabolite Number	Structure[a]	R_f Values[b]	Rat Feces %	Rat Feces μg/g	Rat Urine %	Rat Urine mg/l.	Cow Urine %	Cow Urine mg/l.
XII		0.26[c]	...	...	19.2	43.56	71.4	10.28
XIII		0.17[c]	...	...	2.0	4.54	19.2	2.76
XIV		0.10[c]	...	...	16.7	37.89	...	...

[a]  ; R_1, R_2, R_3, and R_4 are unknowns. [b] Determined on Eastman Chromatogram Sheet 6060 (Silica Gel) developed with the solvent systems: (a) acetone:benzene (5:95), (b) acetone:benzene (25:75), and (c) isopropyl alcohol:ethyl acetate:water (25:65:10).

The metabolites recovered from urine were cochromatographed with synthetic compounds by tlc, and this led to the assignment of structure for the urine metabolites, as shown in Table II. As discussed in the previous section, metabolite IX was not present in Compound A fed rat urine. Metabolites XI, XIII, and XIV have not been identified. However, it has been shown that they are derivatives of 3,5-dichlorobenzoic acid by vigorous treatment with methanol/HCl, which in all cases yielded methyl 3,5-dichlorobenzoate.

Identification of Cow Urine Metabolites. The C^{14} radioactive residue in the cow urine used for metabolism studies was 14.4 ppm. Methanol extract from a freeze dried cow urine sample was chromatographed with three solvent systems, the same as described in the rat urine section. There were only four metabolites, but no Compound A present in the cow urine. These four metabolites gave the same R_f values as the metabolites VII, VIII, XII, and XIII in the rat urine (Figure 2). The identities and the amounts of these four cow urine metabolites are presented in Table II.

Metabolic Pathways. A growing volume of literature of the fate of organic chemicals in soil and plants (Kearney and Kaufman, 1969) and mammals (Williams, 1959) shows that it is necessary to invoke only a few simple reactions, notably oxidation, reduction, hydroxylation, and derivative formation (*e.g.*, sugar or amino-acid "conjugate" formation) to explain most of the observed transformations. Applying these biochemical reactions and those standard chemical changes which we have observed in the laboratory to the observed metabolites of Compound A, it is possible to construct a fairly convincing scheme of metabolite interconversions (Table III and Figure 3). Using this chart it is possible to distinguish a number of possible metabolic pathways. Thus, it has already been shown that metabolites I and II are formed consecutively from Compound A by what are almost certainly simple chemical transformations (Yih *et al.*, 1970). The metabolism of phenylacetylene is reported to proceed *via* terminal hydration of the triple bond (El Masri *et al.*, 1958). Similar hydration of Compound A will give the enol shown in parentheses, which can cyclize to give metabolite III (which can also be formed by direct hydration of metabolite I), and be reduced or oxidized to give metabolites V or VII, respectively. Hydrolysis of metabolite III yields (*in vitro*) metabolite VI, which may be oxidized to metabolite IV (which may be alternatively derived from metabolite II by terminal hydroxylation). Hydroxylation must also be invoked for the transformation of metabolite VII to XII. Metabolites IV, VI, and IX are all interchangeable by oxidation and reduction sequences. We have found that chemically metabolite IX readily decomposes to give metabolite VIII.

Table III. Comparison of the Metabolism of Compound A in Soil, Plants, and Mammals

Metabolite No.[a]	Metabolites[b] Soil	Alfalfa	Rat Feces	Rat Urine	Cow Urine
Compound A	+	+	+	+	−
I	+	+	+	−	−
II	+	+	+	−	−
III	+	+	+	+	−
IV	+	+	+	+	−
V	+	+	+	−	−
VI	+	+	+	+	−
VII	+	+	+	+	+
VIII	+	+	+	+	+
IX	+	+	−	−	−
X	−	+	−	−	−
XI	−	−	+	+	−
XII	−	−	−	+	+
XIII	−	−	−	+	+
XIV	−	−	−	+	−

[a] Structures of the metabolites are given in Table II. [b] Metabolites, (+) = present; (−) = absent.

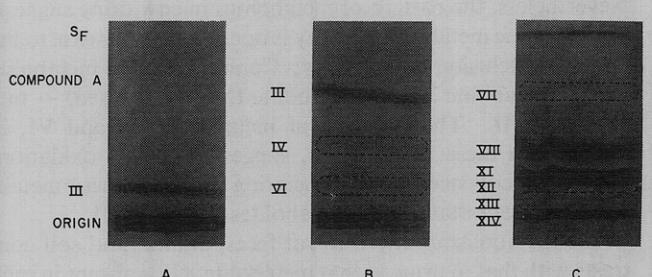


Figure 2. The presence of metabolites in urine of C^{14}-labeled Compound A fed rats. Radioautographs of thin-layer chromatograms (Brinkmann tlc-plates, Silica Gel 254. Thickness 0.5 mm). A. Developed with acetone:benzene = 5:95. B. Developed with acetone:benzene = 25:75. C. Developed with isopropyl alcohol: ethyl acetate:water = 25:65:10

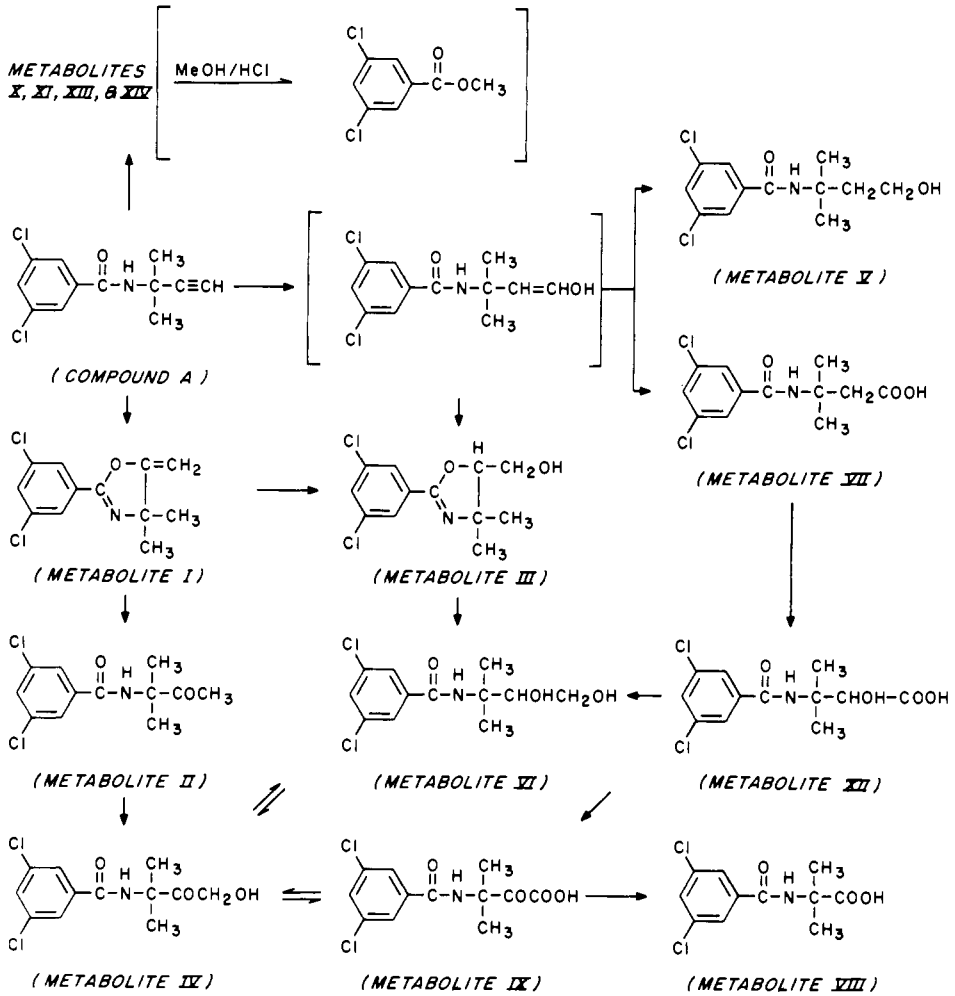


Figure 3. Proposed metabolic pathways of Compound A in soil, alfalfa, rat and cow urine and rat feces

It is most interesting to compare the metabolism of Compound A in soil, plants, and mammals, even though the presence of a particular metabolite may be missed through being present below the detectable concentration. [In particular, the chemically unstable keto-acid (metabolite IX) is required by the proposed scheme to be the sole precursor to metabolite VIII.] Nevertheless, the pattern of metabolites in cow urine suggests that only one metabolic pathway is operating in this case to the practical exclusion of all others; Compound A → metabolite VII → metabolite XII → metabolite IX (not detected) → metabolite VIII. The presence of metabolites IV and VI, in addition to these in rat urine, suggests that the oxidation/reduction sequence may be operating and that these metabolites are not obtained *via* metabolites I, II, and III.

The metabolism pattern in rat feces, alfalfa, and soil contrasts with that in urine in that metabolite XII is absent in each of these cases, suggesting (perhaps) that the enzyme responsible for the hydroxylation of metabolite VII is not available in these cases. The absence of metabolite VI from rat feces has been ascribed to insufficient quantities being present for its detection, since its formation is required by the metabolism sequence as proposed.

In summary, the pattern of Compound A metabolites in soil, alfalfa, and rat feces appears to be essentially similar, following at least two major pathways. Over the period of the investigation the final products in the metabolic chain are metabolites VII and VIII. In striking contrast, a third pathway is uniquely followed in rat and cow urine in which metabolite VII is ultimately converted to metabolite VIII.

It is interesting that all of the identified metabolites are the result of manipulations on the two terminal carbon atoms. We have not been able to identify 3,5-dichlorobenzoic acid as a metabolite, although it is possible that one or more of the unidentified "conjugates" have, in fact, lost the alkyl side chain. We have also been unable to find any evidence of ring hydroxylation (with or without loss of chlorine, or rearrangement) by the absence of compounds with R_f's similar to those of methyl 2- or 4-hydroxy-3,5-dichlorobenzoate in the hydrolyzate of the unknowns, although this evidence is clearly insufficient for a firm conclusion.

ACKNOWLEDGMENT

The authors are indebted to D. Harold McRae and Patrick J. McNulty for their contributions to this work, and to Thomas L. Cummings, Thomas A. McLaughlin, and Stacy T. Satterthwaite for skilled technical assistance.

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Received for review August 20, 1970. Accepted November 16, 1970.