that aliquots containing as much as 3 μg equiv of P exhibited no detectable cholinesterase inhibiting activity. This indicated that the water-soluble fractions contained little or no intact carbamates. Thus, intact carbamates could not be expected to exceed 2.4 ppb in the seed. This proposal was documented by the field studies which showed no detectable carbamate residues at the 5-ppb sensitivity level from cottonseeds grown from seed-treated and in-furrow-treated cotton. These low carbamate levels in cotton (<5 ppb) have been confirmed using two additional gas–liquid chromatography column detection conditions (unpublished results).

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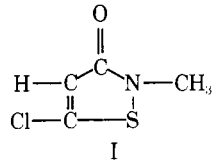
Fate of Microbicidal 3-Isothiazolone Compounds in the Environment: Modes and Rates of Dissipation

Stephen F. Krzeminski,* Charles K. Brackett, and James D. Fisher

Modes and rates of dissipation of two microbicidal 3-isothiazolone compounds—5-chloro-2-methyl-4-isothiazolin-3-one calcium chloride and 2-methyl-4-isothiazolin-3-one calcium chloride—were determined over a range of conditions likely to occur in the environment. Degradation of both compounds at levels near 1 ppm was observed to occur readily by hydrolytic, photochemical, and biological action in the aquatic and terrestrial environments. Hydrolysis increased with increasing pH and increasing temperature. Adsorption by river silt or soil was not rapid; accumulation and subsequent metabolism by certain aquatic ferns were rapid. Metabolism by rats was extensive. Although moderately toxic to fish, at sub-lethal levels fish did not concentrate the compounds within themselves to any appreciable extent upon continuous exposure and rapidly excreted the residues upon withdrawal from the microbiocidal source. The decomposition of both 3-isothiazolones by several chemical and biological mechanisms ensures that the compounds will not persist in the environment.

Recently, certain 3-isothiazolone compounds have been found to exhibit a high degree of biological activity toward a number of microorganisms. Because of this activity, such compounds are effective as slimicides in industrial waters of cooling towers and paper mills and as preservatives for cosmetics, cutting oils, and jet and heating fuels (Lewis et al., 1973). Two such compounds are 5-chloro-2-methyl-4-isothiazolin-3-one (I) and, to a lesser extent, its unchlorinated counterpart (II). While the free bases I and II are inherently unstable, their shelf lives are markedly extended by the formation of adducts with $CaCl_2$, presumably occurring through the oxygen of the carbonyl group, to give III and IV (Lindquist, 1963).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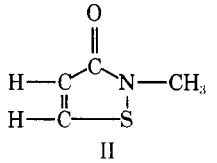
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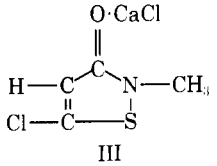
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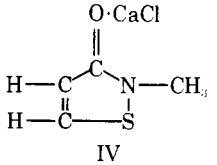
IV

Although both III and IV show biological activity against microorganisms, III is significantly more active than IV. Compound III alone or as a 3:1 mixture with IV, a normal by-product of the synthesis of III, shows promise as a potent, broad spectrum industrial microbicide. Because of high biological activity, these compounds are effective for most uses at low concentrations (several parts per million). Even so, safety considerations dictate that the fate of these compounds be defined, and their impact on the environment be evaluated. We have evaluated the environmental fate of both III and IV in a series of studies. These include measuring accumulation and elimination in rats and fish, defining the modes and rates of dissipation (degradation, translocation, and adsorption) in the environment, and isolating and identifying the transformation products. This paper concerns itself with the rat and fish studies and the modes and rates of dissipation of III and IV; a second paper details the isolation and identification of transformation products of III and IV (Krzeminski et al., 1975).

EXPERIMENTAL SECTION

All work reported below entailed the use of either compounds III or IV or a 3:1 mixture of the two. Each compound was available with and without a carbon-14 label. Synthesis of the free base I, whether labeled or unlabeled, was according to the method of Miller et al. (1971) for the chlorine-induced cyclization of 3,3′-dithiodipropionamides. For the carbon-14 compounds the appropriate 3,3′-dithiodipropionamide was formed from the reaction of ammo-

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nium polysulfide with [2,3-^{14}C]methyl acrylate (Mallinckrodt). The radiolabeled 3-isothiazolone formed was tagged in the 4 and 5 carbon positions. Compound II was formed as a by-product (25% yield) of the synthesis of I. After neutralization, I and II were separated by column chromatography on silicic acid, and then the individual $CaCl_2$ complexes formed by reaction of the corresponding free base with $CaCl_2$ (1:1 ratio) in methanol.

Standard techniques of elemental analysis and ir spectroscopy were used to check the purity of the unlabeled materials. The authenticity of the labeled products (free bases) was confirmed by ir spectroscopy and thin-layer chromatography. The radioactive purity was determined by TLC, radioautography on Kodak No-Screen Medical X-ray Film (NS54T), and subsequent zonal analysis by direct liquid scintillation counting. Products were found to be radio and chemically pure (>98%). Specific activities were 0.76 mCi/g for compound III and 0.95 mCi/g for compound IV.

Two detection techniques were used throughout this work—gas–liquid chromatography using electron capture detection for chemical analysis and liquid scintillation counting for radioassay. The method used for the analysis of III and IV was as follows. The free base was dissociated from the $CaCl_2$ in water and extracted into benzene in the presence of a high salt background of anhydrous Na_2SO_4. An aliquot of the dried benzene phase was then injected into the EC–GLC for quantitation against standards of the free isothiazolone. The GLC used was a Fisher-Victoreen 4000 series with an electron capture detector (^{63}Ni source) and equipped with a Leeds and Northrup Model H 1-mV recorder. Argon with 10% methane was the carrier gas, and its flow rate was 60 ml/min. The column was an 8 ft × 3 mm i.d. Pyrex glass column packed with 10% OV-17 on 80–100 mesh Gas-Chrom Q. The detector was operated in the pulse mode (100 μsec). The various temperatures used were 180, 220, and 265°, for the column, injection port, and detector, respectively. Attenuation was 256×10^2 (7.68×10^{-9} A), and chart speed was 0.25 in./min. Under these conditions the retention time was about 4 min. The demonstrated limit of detectability of the method was 0.01 ppm with recoveries greater than 95%. The limit of detectability was about 1–2 orders of magnitude less for the unchlorinated isothiazolone. In the latter case larger samples were used when needed to overcome this limitation.

Radioassay determinations were made by adding 1–4 ml of ^{14}C-labeled sample to 15 ml of an appropriate liquid scintillator solution in a polyethylene counting vial. Solid samples were previously weighed into zircon combustion boats (Matheson Scientific Co.) and burned in a modified Peets, Florini, and Buyske oven (Peets et al., 1960; Ashton and Kelso, 1971). The $^{14}CO_2$ resulting from the combustion was trapped in 10 ml of a 5 *M* solution of ethanolamine in methyl Cellosolve. After the combustion was completed, the final volume of the trap solution was recorded and a 4-ml aliquot portion was combined with 15 ml of "M" scintillation solution in a polyethylene counting vial for radioassay. ["M" scintillator solution is composed of 1285 ml of methanol, 1715 ml of toluene, 15.0 g of 2,5-diphenyloxazole, and 0.90 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene.] A carbon-14 standard was added to selected samples prior to combustion. Combustion and counting of such samples enabled one to determine the combustion recovery.

All measurements of radioactivity were made using either a Model 3314 or a Model 3320 Packard Tri-Carb liquid scintillation spectrometer. The instrument settings used were discriminator, 50 to 1000, and amplifier gain, 25%. Counting efficiency was determined by the use of an internal standard. Each sample was counted for 5-min periods at least 6 times for a total counting time of at least 30 min.

(A) Hydrolysis Study. Five solutions were made up in deionized, sterile water such that their pH values varied from 3.0 to 11 by using Clark and Lubs buffer solutions (Bates, 1959). To 20 ml of each standard was added nonradioactive compound III to make a solution of 0.94 ppm concentration. Each solution was divided in half, and each 10-ml portion put into a 25-ml glass vial and tightly capped. One vial was stored in a constant temperature bath which was maintained at 40° in the dark. The second vial was stored in a refigerator whose temperature was 7°. (These two temperatures represent the temperature extremes of natural waters.) Periodically, 100-μl portions were removed from the stored solutions and analyzed by electron capture gas–liquid chromatography for the parent compound.

(B) Biodegradation Studies. (*1*) *River Water Die-Away Tests.* To 1 l. of natural water (obtained from the Schuylkill River in Pennsylvania) was added the necessary amount of ^{14}C-labeled compound III or IV to make the concentration of the solution 1.0, 0.1, or 0.01 ppm. Whenever a solution was made up, a fresh supply of water was obtained from the river. In all cases, solutions were held in 1-l. glass filter flasks. To each flask was affixed a one-hole rubber stopper containing a glass inlet tube (3 mm i.d.) which dipped below the surface of the water. The side arm of the flask was used as an outlet tube. The system was connected to an air supply with ambergum tubing. A Mallcosorb indicating carbon dioxide adsorbent trap (Mallinckrodt) was inserted into the air line before the flask. The outlet tube was connected to a CO_2 trap vessel which contained 20 ml of 5 *M* ethanolamine in methyl Cellosolve. Air flow through the solution was 60 ml/min. (A Dry Ice trap before the CO_2 trap was used initially to trap any organic volatiles. After none were found, this trap was removed from the system.)

Once aeration was initiated, trap solutions were sampled at approximately weekly intervals. Two milliliters of each sample was counted with 15 ml of D scintillator solution containing 6% by weight of Cab-O-Sil, a gelling agent, in a polyethylene counting vial. [D scintillator solution is made of 3000 ml of dioxane, 360 g of naphthalene, 12.0 g of 2,5-diphenyloxazole, and 0.15 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene.] All determinations of radioactivity were as described above.

(*2*) *Activated Sludge Test.* The semicontinuous activated sludge method used was the standard method of the Soap and Detergent Association (SDA, 1966). Briefly, the method involved the daily addition of synthetic sewage (nutrients) and the test compound to activated sludge (obtained from the Northeast Philadelphia Sewage Treatment Plant). The 1.5 l. of sewage and sludge was aerated for 23 hr, the sludge was allowed to settle for 30 min, and then 1 l. of supernatant (effluent) was removed. Fresh synthetic sewage and test compound were then added to the sludge remaining in the test unit and the cycle repeated. This sequence was followed Monday through Friday. Over weekends and holidays aeration was continued, but the daily draw and fill were omitted.

The effluent, which represents treated water that would be discharged from a sewage treatment plant, was radioassayed by placing 1 ml of effluent in a polyethylene counting vial with 10 ml of T scintillator solution. [T scintillator solution is 6.0 g of 2,5-diphenyloxazole, 500 ml of Triton X-100 (Rohm and Haas Co.), and 1000 ml of toluene.] Carbon dioxide evolved from the activated sludge unit was trapped in 200 ml of 0.5 *N* NaOH, and a 0.5-ml aliquot part counted with 18 ml of D Cab-O-Sil scintillator solution in a polyethylene vial. Two sludge units were maintained, one for ^{14}C-labeled III and one for ^{14}C-labeled IV. The specific activity was 0.25 mCi/g for the former and 0.32 mCi/g for the latter.

Anticipating a toxic effect from III and IV on the bacteria, initial dosages were made at gradually increasing con-

Table I. Physical Properties of Soils Used for Leaching Study

Soil type	Organic matter, %	pH	Sand, %	Silt, %	Clay, %	Cation exchange capacity, mequiv/100 g
Lakeland sand	0.95	5.0	98	0	2	1.7
Hagerstown silt loam	3.42	6.4	38	34	28	9.4
Cecil clay[a]	0.44	4.7	32	14	54	6.9
Solomon clay loam	15.1	7.4	24	44	32	49.9
Virdan silty clay loam[b]	5.34	6.0	18	64	18	21.4

[a] Kaolinite clay. [b] Montmorillonite clay.

centrations. To preserve the limited supply of ^{14}C-labeled III and IV available, these initial dosages were made using nonradioactive III and IV, respectively. During the first week of the test, added levels were 0.1, 0.2, 0.4, 0.8, and 1.0 ppm. During the second and third weeks, the level was raised from 1.0 to 10 ppm in increments of 1 ppm. No significant change occurred in the total amount of CO_2 released by the bacteria in degrading the added sewage either before any III or IV was added or after the 10-ppm level was reached, indicating no harm to the bacteria (Lenhart, 1971). Thus, the dosing with ^{14}C-labeled III and IV at the 10-ppm level began at this point.

(C) Photolysis Study. Solutions (1 ppm) of III and IV prepared with pond water obtained from the Rohm and Haas Experimental Farm in Newtown, Pa., were irradiated for 48 hr with a system of three 20-W fluorescent sunlamps (Westinghouse FS20) and two 20-W blacklights (Westinghouse F20T12/B1). This system closely simulated the intensity and the uv spectrum of natural sunlight. The initial pH of the water was 8.0 and the temperature during the irradiations was 37°. Controls were placed in the dark at 37°. All irradiations were done with samples in 10-cm glass petri dishes covered with 0.06 in. thick ultraviolet transmissible Plexiglas (UVT II). Each petri dish contained a small cup with 1 ml of 0.1 *N* NaOH for trapping evolved CO_2. All irradiations were done on a revolving turntable.

During the study, control and treated samples were analyzed by radioassay for ^{14}C content and by thin-layer chromatography followed by radioautography and zonal analysis for parent compound.

(D) Adsorption Studies. (*1*) *Adsorption by River Silt.* Ten-milliliter portions of ^{14}C-labeled III and IV solutions at concentrations of 1.0 and 10.0 ppm were added to 1-g samples of Delaware River silt in 1-oz vials. [Silt characteristics were: organic matter, 10.8%; sand, 44%; silt, 48%; clay, 8%; pH 5.7; cation exchange capacity, 8.5 mequiv/100 g.] After the soil–water mixtures were mechanically shaken for 20 hr, each was centrifuged and an aliquot portion of supernatant was taken for radioassay.

(*2*) *Accumulation by Aquatic Plants.* Several experiments investigated the affinity of III and IV for aquatic ferns. In the first experiment, to each of four glass jars was added 100 ml of distilled water and the radioactive test compounds to make two solutions of 0.1 ppm (III) and 0.1 ppm (IV). To each set of two jars, 2 g of duckweed (*Lemma minor*) or salvinia (*Salvinia braziliensis*) aquatic fern was placed on the water's surface. Each of the four systems was then magnetically stirred for 42 hr. Samples of water were radioassayed after 20 and 42 hr of contact time.

In the second plant adsorption study, each test unit consisted of a 1-l. solution of approximately 0.1 ppm of ^{14}C-labeled III or IV to which was added 2 g of salvinia. After 24 hr of contact, under mild stirring, 0.25 g of aquatic plants was removed from the solution of III for solvent extraction studies. Two grams of fresh plants was then added to the test solution of IV and 3 g to the test solution of III and stirring resumed. The aqueous phase was sampled on days 1, 3, and 6, and the concentration of carbon-14 activity in the plants determined by difference.

After the 6 days of contact, 2 g of plant was removed from the test solutions and placed in 135 ml of distilled water and gently stirred for a 6-day period. Samples of plants were removed on days 3 and 6, air-dried, and combusted. Water was sampled on these days also.

(E) Soil Studies. (*1*) *Leaching Study.* ^{14}C-Labeled III was applied to duplicate 5 in. i.d. × 10 in. high metal tubular columns of five representative soil types (Table I) at a level of 2 lb/acre. For each soil type from a stock solution (4.4 mg/ml in acetone) of ^{14}C-labeled III, an aliquot portion of 1.42 ml was added to a 200-g sample of soil in a glass jar. The jar was capped and the sample mixed by hand tumbling for 5 min. After mixing, 100-g portions were spread onto the surface of both the duplicate columns. To each column was added an equivalent of 1 in. of water per week for 8 consecutive weeks. After 58 days the soil columns were dismantled, and each 1-in. soil segment was sampled for combustion and radioassay. Water which eluted from the columns was collected and radioassayed.

(*2*) *Dissipation Study.* ^{14}C-Labeled III was applied at 1 ppm to Hagerstown silt loam soil, and the treated soil placed in sealed biometer flasks equipped with a side arm containing sodium hydroxide for trapping carbon dioxide produced by the soil media (Bartha and Pramer, 1965). The trapping solution was analyzed at frequent intervals for ^{14}C content. Concurrently, the trapping solution was also analyzed for total carbon dioxide. Studies were done in sterile and nonsterile soils.

(F) Animal Studies. (*1*) *Rat Study.* A rat feeding study was conducted with two pairs of male and female adult albino rats. Each rat was housed in a separate Roth metabolism cage (Roth, 1956) and given daily oral doses of ca. 2.5 mg of a 3:1 mixture of III and IV via syringe and stomach tube. One pair received labeled III and unlabeled IV; the other received labeled IV and unlabeled III. Each rat received 20 g of rat chow and an ad libitum supply of water each day. Every 24 hr just before dosing, excrements of breath, urine, and feces were collected. These samples, together with the tissues and organs obtained at the time of sacrifice (after 3 days withdrawal), were analyzed for radioactivity.

(*2*) *Fish Residue Study.* Bluegill sunfish (*Lepomis macrochirus*) were exposed to a 3:1 mixture of III and IV formulated in one case with ^{14}C-labeled III and unlabeled IV (designated formulation I) and in another case with unlabeled III and labeled IV (designated formulation II). The concentrations to which the fish were exposed were nominally 0.02 (the level expected to occur immediately after mixing of cooling tower blowdown with a stream), 0.12, and 0.80 ppm for formulation I and 0.12 ppm for formulation II. (These levels of exposure were all well below the TL_{50} value for bluegill to RH-886.) For each level of exposure, bluegills, initially numbering 100, were continuously exposed to the biocide under dynamic conditions in a flow-through aquarium. Each aquarium contained 30 l. of water and had a turnover rate (mean residence time) of 5 l./hr. After a 2-week exposure period, 30 of the fish were removed from the exposure tank and placed in a second aquarium receiving water containing no biocide. Fish in the original tank continued to be exposed to the biocide for 4–6 weeks.

Fish continuously exposed to the biocide and those on

Table II. Rates of Hydrolysis of Compound III

		Temperature, 7°		Temperature, 40°		
pH	Molarity	k, days^{-1}	$t_{1/2}$, days	k, days^{-1}	$t_{1/2}$, days	$k(40°)/k(7°)$
4.5	0.05			6.91×10^{-3}	100	
8.2	0.01	7.59×10^{-3}	91.3	2.68×10^{-1}	2.59	35.2
	0.10	9.21×10^{-3}	75.2	22.65×10^{-1}	2.62	28.1
9.0	0.01	6.35×10^{-3}	109.0	1.16×10^{-1}	5.97	18.3
	0.05	9.02×10^{-3}	76.8	2.12×10^{-1}	3.27	23.5
11.0	0.01	6.91×10^{-2}	10.0	7.28	9.51×10^{-2}	105.0
	0.05	1.08×10^{-1}	6.42	14.1	4.92×10^{-2}	130.0

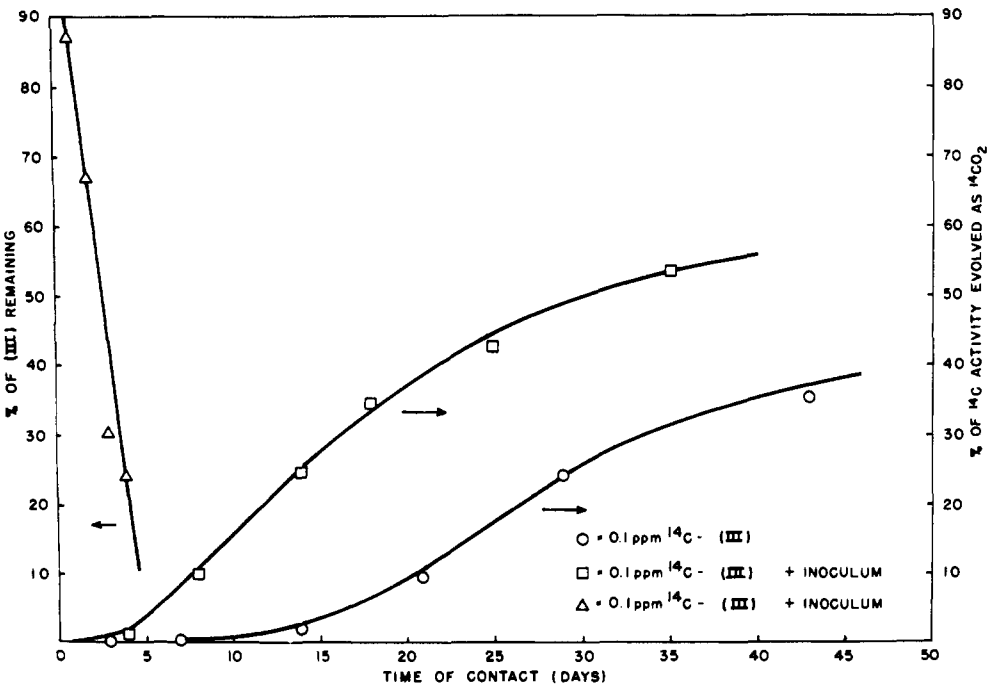

Figure 1. Fate of compound III in river water.

withdrawal were analyzed periodically through the course of experiment. In each case fish were divided into samples of viscera and carcass (tissue) for combustion and radioassay. In two cases, the distribution of ^{14}C residue among the inner tissue, fins and scales, head and gills, and viscera of the fish was determined.

At various times during the study, fish carcass (tissue) was extracted with polar (methanol) and nonpolar (hexane) solvents to ascertain the extent to which ^{14}C residues were bound in the fish. Daily water samples from each aquarium were also taken and radioassayed in order to verify that exposure levels were constant throughout the study. Exposure of fish was carried out at Bionomics, Inc. (Wareham, Mass.) and the analysis at the Rohm and Haas Research Laboratories.

RESULTS AND DISCUSSION

(A) Hydrolysis Study. Pseudo-first-order rate constants and half-lives determined from GLC analysis of III in aqueous environment under various conditions of pH, temperature, and buffer strength are listed in Table II. From the data one can conclude that the rate of hydrolysis for compound III at low concentrations (ca. 1 ppm) increases with increasing pH, increasing temperature, and to only a limited extent, with increasing ionic strength of the buffer. The compound is stable under acidic conditions, but the rate of disappearance increases by a factor of about 2000 in going from pH 4.5 to 11. In going from 7 to 40° the rate increases by 1–2 orders of magnitude.

(B) Biodegradation Studies. *(1) River Water Die-Away Tests.* Several sets of die-away experiments were performed. It was observed that the rate of degradation of compound III by biological mechanisms is concentration dependent, decreasing with increasing concentration. For example, a tenfold increase in concentration of compound III from 0.1 to 1.0 ppm results in a twofold decrease (2.98 to 1.84%) in its rate of degradation to CO_2 after 21 days contact with river water. On the other hand, the rate of degradation of IV increases with increasing concentration at the levels studied (0.1 and 1.0 ppm). After 54 days of contact the 1.0-ppm solution of IV was about sixfold faster in the rate of production of $^{14}CO_2$ than that at 0.1 ppm. Comparing the rates of degradation of III and IV at 0.1 ppm shows only a slight increase in degradation of IV over III (18% vs. 21% as $^{14}CO_2$). But at the 1-ppm level the rate of degradation of IV to CO_2 is about 20 times faster after 21 days. The reason for this difference in degradation rates is caused by the much greater microbicidal activity of III over IV which represses the microbicidal population for a longer period of time.

Table III. ^{14}C Material Balance of Activated Sludge Units

Sample	% of total ^{14}C added	
	III	IV
Effluent	55.1	59.6
Carbon dioxide	23.6	18.3
Sludge	22.5	19.3
Total	101.0	97.2

To verify that the observed degradation in river water was biochemical in origin, it was necessary to demonstrate that microorganisms' acclimation gave rise to increased rates of degradation. Here 1-l. solutions of ^{14}C-labeled III were made up at 1.0, 0.1, and 0.01 ppm, and to each was added 1 ml of the 0.1-ppm ^{14}C-labeled III solution from the previous terminated experiment. These additions represented a maximum of 0.01, 0.1, and 1% addition of ^{14}C products, respectively, to each new solution. In like manner new solutions of ^{14}C-labeled IV were made up and inoculated with 1 ml of the 0.1-ppm ^{14}C-labeled IV solution from the previous experiment.

Comparing the data from the unacclimated and acclimated tests for the first 29–35 days, it was observed that rates of degradation are increased (2 to 8 times) by microorganism acclimation to the subject compounds. Again, the influence of the biological activity of the 1.0-ppm solution of III is demonstrated in a long induction period (2–3 weeks of contact) before degradation. Since the microorganism content of natural waters varies from day to day, as witnessed by the longer induction period in inoculated water, the increase in the rate of degradation for acclimated water is even more pronounced than reported here.

In Figure 1, a comparison is made of the rate of degradation of a 0.1-ppm solution of ^{14}C-labeled compound III by $^{14}CO_2$ production of noninoculated and inoculated solutions and by GLC analysis of parent compound. It is observed that the rate of disappearance of parent compound is significantly faster than the production of $^{14}CO_2$. (Similar behavior for IV was also observed at this level.)

(2) *Activated Sludge Test.* The results for the test are summarized in Table III. Figure 2 illustrates the day-by-day ^{14}C accountability for the compound III test. (Compound IV behaved in a similar fashion.) The indicated amounts added in the sewage were actually added on the previous day when the cycle began while the ^{14}C analyses are for the effluent removed at the end of the cycle on the day shown. CO_2 was trapped throughout the cycle and the value given is for the cycle ending on the day shown.

Figure 2 shows that the amount of $^{14}CO_2$ evolution from the 24-hr cycles increased from 8 to 20% during the first 2 weeks. This indicates that some acclimation was occurring and that some of III was being completely degraded. No further increase occurred during the next 4 weeks. The higher Monday levels show the effect of longer (weekend) exposure times. Effluent values varied between 45 and 58% during these 6 weeks, showing only a very slight upward trend.

Both the $^{14}CO_2$ evolution and the amount of ^{14}C found in the effluent showed a mild increase during the seventh and final week of the test. This may have been caused by saturation of adsorption sites on the sludge or additional acclimation of the bacteria. Perhaps both are occurring, although the previous 4 weeks' results would tend to discount additional acclimation.

The same dosage increases with nonradioactive material as previously described for III were used with IV. Results closely paralleled those obtained with III. The test was terminated after the fourth week when $^{14}CO_2$ evolution and the amount of ^{14}C in the effluent appeared to have reached a steady state.

It is also noteworthy that during the course of the experiment the effluents from both units treated with III and IV never showed detectable (0.01 and 0.1 ppm, respectively) residues of the parent compounds by gas–liquid chromatographic analysis. Apparently the test compounds are almost immediately converted to degradation intermediates.

(C) Photolysis Study. The results of this study appear in Table IV. Although there is apparently some question as to the actual concentration of compound III used, based on the nominal value, only 79% is measured experimentally. In spite of this it is apparent that III and IV are readily photolyzed to other products by the action of ultraviolet radiation. Over the 48 hr of the test there appears a 48% drop in the content of III and a 61% drop in the content of IV. Thus, IV is more easily photolyzed than III. The CO_2 trap

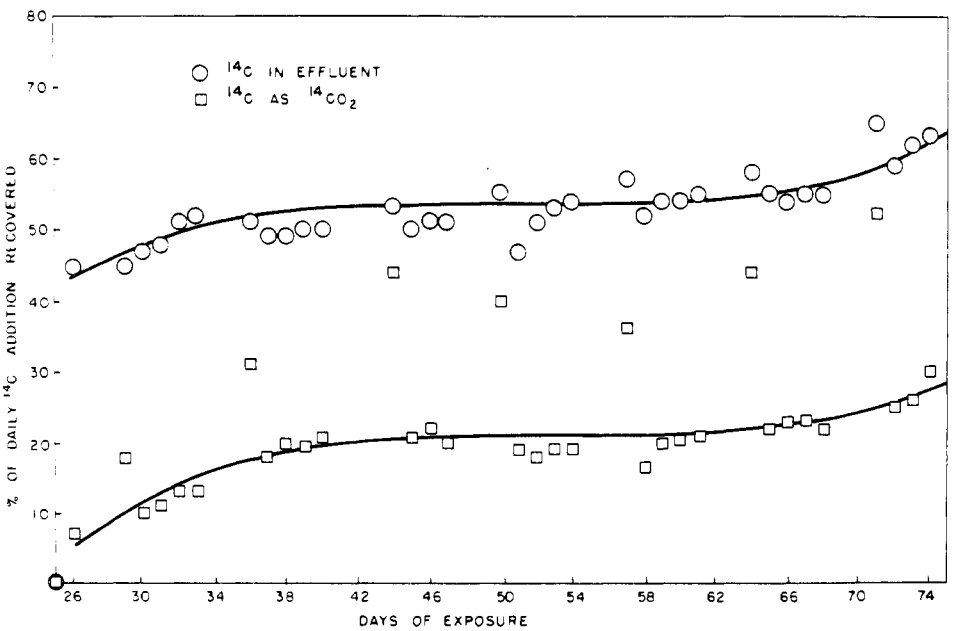

Figure 2. Daily distribution of ^{14}C activity in activated sludge unit treated with ^{14}C-labeled III at 10-ppm level after 24-day buildup.

Table IV. Decrease of Compounds III and IV under Exposure to Uv Light

Time, hr	% of total ^{14}C			
	Compd III		Compd IV	
	Uv	Dark	Uv	Dark
0	78.8	78.8	97.8	97.8
5	59.3	77.6	96.9	
48	31.2	79.1	37.7	95.5
	CO_2 Trapped			
0	0	0		
5	0.12	0.06		
48	2.71	0.19		

Table V. Accumulation of Compounds III and IV by Aquatic Plants

Compd	Aquatic plant	k = ppm in plant/ppm in H_2O	
		20 hr	42 hr
III	Duckweed	40	93
	Salvinia	86	159
IV	Duckweed	25	43
	Salvinia	105	170

data [only available for the test with III] indicate that almost 3% of the starting amount of III is already degraded to CO_2 after 48 hr of irradiation.

(D) Adsorption Studies. *(1) Adsorption by River Silt.* When aqueous solutions of III and IV were separately put in contact with Delaware River silt at room temperature for 20 hr, the following results were obtained. Compound III at concentrations of 1 and 10 ppm was adsorbed on the silt to an extent of 5 and 8%, respectively. Compound IV at concentrations of 1 and 10 ppm was adsorbed to the extent of 11 and 2%, respectively. One thus concludes that III and IV are not readily adsorbed from aqueous solutions onto river silt. This is not unexpected when one realizes that both compounds are quite water soluble.

(2) Accumulation by Aquatic Plants. Contrary to the above results with river silt, compounds III and IV were found to be rapidly taken up by aquatic plants as shown in Table V. In a second study with salvinia alone, uptake and release by the aquatic fern were followed by solvent extraction of the plant as well as by radioassay. The radioassay data for desorption appear in Table VI. Extraction of the fern in contact with ^{14}C-labeled compound III after 1 and 3 days of contact was accomplished with methanol. Sixty percent of the ^{14}C activity was extracted from the salvinia plants exposed to the test compound for 1 day. This decreased to 30% after 3 days exposure. The extracts were analyzed by the GLC method, and no detectable residue as III was found in either extract.

From the above sorption experiments one can draw the following conclusions. First, compounds III and IV are readily adsorbed (or absorbed) by aquatic plants. Second, after uptake the plant quickly metabolizes the test compounds into different substances. Third, the plants are probably metabolizing the compounds to CO_2 in a period of only several days since the ^{14}C material balance decreases with increasing time. A later experiment, using a closed system to enable trapping of $^{14}CO_2$, verified this assumption (Fisher, 1972).

Table VI. Desorption of Compounds III and IV Residues by Salvinia

Test compd	Withdrawal day	^{14}C residue (calcd as ppm of parent compound)	
		Plant[a]	H_2O
III	0	24.1	
	3	9.5	0.008
	6	11.7	0.009
IV	0	34.9	
	3	19.4	0.009
	6	20.9	0.012

[a] These values were determined by combustion.

Table VII. Summary of Carbon-14 Recovery Data in Soil Columns Treated with ^{14}C-Labeled Compound III

Soil type	% of applied activity recovered[a]			
	Top segment	Remaining segments	Drainage water	Total
Lakeland sand	6.78	17.0	0.75	24.5
Hagerstown silt loam	45.0	29.5	0.71	75.2
Cecil clay	12.8	49.5	1.19	63.5
Solomon clay loam	61.6	11.6	0.87	74.1
Virdan silty clay loam	53.6	11.6	0.34	65.5

[a] Calculated assuming that 3.12 mg (5.26×10^6 dpm) of compound III was placed on each column. Each value is an average of two determinations.

Table VIII. ^{14}C Material Balance of Rat-Feeding Study

Sample	^{14}C recovered as % of total dose			
	Compd III		Compd IV	
	Male	Female	Male	Female
Feces	52.0	56.2	29.8	45.7
Urine	37.3	36.5	57.3	44.8
Carbon dioxide	0.36	1.47	0.47	0.47
Organs and tissues	1.72	1.84	2.10	2.10
Residues of cages and food	7.89	2.75	6.53	3.57
Total	99.3	98.9	96.2	96.6

(E) Soil Studies. *(1) Leaching Study.* The leaching pattern of compound III for various soil types is summarized in Table VII. In the five soil types studied, leaching of III, as determined by radioassay, increased as follows: Solomon clay loam < Virdan silty clay loam < Hagerstown silt loam < Cecil clay < Lakeland sand. This order is approximately the order of decreasing organic matter content of the soils (Table I). After 8 weeks the mean recovery of applied ^{14}C

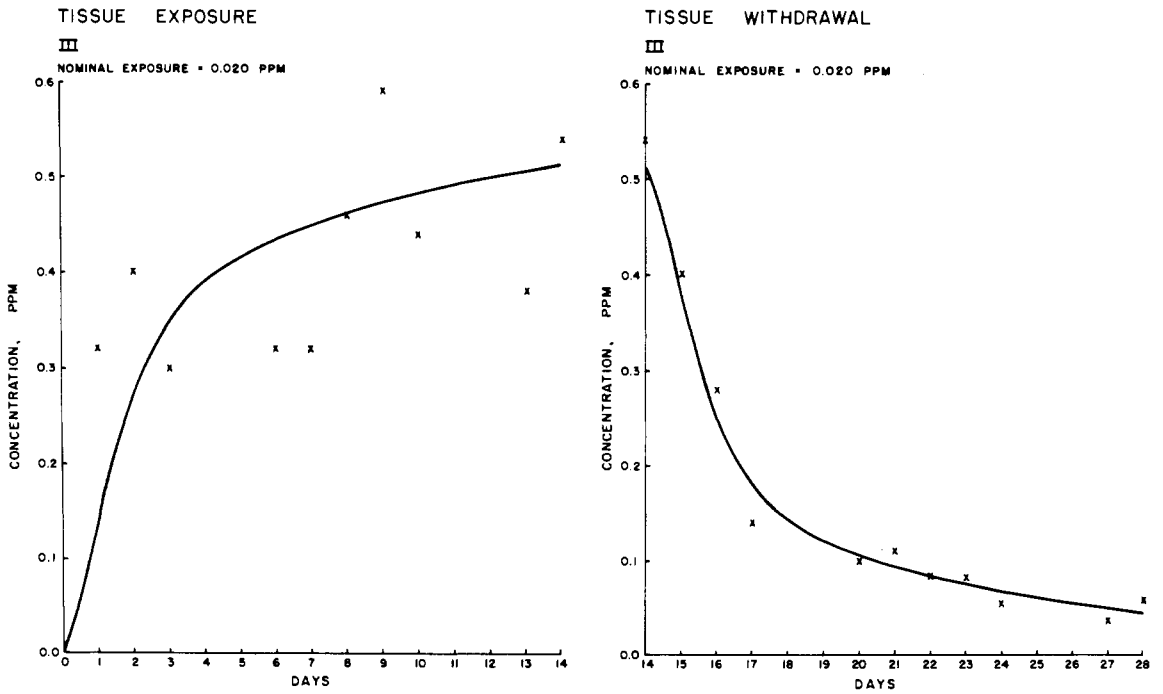

Figure 3. ^{14}C residues of III in fish tissue during accumulation and withdrawal phases.

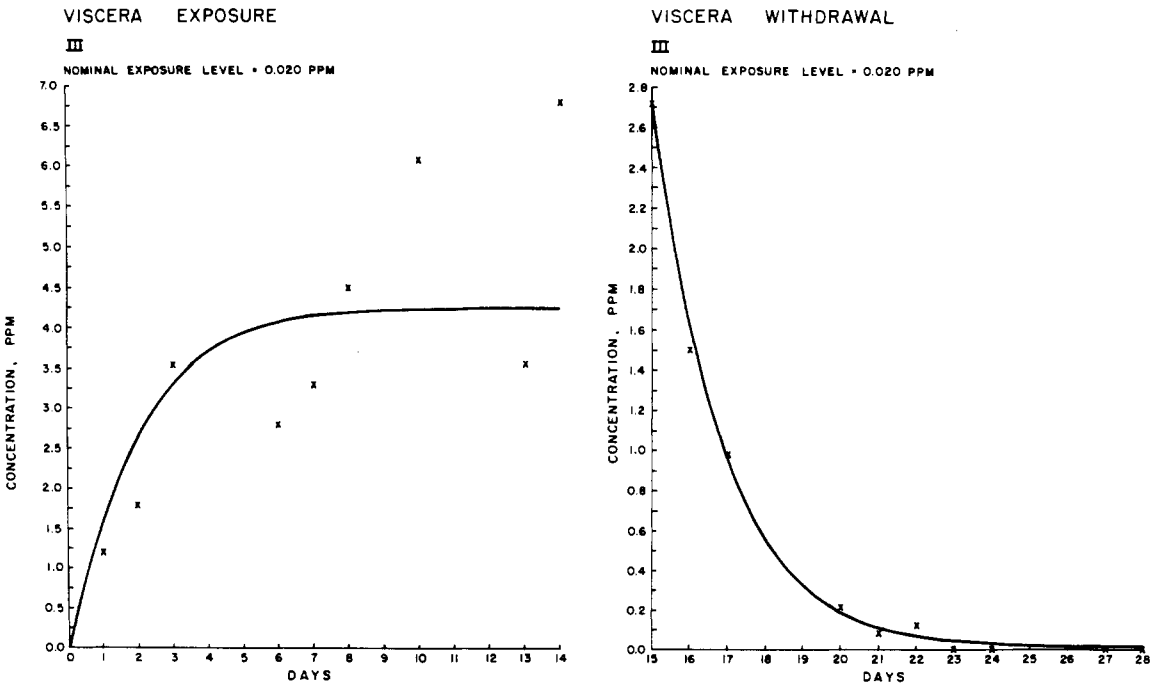

Figure 4. ^{14}C residues of III in fish viscera during accumulation and withdrawal phases.

material was 60% and ranged from a high of 76% for Hagerstown silt loam to a low of 25% for Lakeland sand.

One can conclude the following. First, leaching of compound II, a fairly water-soluble material, is inversely proportional to the organic content of the soil. This is probably caused by increased adsorption of III. Second, metabolism of III in soil is fairly rapid as evidenced by the low ^{14}C recovery data.

(2) *Dissipation Study.* In nonsterile soil to which ^{14}C-labeled III was added, an appreciable level of ^{14}C was released as $^{14}CO_2$ after 41 days, ca. 28%. In sterile soil release of $^{14}CO_2$ was less than 1% over the same time period. The amounts of total CO_2 produced by the control and treated soils (sterile and nonsterile) were approximately equal.

It is concluded from these results that the labeled carbons of III are rapidly degraded by biological mechanisms to CO_2 in soil media. Also, at 1 ppm III has no apparent adverse effect on the overall microbial activity of the soil as measured by total CO_2 production. This observation is not contradictory with findings in water at 1 ppm (see above discussion on river water die-away study) but is merely a result of the much greater microorganism population in soils than water.

(F) Animal Studies. (*1*) *Rat Study.* The results of the

Table IX. Steady-State ^{14}C Residues [Calculated as Parts per Million of a 3:1 Mixture of III and IV]

Formulation and concn	Residue	
	Carcass	Viscera
I, 0.02	0.69	4.08
I, 0.12	3.19	18.8
I, 0.80	17.8	240.0[a]
II, 0.12	0.575	4.81

[a] Maximum, not steady-state, value.

rat-feeding study with a 3:1 mixture of III and IV are summarized in Table VIII. The overall material balance of carbon-14 is excellent for all four rats. Although complete metabolism to CO_2 is slight (1.5% or less), storage in tissues is also minimal (2.1% or less). Moreover, the bulk of the ^{14}C residue is readily excreted ($t_{1/2} < 1$ day) via the feces and urine. There appears to be little difference in the manner in which the rats handle III or IV. If there exists a difference, it might be that, because the residues found in the urine of the rats dosed IV are somewhat larger than the other pair, IV is metabolized or eliminated at a slightly faster rate than III. Also, there appears no significant difference in metabolism between male and female rats dosed III, or between rats dosed IV.

Analysis of some 25 tissues and organs indicated that the carbon-14 in the rats was almost uniformly distributed in the animals, with the largest residues (only several parts per million) found in the digestive system and the excretory organs.

(2) *Fish Residue Study.* Carbon-14 residues for the continuous exposure portion of the fish residue study increased continuously with increasing time and leveled off within 1–3 weeks. Among exposure levels a definite relationship was noted between the concentration of test material and the corresponding residues. Within a given exposure level residues found in the viscera were, on the average, 6–8 times larger than in the carcass (tissue), with residues in viscera showing much greater fish-to-fish variability than residues in carcass. At a given exposure level residues from IV were much less than those from III.

Residues in fish exposed to formulation I [^{14}C-labeled III and unlabeled IV] concentrations of 0.02 and 0.12 ppm appeared to reach a steady-state (intake = output) level after 1 week of exposure (Figures 3 and 4). Such a plateau in residues occurred after 2–3 weeks for fish exposed to formulation II [unlabeled III and ^{14}C-labeled IV] at a nominal concentration of 0.12 ppm. Residues in fish exposed to formulation I at a nominal concentration of 0.80 ppm appeared to reach a steady-state level after a week of exposure, but 2–3 weeks later residues started to decrease in magnitude and continued to do so for the duration of the experiment. (Suspecting degradation of III led us to investigate the initial stock solution of III used in these studies. We subsequently verified that a 30% acetone–70% water solution was used and indeed did cause some noticeable degradation during the latter part of the fish study.) The steady-state values found in the carcass and viscera of fish are given in Table IX. These values were obtained by means of a trend-inversion test (Bendat and Piersol, 1966).

After several weeks of continuous exposure to formulation I, 0.80 ppm, only 30% of the ^{14}C residues found in the carcass was actually present in the inner (edible) tissue. The corresponding value for the formulation II, 0.12 ppm, exposure level was 49%.

Measured exposure concentrations were 0.012, 0.065, and 0.66 ppm for formulation I and 0.10 ppm for formulation II. Day-to-day fluctuation in concentrations at each exposure level was minimal throughout the study. The fact that the measured concentrations were less than the nominal values might be explained in part by adsorption on excreta and various parts of the flow-through system, especially at low concentration levels. The bioconcentration factors (ratio of ^{14}C residue in fish to ^{14}C in water) for the mixture of III and IV in bluegills, based on the above values derived from radioassay, are 50 for carcass and 310 for viscera for fish exposed to ^{14}C-labeled III plus unlabeled IV and 6 for carcass and 48 for viscera for fish exposed to unlabeled III plus ^{14}C-labeled IV, indicating a faster rate of elimination or metabolism for IV than III.

When put in a biocide-free environment, the residue in fish decreased with increasing time of withdrawal. The time for 50% of the carbon-14 residues to be eliminated from the fish was found in all cases to be less than 1 week (Figures 3 and 4). After 1 or 47 days of exposure, less than 4% of the ^{14}C-containing materials from fish carcass at the formulation I, 0.80-ppm, exposure level was extracted with hexane; 50–60% was extracted with methanol. Unextractable ^{14}C materials were 30–40% of the total ^{14}C content. Two possible explanations for the unextractables are as follows. First, III has been metabolized to such an extent that some of the carbon from this molecule enters the general metabolic carbon pool of the fish and is converted to naturally occurring substances and is permanently bound until removed by normal cell turnover. Second, some type of conjugation with a natural product has occurred.

SUMMARY

From the studies reported herein it is apparent that the microbicidal 3-isothiazolones III and IV are readily degraded in aqueous systems by hydrolytic and biological mechanisms at levels which might appear in the environment, 0.01–1 ppm. Compound IV, being less biologically active than III, is biologically degraded in river water or a simulated sewage treatment plant faster than III. Both IV and III are degraded by uv radiation and show little adsorption by river silt but are readily absorbed and metabolized by aquatic ferns. If these biocides find their way into the soil environment, there should be no significant adverse effect since it has been demonstrated that they are readily degraded to simpler products at levels near 1 ppm. Tissue storage of the biocides is minimal in rats and fish. Overall, these studies demonstrate that under recommended use levels (several parts per million), dissipation in the environment of both compounds III and IV will occur rapidly. Thus, one must conclude that the use of these 3-isothiazolones will not produce an undue ecological disturbance to the environment.

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

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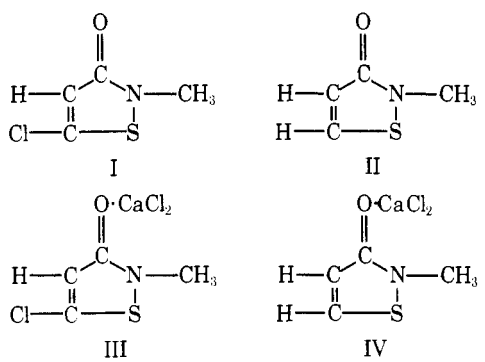
The major degradative pathway in the environment has been defined for two similar microbicidal 3-isothiazolones, 5-chloro-2-methyl-4-isothiazolin-3-one calcium chloride and 2-methyl-4-isothiazolin-3-one calcium chloride. In eight systems, covering chemical, biochemical, and photochemical aspects of environmental 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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