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Fate of Microbicidal 3-Isothiazolone Compounds in the Environment: Products of Degradation

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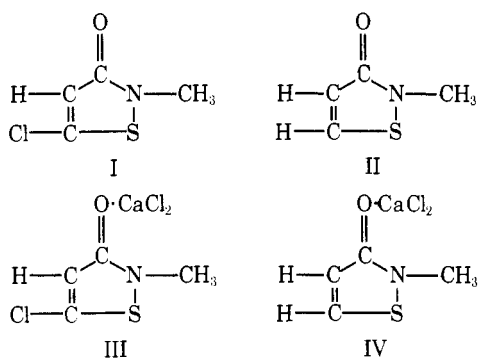
The major degradative pathway in the environment has been defined for two similar microbicidal 3-isothiazolones, 5-chloro-2-methyl-4-isothiazolin-3-one calcium chloride and 2-methyl-4-isothiazolin-3-one calcium chloride. In eight systems, covering chemical, biochemical, and photochemical aspects of environmental degradation, the disappearance of the two compounds was rapid with both compounds generating, qualitatively and quantitatively, a similar distribution of degradation products. The principal degradative pathway involved the dissociation from $CaCl_2$, ring opening, and loss of Cl and S, and led to *N*-methylmalonamic acid. The degradation then proceeded through malonamic, malonic, acetic, and formic acids to CO_2. Other products along the degradative pathway were tentatively identified as 5-chloro-2-methyl-4-isothiazolin 1-oxide, *N*-methylglyoxylamide, ethylene glycol, and urea.

Certain 3-isothiazolones demonstrate strong microbicidal properties. An earlier work (Krzeminski et al., 1975, hereafter referred to as paper I) discussed the modes and rates of degradation in the environment of two such compounds, 5-chloro-2-methyl-4-isothiazolin-3-one and 2-methyl-4-isothiazolin-3-one, designated I and II, and their corresponding, and more stable, $CaCl_2$ adducts designated III and IV.



Since these compounds show usefulness as microbicides, in some applications (e.g., as cooling tower microbicides) they might be discharged directly to natural waters. Therefore, the objectives of the present work were to characterize, isolate, and identify the major transformation products of III and IV, in several environmental systems and, by so doing, define their degradative pathway in the aquatic environment.

EXPERIMENTAL SECTION

The studies described below were performed on degradation products generated from systems detailed in paper I. Those were: (1) an activated sludge system, (2) a river water system, (3) an acetone–water (30:70, v/v) system, (4) a basic hydrolysis system, (5) a photolysis system, (6) rat urine, (7) extract of rat feces, and (8) extract of aquatic plants. All eight systems were generated from 4,5-^{14}C-labeled compound III and several were also generated from 4,5-^{14}C-labeled compound IV.

Two techniques were used throughout this study to separate the degradation products of III or IV from each other and from natural products. They were thin-layer chromatography (TLC) and high-voltage electrophoresis (HVE). Qualitative identification was carried out by the use of cochromatography of known standards and unknown degradation products using TLC and HVE, by solids-probe mass spectrometry and by the reverse isotope dilution technique. Quantitation of the components of degradation was by liquid scintillation counting of carbon-14 zones on TLC plates and HVE paper. Various extractions and cleanup techniques were used, depending on which degradation system was being investigated.

(1) Extraction and Cleanup. Concentrated samples of ^{14}C-labeled III and IV effluents from a laboratory semicontinuous activated sludge unit, rat excrements of urine and feces, and aquatic plants were extracted and cleaned up as follows. Samples of treated effluent were filtered using Gelman Metricel TM alpha-6 0.45-μ membrane filters to remove organic material. Effluent was then concentrated in a beaker on a steam bath under nitrogen purge, followed by concentration in an N-Evap (Model 10, Organomation Assoc.). Temperature of the effluent was maintained under 40° to prevent loss of ^{14}C activity. After evaporation, filtration, as described above, was used to remove inorganic salts of calcium and magnesium which had precipitated out during the concentration step. Urine samples were first freeze-dried and then the dried solids picked up in methanol. Feces samples were Soxhlet extracted with methanol. The recovery of ^{14}C was about 90% for the urine and 70–80% for the fecal samples. Soxhlet extraction with methanol was also used to bring degradation products of III in aquatic plants into solution. Extraction efficiencies were ca. 70%.

(2) Thin-Layer Chromatography. All thin-layer chromatography was performed on 250-μ thick silica gel, glass-backed plates of commercial origin (Brinkman). Plates

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were either 5 × 20 or 20 × 20 cm in size. Extracts were spotted 1 or 2 cm from the bottom edge of the plate in a tight spot or elongated band about the origin. Development was done in ascending fashion in a covered glass tank and was carried out to approximately 10 cm from the origin of spots. Two tertiary solvent systems were used for development. The less polar was ethyl acetate–2-propanol–water (65:25:10), designated solvent system A; the more polar was ethyl acetate–methanol–water (60:30:10), designated solvent system B. After development, the solvent was removed from the plate by air drying. When co-chromatography was performed, a spot of the standard was placed on the same plate as the unknown sample and the plate developed.

(3) High-Voltage Electrophoresis. For all the high-voltage electrophoresis work done in this study a Camag HVE system (No. 60-560) was used. (When a volatile buffer was used, a low-voltage Gelman electrophoresis unit was used.) Electrophoresis separation was performed on 200- and 400-mm electrophoretic paper (Camag 68-011 paper) or divisions thereof. For each run a narrow tight band, about 1 in. wide, of sample was applied 3 in. from one end of the paper which previously had been soaked in buffer and damp-dried. (Preliminary studies indicated that the charged degradation products moved in only one direction—toward the anode—and spotting in the center of the paper was not necessary.) The Camag recommended procedure was used with two exceptions. First, electrophoretic paper was used to replace the plastic-coated current transfer flap (to avoid phthalates in the mass spectrometer). Second, excess wetting of the HVE paper by the buffer, normally prevented by the flap, was curtailed by using a higher than recommended pressure in the inflatable bag of the HVE apparatus, that is, the air-bag bulb distention was 2 in. rather than the recommended 1 in.

Spotting of 5 μg/in. of ^{14}C-labeled III or its metabolites enabled optimum HVE separation. For cleaner systems larger quantities (up to several milligrams) could be spotted. Several buffers were used throughout this work. One was an ammonium formate–formic acid buffer (0.02 *M*, pH 4) which provided the best separation of decomposition products. A second was a KH_2PO_4–phosphoric acid buffer (0.02–0.04 *M*, pH 4.6). The latter buffer did not provide as clear-cut a separation as the former, but it did provide a cleaner system for use in the preparation of samples for mass spectrometry.

Optimum resolution for the KH_2PO_4 buffer was obtained with HVE cell at a constant, controlled temperature of 14°. Other operational conditions were 4000 V, 104 V/cm, ca. 65 mA/4 in. wide strip; run time, 20–25 min; and buffer ionic strength, 0.02 to 0.04. Optimum resolution for the ammonium formate buffer was obtained at 14°, 3000 V, 78 V/cm. Two other buffer systems used were the Gelman HR buffer, a Tris–barbituate buffer (pH 8.9, 3000 V, μ = 0.05, 78 V/cm, 30 min), and NH_4HCO_3 buffer (pH 7.7, 400 V, 31 V/cm, 25 min, μ = 0.01).

(4) Radioassay. TLC plates, after identification of carbon-14 areas by radioautography (Kodak No-Screen X-ray film No. NS54T), were divided into the appropriate zones with a spatula. The silica gel from each zone was scraped off the glass and put into a polyethylene counting vial containing 15 ml of Aquasol (New England Nuclear) scintillation solution and 2 ml of water for deactivation. HVR papers were cut up into 0.5 in. wide segments starting at the origin. Each 0.5-in. strip was put into a polyethylene counting vial containing 1 ml of water and 15 ml of Aquasol.

Carbon-14 was determined in all cases by the use of a Packard Tri-Carb liquid scintillation spectrometer (Model No. 3314 or 3320). The instrument settings used in this study were: discriminator, 50 to 1000, and amplifier gain, 25%. Counting efficiency was determined by the use of an interval standard and averaged 80–85%. Each sample was counted for 5 or 30 min in increments of 1 or 5 min duration.

(5) Gas–Liquid Chromatography. In several of the systems studied an analysis of III was made by an electron capture/gas–liquid chromatographic method described previously in paper I.

(6) Mass Spectrometry. All mass spectra were obtained on either a Hitachi high-resolution mass spectrometer (Model RMUM 7, Perkin-Elmer Corp.) or a Finnegan quadruple mass spectrometer (Model 1015 S/L system 150 GC–MS computer system, Finnigan Corp.). Samples were normally entered into the spectrometer via the solids' probe, but in one case the GLC inlet was used for sample introduction.

Samples which showed a lack of volatility in the mass spectrometer were treated as follows. After HVE, the sample was extracted from the paper with water (90% efficiency) and concentrated to a small volume on a vacuum rotary evaporator in a heated water bath. The resulting concentrated sample of 25–100 μl was then passed through a 76 mm × 2 mm i.d. Chromatronix glass column containing Bio-Rex 70 (Bio-Rad Labs) 200–400 mesh cation exchange resin in the hydrogen form. This converted the metabolites into their more volatile acid form and removed some of the buffer cations.

(7) Reverse Isotope Dilution. The reverse isotope dilution method used is described in a publication by Raaen (Raaen et al., 1968). Approximately 1 g of unlabeled carrier was equilibrated with the carbon-14 solution and consecutive recrystallizations performed. The criterion of constant specific activity was attained after four–five recrystallizations.

RESULTS AND DISCUSSION

Eight systems of degradation products of III were studied and characterized because of their environmental importance or because they were clean and/or rich sources of degradation products. Four systems of IV were also investigated.

At first isolation and direct identification of degradation products of III were attempted using the effluent from the semicontinuous activated sludge test. This system offered a broad spectrum of degradation products in fair abundance (ca. 5 ppm before concentration) and was important from an actual use standpoint. Although direct identification of degradation products in this system was not attained, it did emerge as the standard to which all degradation systems of III were compared. Results of radioassay of the HVE electrophoregram of it are given in Figure 1. The coding of peaks given in the figure is used for all other systems reported herein.

Degradation Products along the Major Degradative Pathway. *(1) Isolation and Direct Identification of Degradation Products of III.* After a fish residue study reported in paper I was completed, it was determined that a stock solution of ^{14}C-labeled III in acetone–water (30:70) which was used to make up less concentrated solutions of III for that study had degraded somewhat during the course of the test. This was noted when a precipitate was found in the prime stock solution several months after the fish residue study was terminated. The first concern was for the effect on the conclusion of the fish exposure study. Duplication of the concentrations of III and IV actually exposed to fish indicated that the maximum amount of acetone present was 1.1%. Total carbon-14 measurements, together with GLC determinations of parent compound, indicated that less than 10% of III degraded after 2 weeks of the fish test. Since the steady state of ^{14}C residues occurred before 2 weeks, the original conclusions drawn from that study remain valid. (Thus, later in the first study, fish were exposed to not only III but also some of the degradation products of III.)

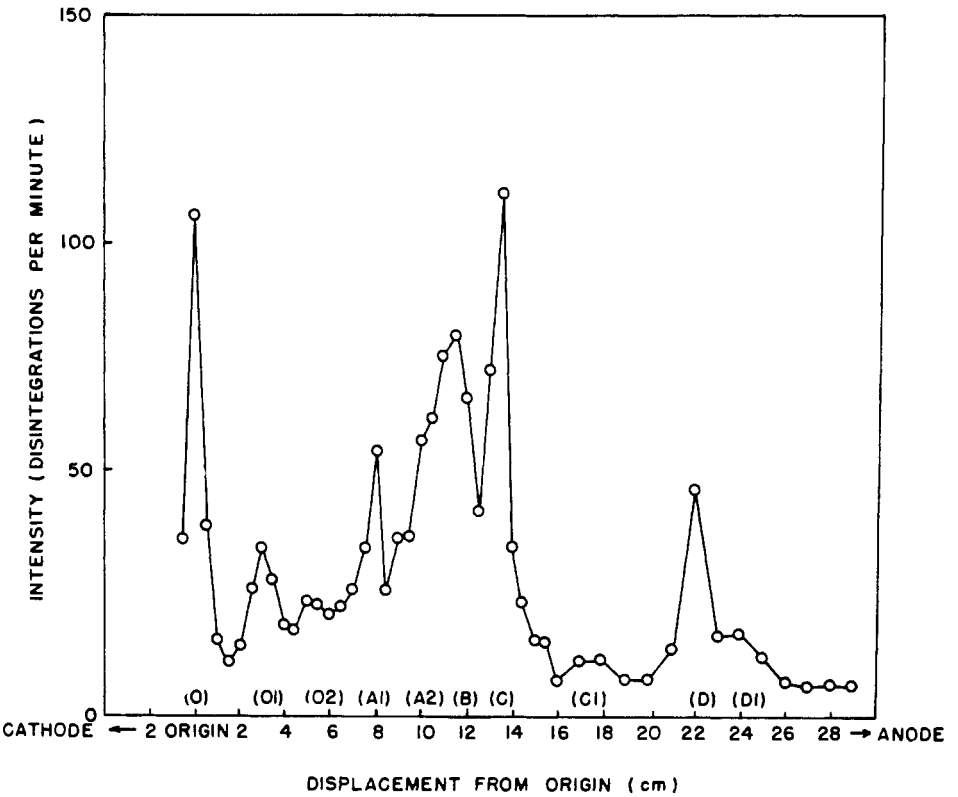


Figure 1. Metabolites of ^{14}C-labeled III in sludge effluent as determined by high voltage electrophoresis (HVE) in Gelman HR buffer and by radioassay.

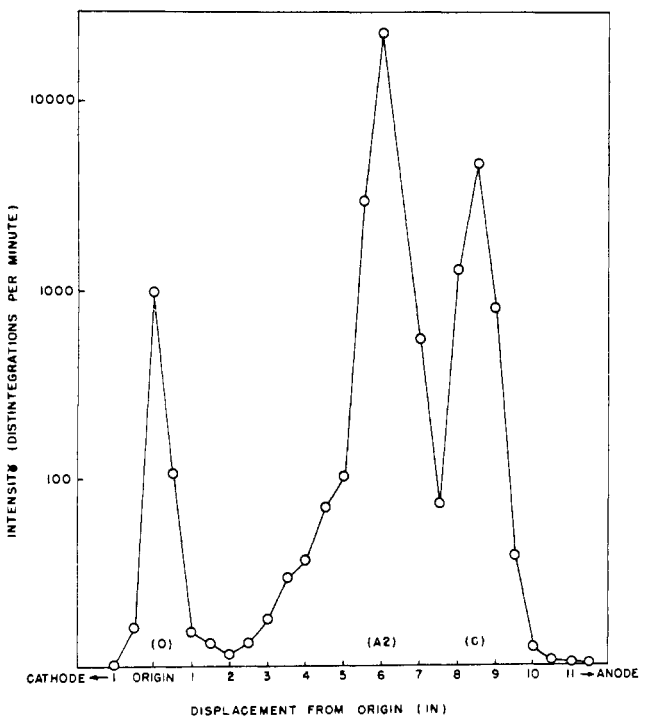


Figure 2. Degradation products of ^{14}C-labeled III in an acetone–water (30:70) system as determined by HVE in KH_2PO_4 buffer and by radioassay.

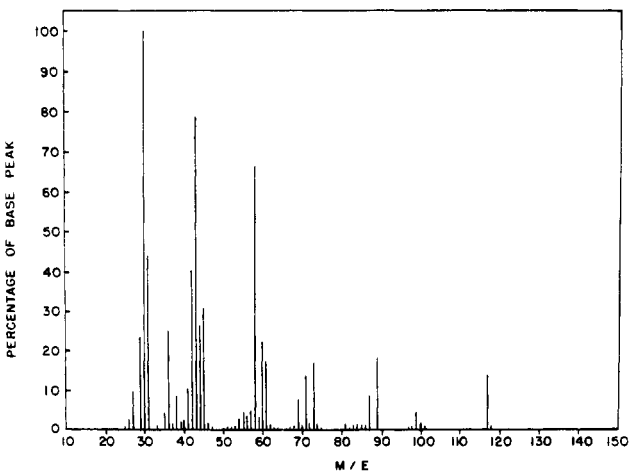


Figure 3. Mass spectrum of *N*-methylmalonamic acid (background subtracted)

After resolving this complication, it appeared that the stock acetone–water solution of degraded ^{14}C-labeled III (1 year old) would be a clean, rich source from which degradation products might be isolated and identified. Preliminary investigation showed that all the ^{14}C activity was in solution and in a more polar form than III because it was nonextractable with benzene while the free isothiazolone (I) is. The pH of the solution was found to be acidic (pH 2), and the precipitate was found to be elemental sulfur. Electrophoresis (Figure 2) indicated the presence of three carbon-14 components in solution, one major and two minor. Each of the three components had a mobility which matched a component from the sludge effluent system.

Samples of the major acetone–water degradation product were prepared as follows. The acetone–water fractions were separated by HV electrophoresis using KH_2PO_4 buffer. After location of the fraction of interest by radioassay of the edge of the phorogram, the fraction was extracted with water and concentrated. The sample was divided into two equal portions and one portion re-electrophoresed as above. The second was re-electrophoresed in a H_3PO_4 solution (0.18 *M*, pH 1.4, HVE = 1000 V, 26 V/cm, t = 20 min, T = 14°). After extraction for the second time, the samples

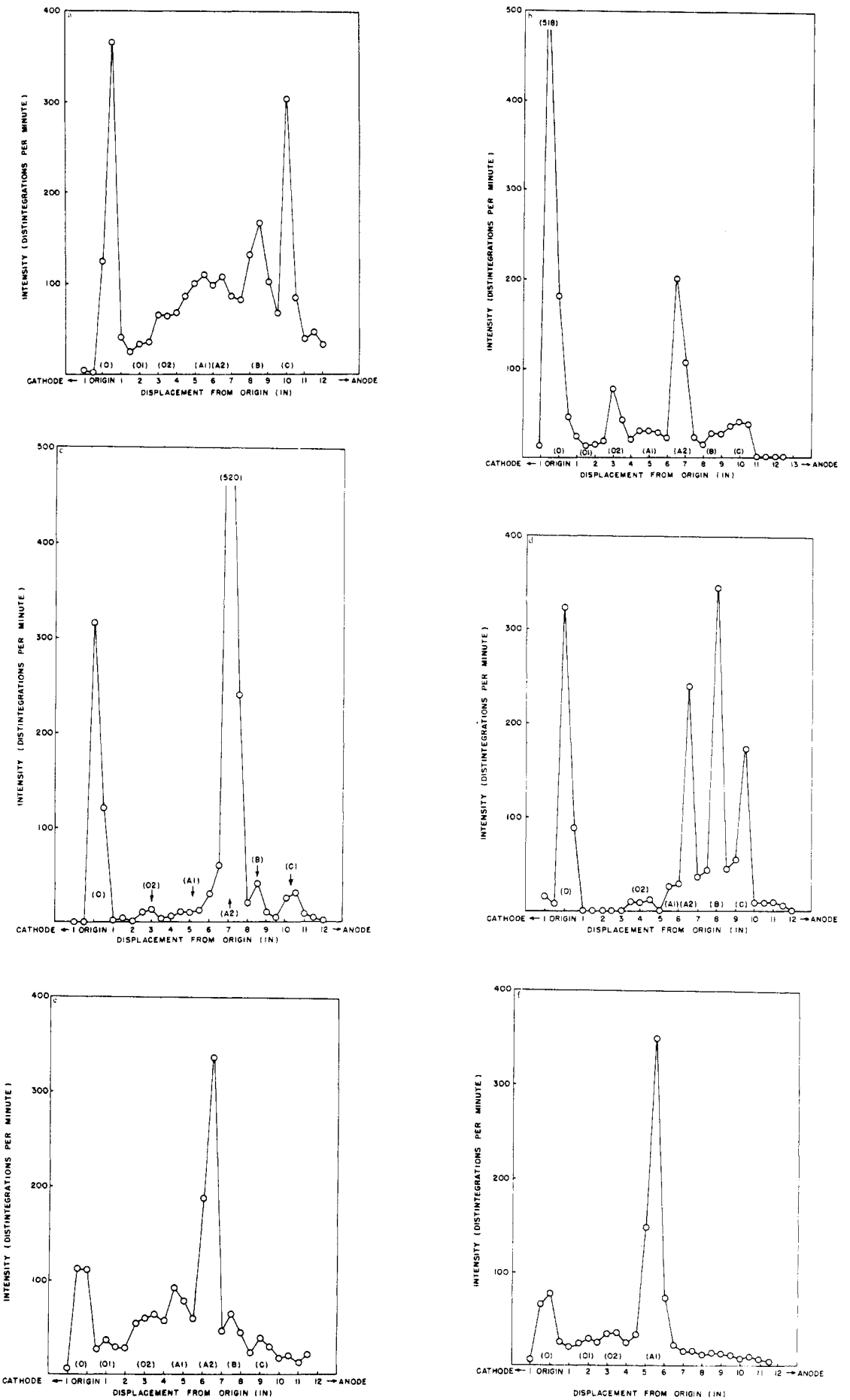


Figure 4. Electrophorograms (KH_2PO_4 or NH_4COOH buffer) of metabolites of III in: (a) sludge effluent system; (b) river water system; (c) basic hydrolysis; (d) photolysis system; (e) rat urine; and (f) methanol extract of rat feces.

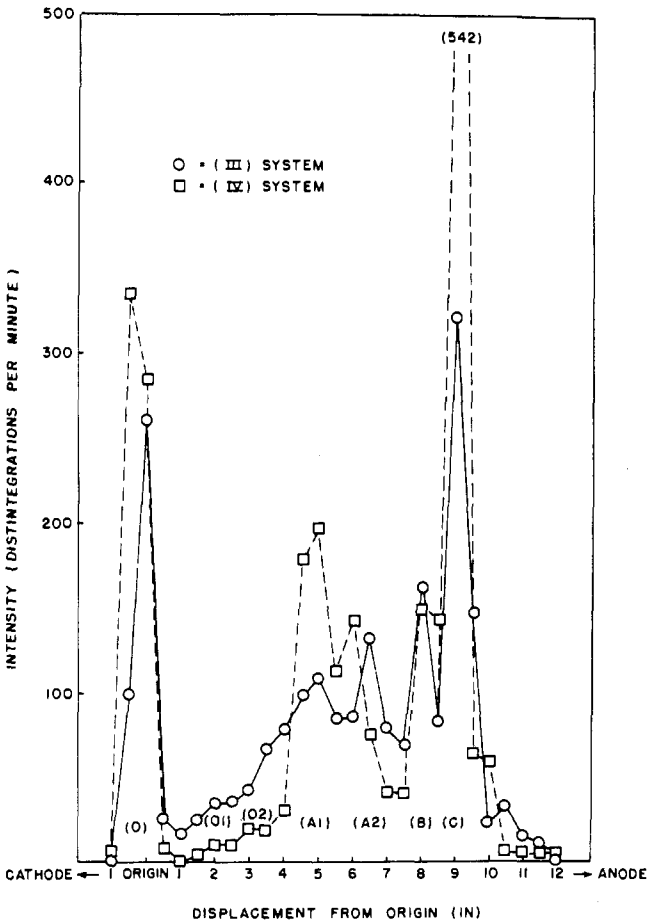


Figure 5. Comparison of III and IV sludge effluent metabolites by HVE in KH_2PO_4 buffer: (O) III; (□) IV.

were concentrated and passed through a Bio-Rex 70 cation exchange minicolumn.

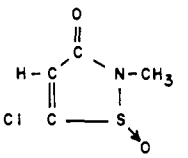
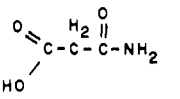
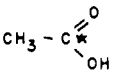
These samples together with an appropriate blank for each method of sample work-up were analyzed by mass spectrometry. The spectrum obtained from both work-ups is shown in Figure 3. The compound was found to be *N*-methylmalonamic acid.

A second degradation product (C) was isolated from the acetone–water system by the above HVE–ion exchange technique and subjected to mass spectrometry on the Finnigan MS unit. The major *m/e* peaks (intensity as percent) found were: 87 (5), 69 (3), 60 (78), 45 (66), 44 (18), 43 (40), 42 (100), 41 (10), 31 (21), 29 (20), 15 (17). The compound was identified as malonic acid. (RID studies using ethanol as a recrystallization solvent also indicated the presence of these two degradation products.)

(2) *Isolation and Indirect Identification of Degradation Products of III.* With the knowledge of two of the major metabolites in hand, the degradative pathway began to unfold. Numerous experiments were carried out by HVE and TLC to characterize and compare the degradation profiles for all the systems available. The electrophorograms for all these systems as defined by zonal analysis of radioactivity are given in Figure 4. By and large it was found that the mobilities of the labeled degradation products coincide from one system to another, within the 0.5 in. resolution afforded by the method of radioassay. Although the systems differed from one another in total number or quantity of products, it was evident that a common denominator of several products linked them together.

Knowing the identity of two major products and realizing that degradation of such a small molecular weight compound such as III would probably lead to even smaller, simpler products, a number of candidate degradation products were postulated. These were either impurities found in the technical product, or compounds related to the known degradation products, such as low molecular weight aldehydes, ketones, alcohols, and acids of mono- or difunctionality. In this manner those degradation products listed in Table I were postulated and then were found to be present in various systems by co-HVE, co-TLC, and, in applicable cases, RID.

Table I. Materials Characterized by Co-HVE, Co-TLC, or RID

Compound[b]	Identification Method
 5-chloro-2-methyl-4-isothiazolone 1-oxide	Co-TLC
 malonamic acid	Co-TLC, RID[a]
 acetic acid	Co-HVE
 formic acid	Co-HVE

[a] Recrystallized from 2-propanol. [b] The asterisk indicates the site of the ^{14}C label.

Acetic and formic acids were only seen in the Gelman buffer system because in the other buffers they were run off the HVE paper into the buffer reservoir (see Figure 1). Identification of such materials was accomplished using standards of acetic and formic acid which were ^{14}C labeled.

(3) *Indirect Identification of Degradation Products of IV.* A sludge effluent sample of ^{14}C-labeled IV, generated in identical manner as was the sludge effluent of III, was processed and analyzed by HVE. Figure 5 is a comparison of these sludge effluent samples. It is observed that the ^{14}C-labeled product distribution of III and IV is qualitatively and quantitatively very similar.

(4) *Other Degradation Products.* The components "O" (immobile metabolite fraction) and "A1" were looked for by means of GLC–MS and TLC. O material from a 144-hr photolysis study was separated from the other degradative fractions by HVE in KH_2PO_4 and NH_4HCO_3 buffers. A GLC approach to the separation of components of the O fraction was undertaken. A 2% Carbowax 20M (4 ft) column on Gas Pack F (Chemical Research Services, Inc.) was used to separate two components of the O fraction. Carbon-14 activity was found to elute at 1.5 min and at 2.5 min at 160°. Seven percent of the available carbon-14 was trapped in the first fraction and 6% in the second. Trapping was accomplished by means of a U tube at liquid nitrogen temperature. Scintillator solution was used to rinse out the trapping tube, and samples were counted directly.

Once it was determined that the peaks in question contained ^{14}C products, the mass spectrum of each was obtained via GLC–MS. The early eluting peak was identified as *N*-methylglyoxylamide. Its spectrum appears in Figure

Table II. Degradation Products of III as Separated by TLC and HVE (as Percent of Total)

Identified component	TLC coding[a]	HVE coding[b]	Sludge effluent	River water	Basic hydrolysis	Photolysis	Acetone–water	Rat urine	Rat feces	Aquatic plants
		O	14–26	19	14–22	0–11	3	15	17	
	P(1)					5				
	P(2)									8
1-Oxide of III	P(3)		3			8		1	1	28
III	P(4)		2	25	8–34	2–31		2	1	7
	P(5)		3					2	1	2
	P(6)		3					2	1	
IV	P(7)		3			2		1	1	3
	P(8)									8
	P(9)		3					2	2	
	P(10)									2
	P(11)	O1	2–7	2		0–1		1–5	1–8	5
	P(12)	O2	4–10	8	2	0–1		1–13	1–14	5
	P(13)	A1	14–18	6	3–7	1–13		8–14	46–61	3
	P(14)							2		4
	P(15)		9					7	8	4
Malonic acid	P(16)	C	10–20	7	3–4	0–7	17	5–6		3
N-Methylmalonamic acid	P(17)	A2	8–10		31–57	30–47	80	35–38	14	2
Malonamic acid	P(18)	B	5–19	3	4–8	2–18		7–9	7	5
	P(19)							5		2
	P(20)		5					8	5	4
Acetic acid		C1	0–2			3				
Formic acid		D	4–9			10				
	P(21)		0–17	10	0–2	7–25		11	12	7

[a] P(1) to P(21) represents increasing R_f values. [b] O to D represents increasing mobility from origin.

Table III. Degradation Products of IV as Separated by TLC and HVE (as Percent of Total)

Identified component	TLC coding	HVE coding	Sludge effluent	Rat urine	Rat feces
		O	20		
	P(5)			2	1
	P(6)			2	2
IV	P(7)			2	2
	P(9)			8	5
	P(11)	O1	1	1	1
	P(12)	O2	1	1	2
	P(13)	A1	15	16	44
	P(14)			5	
	P(15)			4	7
Malonic acid	P(16)		26	11	
N-Methylmalonamic acid	P(17)		9	21	12
Malonamic acid	P(18)		6	3	5
	P(19)			3	
	P(20)			3	5
	P(21)		22	15	10

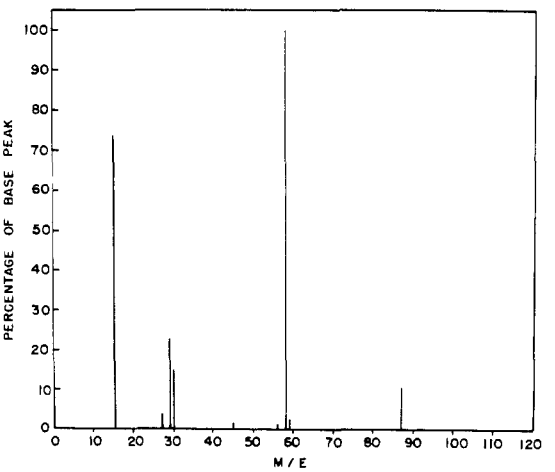


Figure 6. Mass spectrum of *N*-methylglyoxylamide (background subtracted).

6. The late eluting peak gave the following *m/e* peaks: 62 (2), 43 (2), 42 (2), 33 (35), 31 (100), 30 (3), 29 (2), 15 (6). It was identified as being ethylene glycol.

Mass spectrometry and thin-layer chromatography of the immobile HVE fractions also indicated that urea might be a component of the O fraction. The latter stems from co-TLC of unlabeled urea vs. the O fraction. Of course the carbon in urea is not carbon-14 labeled and any identification must be tentative. Identification of the A1 fraction of rat feces extract failed since the material, in freezer storage for 2 months, was found to have degraded to *N*-methylmalonamic acid (A2), malonic acid (B), and the O fraction.

Degradative Scheme. The summary of the degradation products formed as determined by TLC and HVE and quantitated by radioassay following zonal analysis is given in Tables II and III. (The O fraction components are not listed individually in the tables.) As these systems are dynamic in nature, the percentage values presented in the table reflect the concentration range observed rather than the accuracy of measurement. (The products separated by TLC were designated P(1), etc.; the products separated by HVE followed the coding first given to the sludge effluent metabolites given above.)

The eight identified components of sludge effluent from III, probably the most important and complex system studied, represent 55% of the ^{14}C activity in solution (55%) or

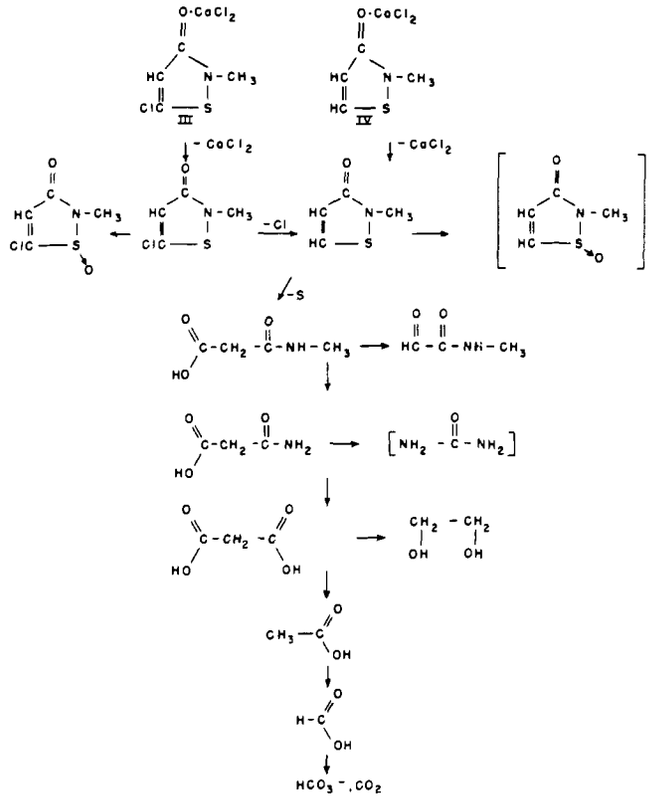


Figure 7. Major degradative pathway of III and IV (bracketed structures are postulated).

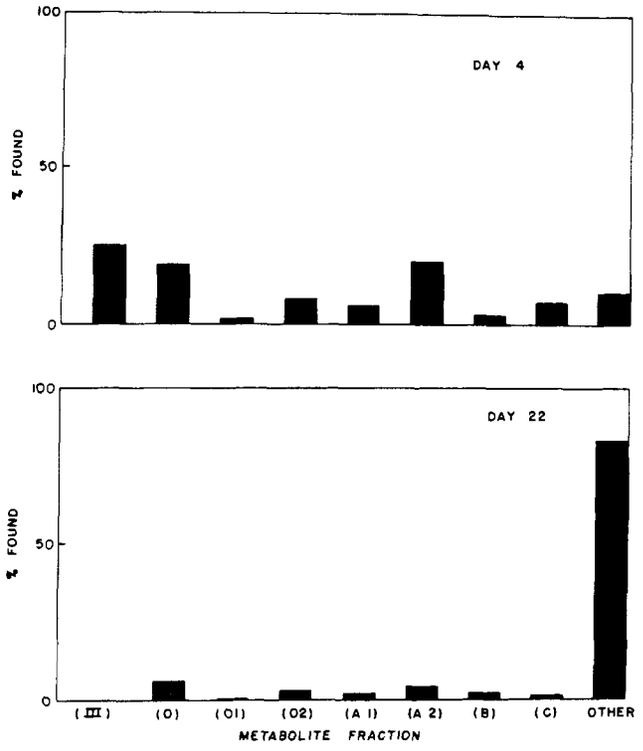


Figure 8. Metabolite–time profile of the metabolites of III in Delaware River water.

30% of the daily ^{14}C charge. It has already been reported (paper I) that 24% of the ^{14}C is lost as CO_2 during the 24-hr period of the sludge unit operation, and that another 23% of the activity remains on the sludge itself. Thus, 77% of the original ^{14}C activity of III has been accounted for: 54% (24 + 30%) as identified metabolic products and 23% as sludge adsorbed materials.

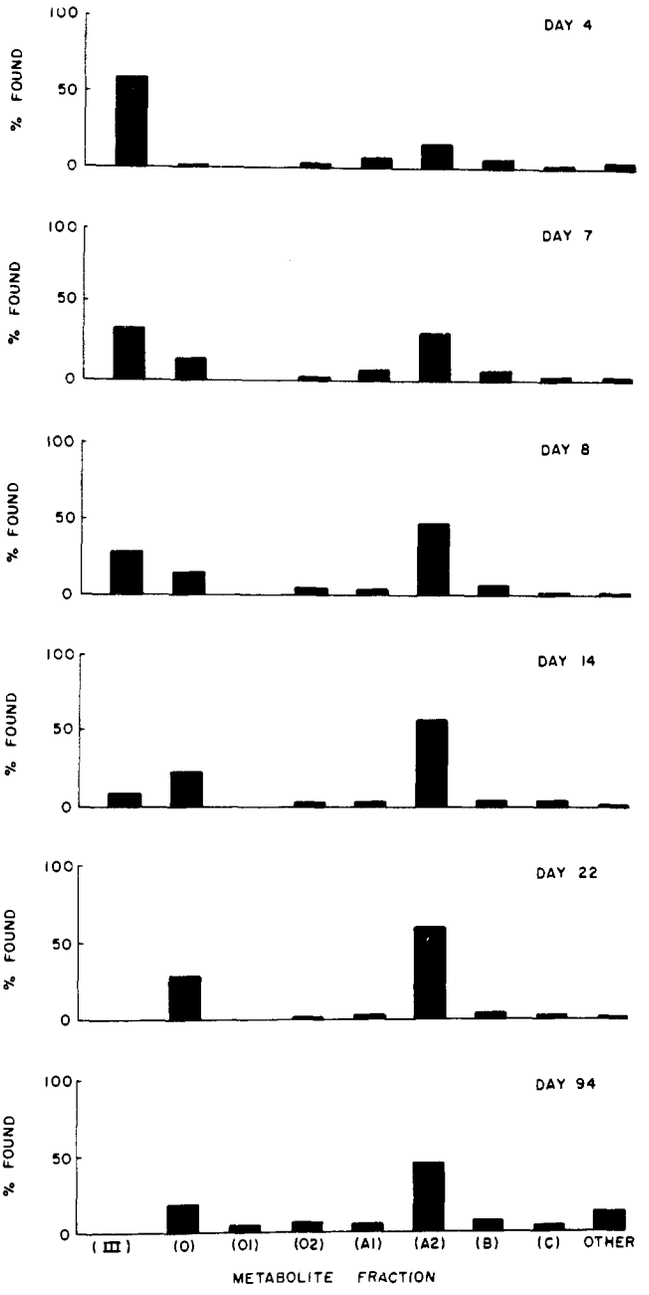


Figure 9. Metabolite–time profile of the degradation products of III in a basic hydrolysis system.

The observation that more metabolites of III and IV are found in biological systems is, more than likely, related to the formation of degradation product adducts (Ca^{2+} complexes, conjugates in urine) or the ^{14}C products degrading sufficiently to allow incorporation of ^{14}C into natural products in the degradation system, as P(2) and P(10) are observed only in the aquatic plant study.

Figure 7 diagrams the degradative scheme of III and IV. The pathway for III involves the dissociation from $CaCl_2$, formation of an oxide of III, loss of chlorine, ring opening, and loss of sulfur, leading to *N*-methylmalonamic acid, malonamic acid, malonic acid, acetic and formic acids, and formation of CO_2. Nonpolar ^{14}C material generated in the degradation of III is not mobile in the HVE systems studied. These materials are thought to be either aldehydes, ketones, or alcohols. The identification of *N*-methylglyoxylamide and ethylene glycol reinforces this hypothesis.

The above outlined scheme of the degradation of III is reasonable since it has been reported (Crow and Gosney, 1969) that the S–N bond is an ambiphilic reaction center,

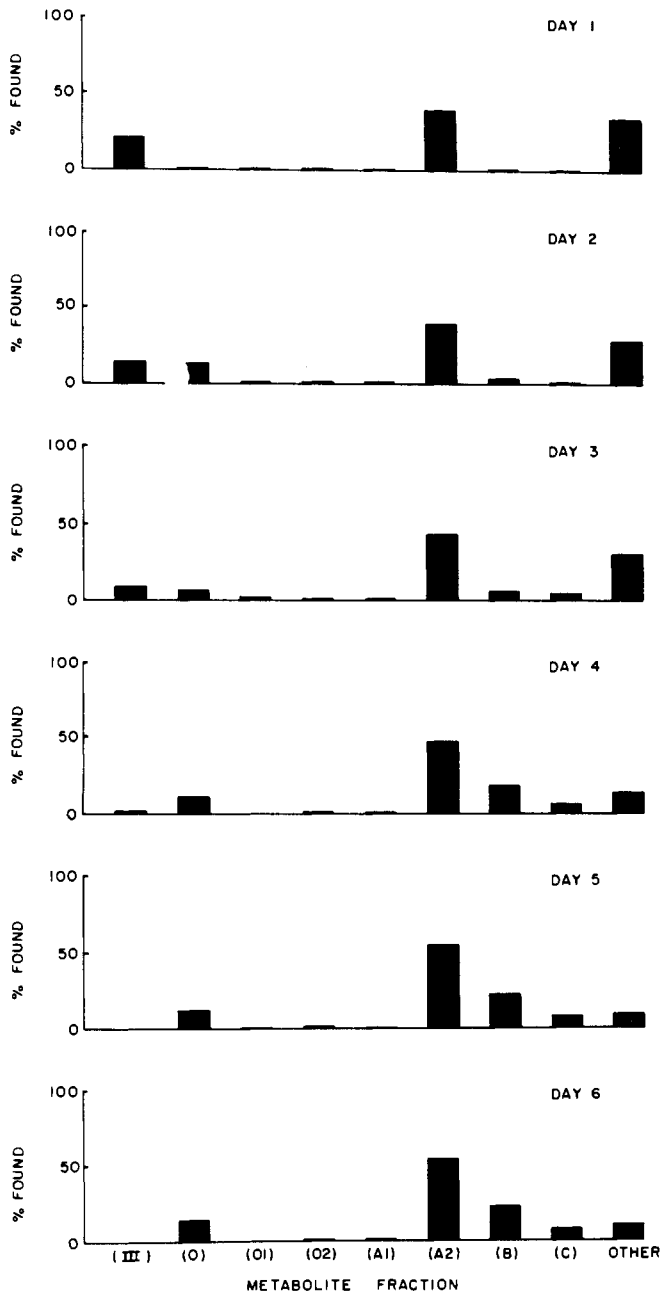


Figure 10. Metabolite–time profile of the photolysis products of III in a 1000-ppm aqueous solution.

in that both electrophilic attack at nitrogen and nucleophilic attack at sulfur are possible. The result in both cases is ring opening. It is also known that ring opening of 2-methyl-4-isothiazolin-3-one occurs rapidly when a complexing agent, like $CaCl_2$, is absent (Weiler, 1973).

Metabolite–Time Profiles. Although some parent compound rate studies with III were reported earlier (paper I), they were repeated and expanded to include not only the parent compound but also the various degradative products. This was accomplished by measuring the disappearance of III by EC–GLC and the formation and subsequent disappearance of the various degradation products by HVE and radioassay techniques.

(*1*) *River Water Die-Away System.* From the data of Figure 8 it is observed that the rate of degradation of III is fairly rapid, 75% being degraded in 4 days at 0.1 ppm in Delaware River water. After 22 days the majority (83%) of the ^{14}C is already converted to CO_2.

(*2*) *Basic Hydrolysis System.* The results of a 38-ppm solution of ^{14}C-labeled III in 0.05 *M* K_2HPO_4 buffer (9.18) are given in Figure 9. It can be seen that III degraded fairly rapidly in this system, mainly to *N*-methylmalonamic acid (A2). After 22 days, III is depleted.

(*3*) *Photolysis System.* The metabolite–time distributions for the photolysis study are given in Figure 10 for a 1000-ppm solution. It is observed that in the 1000-ppm solution, 80% of III is lost in 1 day.

It is apparent that the degradation of III and IV is rapid and can be caused by chemical hydrolysis, photochemical action, and biochemical mechanisms. The biochemical mechanism would probably be the most pronounced route of degradation. Photochemical action would be limited to the areas of water near the surface where light quanta could easily come in contact with the biocide. Chemical hydrolysis would become less important at the normally lower pH values of natural waters. Biochemical degradation could occur at any depth of surface waters and would be very effective in a sewage treatment plant. Once acclimation of the microorganisms to III or IV occurred, degradation would proceed at an even faster rate.

CONCLUSION

From the ease of metabolism, the nature of the identified metabolites, their decreasing toxicity (*N*-methylmalonamic acid is ca. $\frac{1}{50}$ as toxic as III), and previous fish exposure studies (paper I), it can be concluded that III or IV, when discharged to the aquatic environment, should present no undue ecological disturbance.

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