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Aqueous Photolysis of Niclosamide

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The photodegradation of [^{14}C]niclosamide was studied in sterile, pH 5, 7, and 9 buffered aqueous solutions under artificial sunlight at 25.0 ± 1.0 °C. Photolysis in pH 5 buffer is 4.3 times faster than in pH 9 buffer and 1.5 times faster than in pH 7 buffer. In the dark controls, niclosamide degraded only in the pH 5 buffer. After 360 h of continuous irradiation in pH 9 buffer, the chromatographic pattern of the degradates was the same regardless of which ring contained the radiolabel. An HPLC method was developed that confirmed these degradates to be carbon dioxide and two- and four-carbon aliphatic acids formed by cleavage of both aromatic rings. Carbon dioxide was the major degradate, comprising ∼40% of the initial radioactivity in the 360 h samples from both labels. The other degradates formed were oxalic acid, maleic acid, glyoxylic acid, and glyoxal. In addition, in the chloronitroaniline-labeled irradiated test solution, 2-chloro-4-nitroaniline was observed and identified after 48 h of irradiation but was not detected thereafter. No other aromatic compounds were isolated or observed in either labeled test system.

KEYWORDS: Niclosamide; lampricide; photolysis; half-life; carbon dioxide; aliphatic acids; pH

INTRODUCTION

The approval of the marketing and use of pesticides is wholly dependent upon their environmental fate and metabolism. Not only must the efficacy of the compound be proven, but the kinetics of its degradation mechanism must be characterized in order to ensure public safety and environmental protection. Aqueous photolysis is a significant process by which pesticides are degraded in the environment (*1*). The added mode of degradation by photolysis will lead to faster dissipation than by hydrolysis alone, and so understanding these kinetics becomes important in identifying degradates that may further affect the environment. Aromatic pesticides generally degrade by substitution, removal, or cleavage of functional groups attached to the benzene rings. Complete breakdown of the aromatic ring into carbon dioxide and organic acids as major products has been known to occur (*2*−*15*). The identification by HPLC of aliphatic acids and carbon dioxide formed from a two-ringed pesticide is presented here.

Niclosamide, 5-chloro-*N*-(2-chloro-4-nitrophenyl)-2-hydroxybenzamide, is the active ingredient of Bayluscide, a chemical used to control sea lamprey (*Petromyzon marinus*) in the Great Lakes region of the United States (*16*), where they have fed on the native fish populations (*17*). Adult sea lampreys are ∼15 cm long parasites that attach themselves onto other fish and drain blood and other body fluids from the host. The Welland Canal around Niagara Falls exposed the Great Lakes to the Atlantic Ocean when it was opened in 1829, and sea lamprey were first reported in Lake Erie in 1921. In succeeding years, sea lampreys migrated to the western Great Lakes and reached Lake Superior in 1946. From 1930 to 1952 lake trout production in Lake Superior was consistent at 1.8 million kg, but in the following decade production decreased by 90%, while the number of sea lampreys caught increased from 1000 to 70000 (*18*). To combat this infestation, salicylanilide compounds are often used as molluscicides, and specifically niclosamide shows the most beneficial characteristics (*19*). Niclosamide is rapidly eliminated from fish beginning 12−18 h after exposure as the compound is transformed to niclosamide glucuronide (*20*). Its efficacy appears to be similar to that of 3-(trifluoromethyl)-4-nitrophenol (TFM), the primary chemical used for sea lamprey control in the Great Lakes. Lampreys have a lower glucuronyltransferase activity than fish, so more of the compound is accumulated in lampreys (*20*, *21*).

The purpose of this study was to determine the rate constants and half-lives of niclosamide undergoing photolysis in buffered solutions under a xenon lamp and to identify and quantify niclosamide and its degradates.

MATERIALS AND METHODS

Test Substances and Reference Substances. [^{14}C]Niclosamide test substances were synthesized by DuPont/New England Nuclear. Two separate ^{14}C-labeled aromatic ring test substances were used (**Figure 1**). The radiochemical purity of the test substances was verified by HPLC (99.5% for label A, 97.2% for label B) prior to use in the study. The solubility of niclosamide in water is reported as 5−8 μg/mL (*16*). In the current experiments, niclosamide solubility was found to decrease

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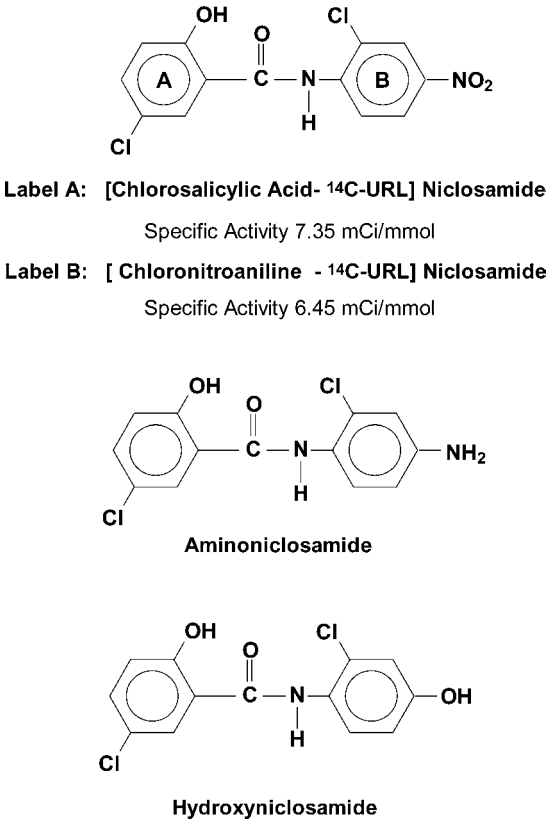

Figure 1. Structures of test and reference substances.

with decreasing pH. At pH 9 the experiment was performed at 2.5 μg/mL, at pH 7 at 0.5 μg/mL, and at pH 5 at 0.05 μg/mL.

Nonlabeled reference standards of aminoniclosamide (2′,5-dichloro-4′-aminosalicylanilide) and hydroxyniclosamide (2′,5-dichloro-4′-hydroxysalicylanilide) were synthesized by Derse and Schroeder Associates Ltd. (Madison, WI). Radioactive sodium carbonate was obtained from Sigma Radiochemicals (St. Louis, MO). Additional reference substances were obtained from Aldrich Chemical Co. (St. Louis, MO) (**Table 1**). Each reference substance was accompanied with a certificate of analysis confirming its identity and purity. Stock solutions were prepared in acetonitrile or methanol and stored refrigerated or at room temperature. Spiking solutions were prepared in methanol and stored at <-20 °C. Under these preparation and storage conditions, the reference substance stock solutions were found to be stable throughout the duration of the studies.

Reagents, Solvents, and Glassware. All chemical reagents and solvents were acquired from VWR Scientific and met or exceeded ACS standards. An oven was used to sterilize the glassware at 180 °C for 4 h. The buffer solutions were prepared at a final concentration of 0.05 or 0.0125 M according to American Society for Testing and Materials specifications (*23*). For pH 5.00, 250 mL of 0.1 M potassium hydrogen phthalate was mixed with 113 mL of 0.1 M NaOH diluted to 0.5 L. For pH 7.00, 500 mL of 0.1 M potassium dihydrogen phosphate and 297 mL of 0.1 M NaOH were diluted to 1 L. The pH 9.00 buffer was prepared by diluting 250 mL of 0.025 M sodium tetraborate and 23 mL of 0.1 M HCl to 0.5 L. The buffer solutions were sterilized by filtration through 0.20 μm membrane filters (Nalge Nunc International, Rochester, NY).

Liquid Scintillation Counting. Liquid scintillation analyses were conducted using a TriCarb model 1900TR liquid scintillation analyzer (Packard Instrument Co., Meriden, CT). Aliquots (100 or 200 μL) of the pH 7 and 9 buffer were analyzed using 4 mL of Ultima Gold scintillant (Packard). In the pH 5 study, 1 mL aliquots were assayed. Trapping solutions were also analyzed in 1 mL aliquots. For select samples, fractions from the output of the HPLC were also collected and analyzed by LSC. The samples were counted for at least 2 min. All counts were automatically corrected for instrument background (~25 cpm) and efficiency (>96%). LSC results were reported in disintegrations per minute (dpm), and total activity and mass amounts are reported as a percentage of the total initially applied radioactivity. Percent relative standard deviations averaged 7.55 for pH 5, 3.88 for pH 7, and 3.59 for pH 9.

Characterization of Volatile Radioactivity. Trapping solutions containing significant levels of radioactivity (≥10%) were further characterized by treatment with a saturated barium chloride solution. Any $^{14}CO_2$ present in the traps was precipitated as $Ba^{14}CO_3$. Loss of radioactivity in the supernatant was determined by LSC analysis, which yielded the fraction of trapped radioactivity due to $^{14}CO_2$.

HPLC Analysis. An HPLC system (Waters, Milford, MA), configured with two model 501 pumps, a model 715 WISP, and Maxima 820 data acquisition software, was used for the primary method of analysis. An Ace 5 C18 4.6 × 250 mm column (Mac-Mod Analytical, Chadds Ford, PA) with a mobile phase of 20 mM potassium phosphate monobasic, pH ~3.4 (A) and acetonitrile (B) achieved the separation beginning with 10% B from 0 to 3 min, a gradient to 70% B at 14 min and 80% B at 15 min, and maintaining 80% B until 35 min. The flow rate was 1.0 mL/min (method 1). Detection was accomplished with a Waters 484 tunable UV detector at 220 or 254 nm and a Packard FLO-ONE/*βeta* radioflow detector. The scintillant was FLO-SCINT II (Packard) at 3.0 mL/min. Those radiolabeled peaks that cochromatographed with reference substances were considered to be tentatively identified and selected for further analysis by TLC. The half-lives ($t_{1/2}$) of [^{14}C]niclosamide in the test systems were determined by performing linear regression analysis on the natural logarithm of the average ratio of test substance concentration (C_t) to the time 0 concentration (C_0) observed at each sampling interval t:

$$\ln(C_t/C_0) = kt \qquad t_{1/2} = \ln(0.5)/k$$

HPLC method 2 was developed to identify polar unknown degradation products and carbon dioxide, utilizing a 7.8 × 300 mm Aminex HPX-87H organic acid ion exclusion column (Bio-Rad, Richmond, CA) and 0.008 or 0.02 N sulfuric acid running at 0.8 mL/min. A GPM-I gradient pump module and variable-wavelength detector (Dionex, Sunnyvale, CA) was used in conjunction with a Packard series A-100 radioflow detector for analysis. Radioactive sodium carbonate was used as the reference standard for carbon dioxide in pH 9 buffer.

To more accurately quantify the degradate peaks, selected samples were also quantified by HPLC fractionation and LSC analysis. Fractionation was also used to isolate and collect unknowns for identification. Throughout the study, the HPLC column recoveries were ≥90%.

TLC Analysis. TLC was utilized as a secondary analytical method for identification and confirmational work. Aliquots of test samples were spotted along with reference substances on silica gel 60F (EM Science, Gibbstown, NJ) plates and developed in dichloromethane/ethyl acetate/acetic acid (80:20:2 v/v/v; solvent system 1), dichloromethane/methanol/10% NH_4OH (80:20:3 v/v/v; solvent system 2), or chloroform/methanol/water/acetic acid (13:7:1:2 v/v/v/v; solvent system 3).

Following TLC development, the plates were dried and the areas where the reference substances had migrated were visualized with a hand-held UV lamp at 254 nm or by staining with iodine vapor. The plate was then scanned for radioactivity using a radioanalytical imaging system (AMBIS, San Diego, CA). Those radiolabeled peaks that cochromatographed with reference substances by both HPLC and TLC were considered to be confirmed.

Aqueous Photolysis Rate Determination. Test solutions for irradiation were incubated in 300 mL airtight, sterile, silanized quartz glass photolysis vessels. The vessels contained a magnetic stir bar and a glass coil through which cooling water was circulated from a water bath to maintain the test solutions at 25 ± 1 °C during irradiation. A port sealed with a septum was used for sample removal by way of syringe. The headspace was purged with sterile air for volatile degradate collection. The trapping system consisted of a 100 mL Pyrex bottle containing 50 mL of 0.1 M sodium hydroxide connected to the test vessel with Teflon tubing. The dark control test solutions were incubated in sterile, silanized 500 mL Pyrex bottles with caps containing tubing for headspace purging prior to sampling. The dark control test vessels were wrapped in aluminum foil and incubated in the dark at 25.0 ±

Table 1. Reference Standards

niclosamide **1**[a]	(26.0)[b]	aminoniclosamide	(24.0)	hydroxyniclosamide	(24.2)
5-chlorosalicylic acid	(18.7)	2-chloro-4-nitroaniline	(23.0)	maleic acid	
salicylic acid	(15.1)	2-chloro-4-nitrophenol	(23.3)	oxalic acid	
2,3-dihydroxybenzoic acid	(4.1)	4-amino-3-chlorophenol	(9.8)	glyoxylic acid monohydrate	
2,4-dihydroxybenzoic acid	(2.6)	4-nitrocatechol	(19.3)	glyoxal	
2,5-dihydroxybenzoic acid	(4.7)	2-amino-5-nitrophenol	(19.6)	sodium carbonate-^{14}C	
3-hydroxybenzoic acid	(2.5)	4-aminoresorcinol	(4.0)	DL-tartaric acid	
3-chlorobenzoic acid	(3.2)	1,2,4-benzenetriol	(4.2)	glyoxal trimeric dihydrate	
chlorohydroquinone	(5.3)	4-chlorophenol	(21.0)	diglycolic acid	
hydroquinone	(3.1)	2-aminophenol	(3.2)	diglycolic anhydride	
2,4-dihydroxybenzoic acid methyl ester		4-aminophenol	(3.0)	fumaric acid	
2,5-dihydroxybenzoic acid methyl ester		2-chloroaniline	(22.8)	succinic acid	
2,6-dihydroxybenzoic acid methyl ester		4-nitroaniline	(20.7)		

[a] Numbers in boldface type refer to **Figure 8**. [b] Numbers in parentheses represent the retention times in minutes by HPLC method 1.

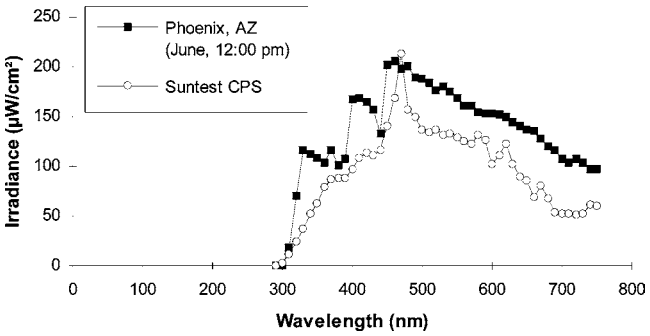

Figure 2. Comparison of xenon lamp to natural sunlight in Arizona at 12:00 p.m. in June. λ_{max} = 460–480 nm. Data courtesy of Atlas Weathering Services Group (DSET Laboratories, Phoenix, AZ).

Table 2. Application Rates and Degradation Rate Constants

	μg/mL		rate constant (h^{-1})	
test conditions	label A	label B	label A	label B
		pH 5		
irradiated		0.049		10.2×10^{-2}
dark control		0.049		5.8×10^{-3}
		pH 7		
irradiated		0.45		6.5×10^{-2}
dark control		0.46		nd[a]
		pH 9		
primary irradiated	2.45	2.43	2.4×10^{-2} [b]	2.3×10^{-2} [b]
secondary irradiated	2.37	2.34		
dark control	2.52	2.51	nd	nd

[a] Not detected. [b] Combined primary and secondary experiments.

1.0 °C. Aseptic techniques were used during the study to maintain sterility. Sterility was verified by microbial assay in which the 360 h sample was plated on standard methods and anaerobic blood agars. No microbial growth was observed on the agars after 72 h of incubation.

An Heraeus Suntest CPS photounit (DSET Laboratories, Inc., Phoenix, AZ) with a xenon lamp (Atlas, Linsengericht, Germany) was used as the artificial sunlight source. A comparison of xenon lamp emission to that of natural sunlight in Arizona is presented in **Figure 2**. The vessel containing the test solution was irradiated continuously directly from above. The elapsed time of irradiation was monitored using the Suntest chronometer. Lamp intensity was measured before initiation and after completion of the exposure phases using an International Light, Inc. (Newburyport, MA) radiometer and photodetector assembly with 280, 365, and 440 nm high-pass filters. The lamp intensity did not change in intensity during the course of the study.

Spiking solutions of the two test substances were prepared in methanol. Dilutions of the spiking solutions were made in methanol, and triplicate aliquots were assayed to determine concentrations on the basis of the test substance specific activity. Aliquots of the sterile buffer solutions (250 mL) were dosed and aseptically transferred to the sterile test vessels. The methanol cosolvent from spiking represented ≤1% of the test solution volume. Following spiking, each test system was mixed thoroughly, and duplicate time 0 samples were taken. The exact application rate was based upon the concentration of the time 0 samples. Due to the solubility of the test substance, the application rates were ∼0.05, 0.45, and 2.5 μg/mL for the pH 5, 7, and 9 test solutions, respectively.

In the pH 9 study, four primary test solutions were initially dosed at ∼2.5 μg/mL with test substance (14 μCi of label A, irradiated and dark control; and 12 μCi of label B, irradiated and dark control). For these test systems, the headspace inlet and outlet port tubings of the test vessels were clamped during incubation. To more accurately define the photolysis kinetics at pH 9, two additional secondary test solutions were dosed at ∼2.5 μg/mL for irradiation (labels A and B). No volatiles were collected from these test systems. The primary test solutions at pH 9 were sampled at 0, 24, 48, 116, 168, and 360 h. A secondary test system was initiated for sampling at 0, 2, 4, 6, 24, and 30 h. Both data sets were combined to determine the rate of degradation. Because of differences in solubility and the rate of photolysis of the test substance, different application rates (presented in **Table 2**) and sampling schedules were used for each pH.

In the pH 7 study, two test solutions were dosed at ∼0.45 μg/mL with test substance (3.0 μCi of label B, irradiated and dark control). Similarly, in the pH 5 study, two test solutions were dosed at ∼0.05 μg/mL with test substance (0.6 μCi of label B, irradiated and dark control). For the irradiated test solutions, the headspace was purged continuously with air through the inlet and outlet port tubings into a 50 mL sodium hydroxide trapping solution. Two trapping solutions were used at the final 48 h sampling for the pH 7 study, and two trapping solutions were used at each sampling for the pH 5 test solution. The dark control test solutions were incubated in sterile, silanized bottles. Volatiles were not collected from the dark controls. Sampling occurred in the pH 7 study at 0, 2, 4, 6, 8, 10, 24, and 48 h and in the pH 5 study at 0, 1, 2, 4, 6, and 24 h.

Prior to sampling of the pH 9 test solutions, the headspace of each test system was purged into the trapping solution with humidified sterile air at a flow rate of ∼10 mL/min for 20−45 min. Because continuous purging was used at pH 5 and 7, samples were simply taken at the appropriate time. Duplicate samples (5 mL from the pH 7 and 9 test solutions, 15 mL from the pH 5 test solutions) were removed aseptically via sterile tubing in the sample access port, and the trapping solutions were changed.

The pH 9 and 7 samples were analyzed directly. Because the pH 5 portion of the study was conducted at a low application rate, the test solution was extracted three times with dichloromethane. The extracts were pooled and concentrated by nitrogen evaporation to ∼1 mL. Two milliliters of methanol was added, and the sample was reconcentrated to 0.5 mL. Concentration efficiencies averaged 99%. An aliquot of each concentrated extract was analyzed by HPLC.

Table 3. Photolytic Decline of Percent Niclosamide Concentrations from pH 9 Buffered Solutions

sample interval (h)	label	irradiated %[a]	dark control %
0	A	100	100
	B	100	100
2	A	88	ns[b]
	B	97	
4	A	74	ns
	B	97	
6	A	70	ns
	B	95	
24	A	46	100
	B	62	99
30	A	18	ns
	B	57	
48	A	43	100
	B	33	100
116	A	0.0	100
	B	0.0	100
168	A	0.0	100
	B	0.0	100
360	A	0.0	100
	B	0.0	100

[a] Percent of time 0 radioactivity. [b] Not sampled.

RESULTS AND DISCUSSION

Aqueous Photolysis at pH 9. In all of the pH 9 test systems, essentially all of the applied radioactivity remained in the test solutions. Only a small amount of volatile radioactivity (0.18% in label A, 0.02% in label B) accumulated in the sodium hydroxide traps after 360 h of irradiation. The mass balance in the pH 9 irradiated and dark control test solutions averaged 93.5 $\pm$ 6.4% (n = number of observations = 22) and 100.7 $\pm$ 0.9% (n = 12), respectively.

The decline of [^{14}C]niclosamide activity from the pH 9 irradiated solutions compared to the dark control test solution is presented in **Table 3**. The data from the primary and secondary irradiated test solutions are combined. [^{14}C]Niclosamide represented 100% of the initial radioactivity at time 0 and declined to 43% (label A) and 33% (label B) after 48 h of irradiation. No [^{14}C]niclosamide was detected in the later sampling intervals. There was no indication of degradation of [^{14}C]niclosamide in the pH 9 dark controls after 15 days of incubation. Regression analysis of niclosamide concentration resulted in irradiated half-lives of 29 h (k = 0.024 h^{-1}) for label A and 31 h (k = 0.023 h^{-1}) for label B [^{14}C]niclosamide. The results are presented in **Figure 3**. A half-life for [^{14}C]-niclosamide was not determined in the dark control test solutions because no degradation was observed.

In the label B irradiated test solution at pH 9, a degradate was formed but did not persist. This degradate (eluting at 23 min by HPLC) reached ~16% of initial radioactivity after 48 h of irradiation, but was not detected thereafter. This degradate was identified and confirmed by HPLC and TLC cochromatography as 2-chloro-4-nitroaniline, and it was the only aromatic degradate observed in the study. The formation of this degradate in the irradiated label B test solutions and not the irradiated label A test solutions is consistent with the site of the ^{14}C-label on the test substances.

In the label A photolysis, two major HPLC degradate peaks were formed by 24 h of irradiation and persisted throughout the study, representing 40% (unknown 1, 2.7 min) and 54% (unknown 2, 3.7 min) of the initial radioactivity after 360 h. Label B [^{14}C]niclosamide showed the same two degradate peaks at 46% (2.7 min) and 47% (3.7 min). These unknowns were the only peaks observed after 116 h of irradiation. A variety of potential degradate reference substances (**Table 1**), derivatives of chlorosalicylic acid and 2-chloro-4-nitroaniline, were mixed with the degradate peaks and analyzed by HPLC and TLC. None of the reference standards coeluted in both analytical methods with the degradates. The fact that unknowns 1 and 2 did not match any of the possible aromatic compounds presented us with the possibility that the unknowns are formed by the degradation of the aromatic ring. This is the only explanation as to how both the chlorosalicylic acid label and the chloronitroaniline label can generate identical degradate patterns. This degradation of aromatic rings will yield two-, three-, and four-carbon aliphatic acids (*2*). To characterize the unknowns, the 360 h sample was analyzed using an organic acid column

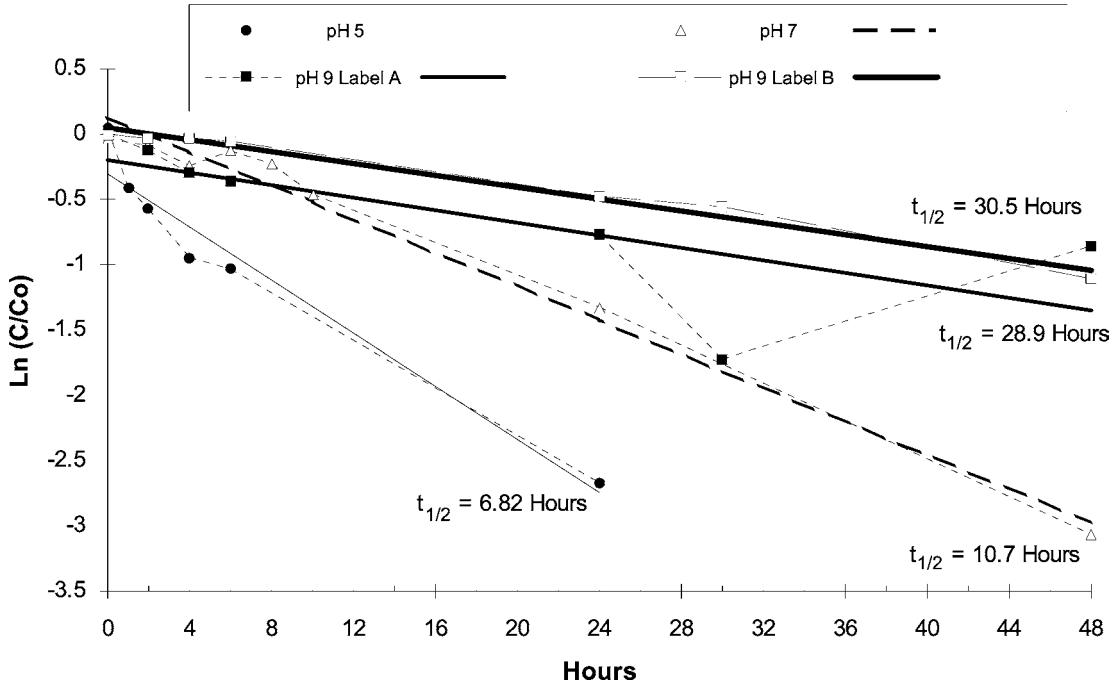

Figure 3. Photolytic decline of niclosamide concentrations in pH 5, 7, and 9 buffered solutions.

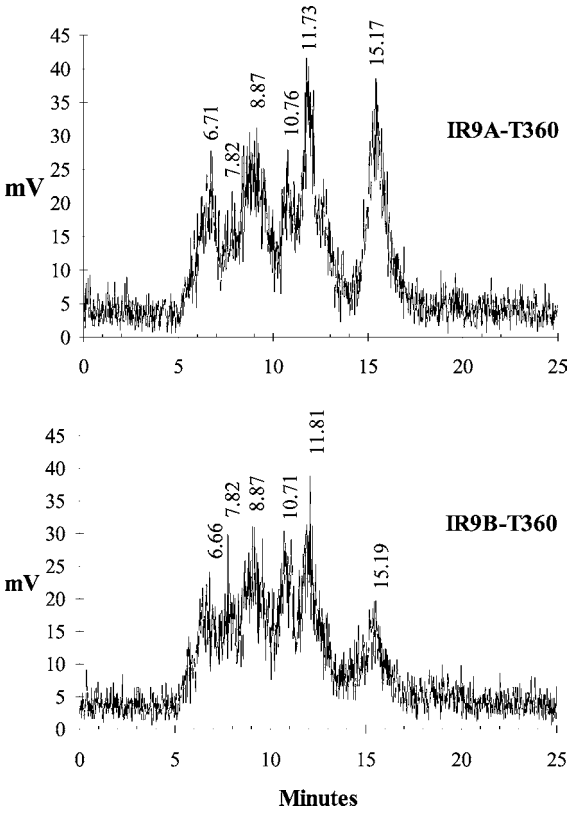

Figure 4. HPLC radiochromatogram of pH 9 irradiated 360 h test solutions on the Aminex organic acid column.

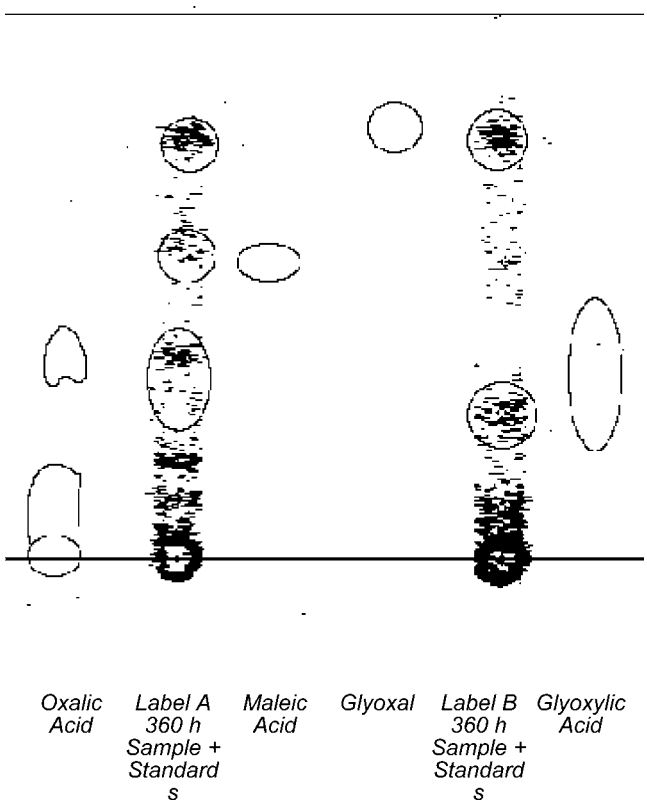

Figure 5. TLC cochromatography of aliphatic acids with pH 9 irradiated 360 h test solutions. The solvent system was chloroform/methanol/water/acetic acid 13:7:1:2 (v/v/v/v). Nonlabeled reference standards are indicated by the circles.

(HPLC method 2). The two unknowns observed in the 360 h photolysates using the primary HPLC method chromatographed into six radioactive peaks (**Figure 4**) using method 2. Hence, there was better resolution of the photolysate on the Aminex column than on the primary HPLC column. In addition, the chromatographs from both photolysates were again identical. The standards oxalic acid, maleic acid, glyoxylic acid, and glyoxal were chromatographed with both label A and label B test substance photolysates. These compounds were found to be present in the photolysates. Their identities were confirmed by TLC. A mixture of oxalic acid, maleic acid, glyoxylic acid, and glyoxal was spiked into aliquots of both label A and label B photolysates. The samples were applied to a silica gel TLC plate along with the individual standard solutions in pH 9 buffer and analyzed using TLC solvent system 3. Each standard matched radioactivity in both samples, confirming their identity (**Figure 5**).

During the course of the study, radioactivity was gradually lost from the pH 9 photolysates during storage at <-20 °C and subsequent thawing and refreezing. The initial concentration of radioactivity of the label A 360 h sample was 114,000 dpm/mL. After 14 months, assay of the label A photolysate was determined to be 75030 dpm/mL, a 34% loss of radioactivity from the initial 360 h assay. Label B showed a similar loss. The initial concentration of radioactivity was 98400 dpm/mL, and after 14 months, a 30% loss of radioactivity from its initial assay was observed. To determine the nature of the radioactive material lost, an aliquot of the label A 360 h photolysate was purged with nitrogen into a 40 mL solution of 1 N NaOH. The photolysate was titrated to pH 7 while trapping the volatiles, and the trapping solution was treated with a saturated solution of $BaCl_2$. Approximately 75% of the radioactivity was precipitated as $Ba^{14}CO_3$. The loss in radioactivity was due to the volatilization of $^{14}CO_2$. It is interesting to note that the loss is from a pH 9 buffer photolysate. Carbon dioxide is expected to be trapped as sodium carbonate and not easily lost in basic solution.

To determine the total amount of $^{14}CO_2$ in the photolysate, HPLC method 2 was used to analyze carbon dioxide as carbonate. The HPLC chromatogram of radioactive sodium carbonate reference standard is compared to the chromatogram of the label B photolysate in **Figure 6**. The elution time of the [^{14}C]carbonate standard matches with the peak at 15.71 min. This radioactivity due to carbon dioxide still in solution is in addition to the radioactive carbon dioxide lost from the sample during storage. Thus, the major portion of the activity in the photolysate is due to carbon dioxide.

To confirm this result, the Aminex HPLC method was modified by using 0.02 N sulfuric acid mobile phase. The $Na_2{}^{14}CO_3$ standard is compared to the photolysates from labels A and B in **Figure 7**. The analyses using 0.02 N H_2SO_4 revealed seven peaks in both the label A and label B photolysates, and 1 min fractions were collected from the column effluent for quantitation by LSC. Overall recovery from the Aminex column of both photolysates was >94%. The $Na_2{}^{14}CO_3$ standard peak at 16.08 min matches precisely the peak found at 16.08 min in both photolysates. This confirms the presence of CO_2 in the pH 9 photolysates. The significance of the loss of radioactivity as CO_2 from the stored sample is that the degradate pattern of the Aminex column HPLC analysis represents ~66% of the radioactivity initially present in the sample. Recovery of this radioactivity from the column was quantitative. The total amounts of $^{14}CO_3{}^{2-}$ in the pH 9 360 h samples were 44% for label A and 37% for label B. The distribution of radioactivity among the seven HPLC peaks (as well as their identities) including carbon dioxide is given in **Table 4**. A pathway for

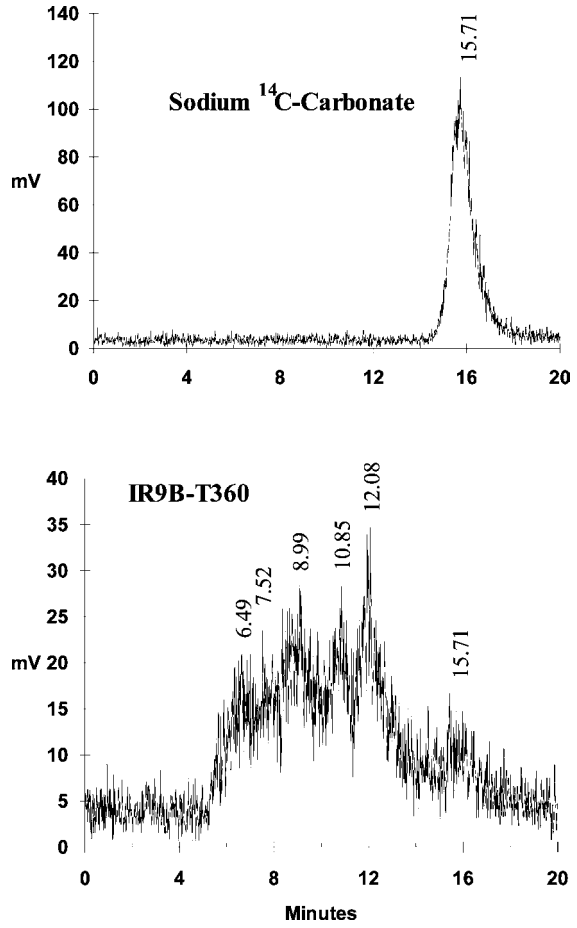

Figure 6. HPLC radiochromatograms of sodium [^{14}C]carbonate reference standard (top) with pH 9 label B 360 h photolysate (bottom) on the Aminex organic acid column using 0.008 N H_2SO_4 mobile phase.

the formation of aliphatic acids and carbon dioxide by photolysis of niclosamide is proposed in **Figure 8**.

Aqueous Photolysis at pH 7. The pH 7 portion of the study was conducted using only label B [^{14}C]niclosamide test substance at application rates of 0.45 and 0.46 μg/mL for the irradiated and dark control test solutions, respectively. Due to the low concentration, only the decline of niclosamide was followed.

The distribution of initial radioactivity and material balance from the pH 7 label B irradiated and dark control test solutions is presented in **Table 5**. In the irradiated pH 7 test system, radioactivity in solution declined from 100% at time 0 to 77% after 48 h. Concurrent with this decline was the formation of volatile radioactivity captured in the sodium hydroxide trapping solutions. After 48 h of irradiation, the volatile radioactivity represented ~21% of initial radioactivity. In the dark control test system at pH 7, all of the radioactivity remained in solution. The material balance in the pH 7 label B irradiated and dark control test solutions averaged 98.8 ± 3.7% (n = 8) and 102 ± 1.9% (n = 8), respectively.

The decline of label B [^{14}C]niclosamide concentration from the pH 7 irradiated solution compared to the dark control test solution is presented in **Table 6**. In the irradiated pH 7 test solutions, [^{14}C]niclosamide represented 97% of the initially applied radioactivity at time 0 and declined to 4.7% after 48 h of irradiation. Regression analysis of the values in **Table 6** resulted in a half-life of 10.7 h (k = 0.065 h^{-1}) for label B niclosamide in the irradiated pH 7 test solution. The results are

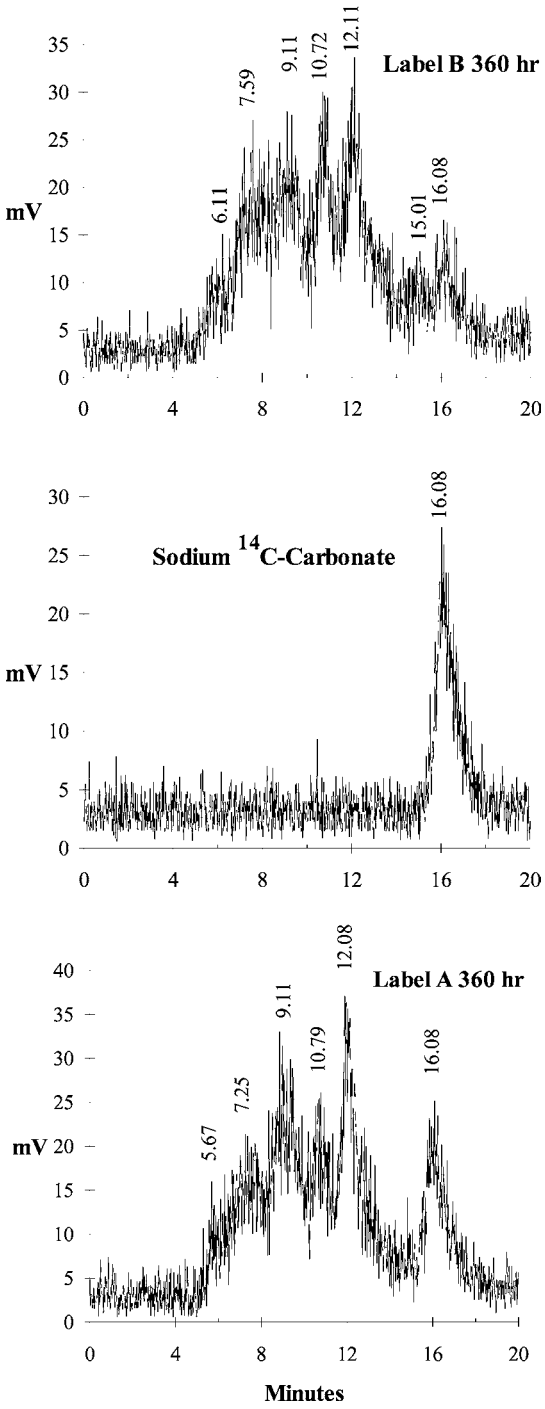

Figure 7. HPLC radiochromatogram of sodium [^{14}C]carbonate reference standard (center) with pH 9 360 h photolysates (label A, top; label B, bottom) on the Aminex organic acid column using 0.02 N H_2SO_4 mobile phase.

presented in **Figure 3**. In the dark control test solution, no degradation of [^{14}C]niclosamide was observed.

The volatile radioactivity, which represented ~21% of the initial radioactivity after 48 h of irradiation, was further characterized by barium chloride precipitation. About 94% of the radioactivity in the sodium hydroxide trapping solution was precipitated and thus identified as $^{14}CO_2$.

Aqueous Photolysis at pH 5. The pH 5 portion of the study was also conducted using only label B [^{14}C]niclosamide test substance, at an application rate of 0.049 μg/mL. As with the pH 7 study, the decline of only the niclosamide test substance was followed due to the low concentration.

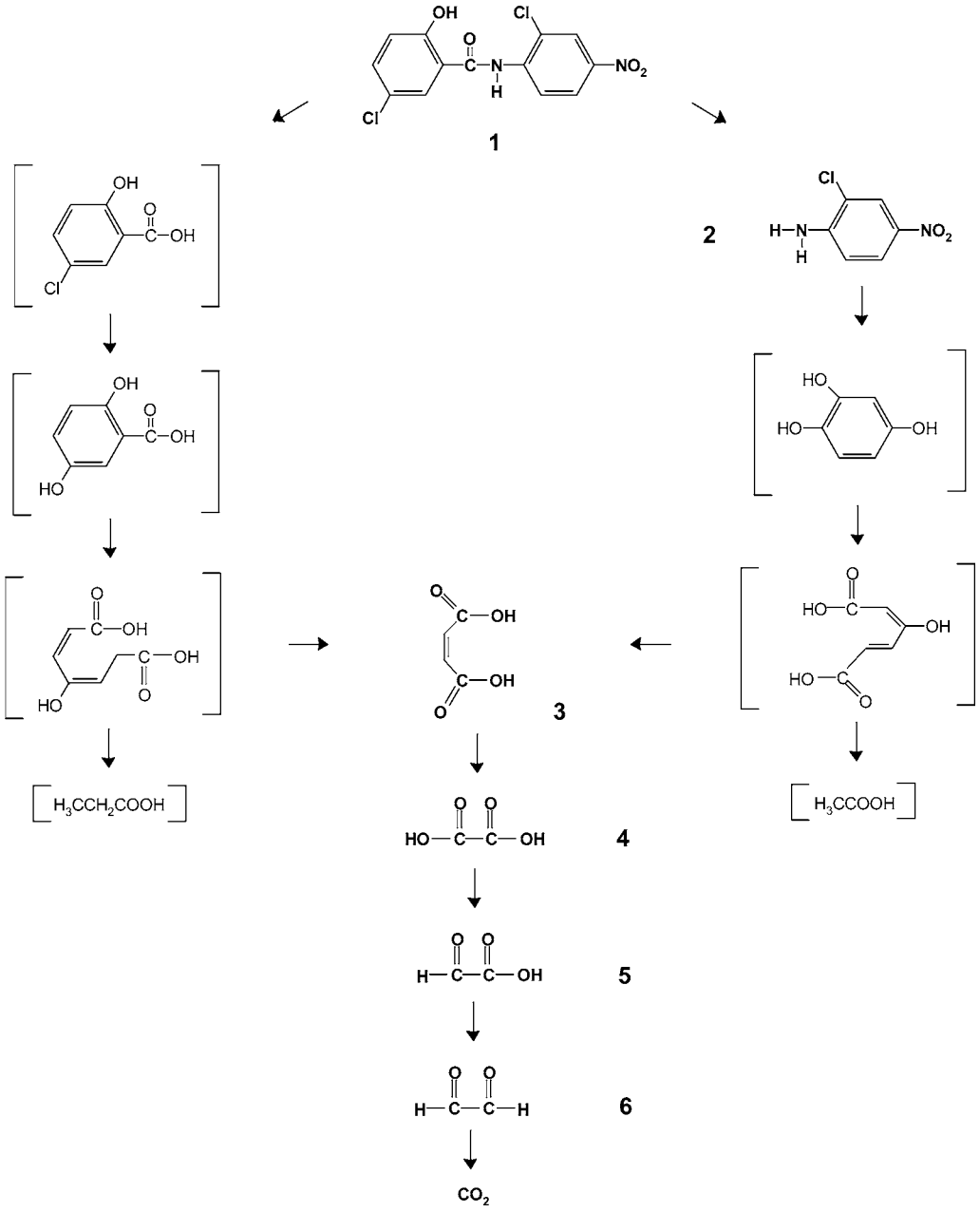

Figure 8. Proposed pathways for the formation of two-, three-, and four-carbon aliphatic acids and CO_2 from aqueous photolysis of [^{14}C-*chlorosalicylic acid* URL]-niclosamide and [^{14}C-*chloronitroaniline* URL]-niclosamide.

The distribution of initial radioactivity and material balance from the pH 5 label B irradiated and dark control test solutions are presented in **Table 5**. In the irradiated pH 5 test system, the radioactivity in solution declined from 100% at time 0 to 76% after 24 h. Concurrent with this was the formation of volatile radioactivity captured in the sodium hydroxide trapping solutions representing ~23% of initial radioactivity. In the dark control test system at pH 5, all of the radioactivity remained in solution. The material balance in the pH 5 label B irradiated and dark control test solutions averaged 89.7 ± 8.0% (n = 6) and 97.2 ± 2.0% (n = 6), respectively.

The decline of label B [^{14}C]niclosamide from the pH 5 irradiated and dark control test solutions is presented in **Table 6**. In the irradiated pH 5 test solutions, [^{14}C]niclosamide represented 104% of the initially applied radioactivity at time 0 and declined to 6.9% after 24 h of irradiation. A half-life of 6.82 h (k = 0.102 h^{-1}) was calculated for label B [^{14}C]-niclosamide in the irradiated pH 5 test solution. The results are presented in **Figure 3**. In the dark control test solution, label B [^{14}C]niclosamide declined slightly to ~87% after 24 h. This results in a half-life of 120 h with $k = 5.8 \times 10^{-3}$ h^{-1}. Taking the difference in rate constants between the pH 5 irradiated and dark control degradations results in a rate constant due to photolysis of 9.6×10^{-2} h^{-1}.

The volatile radioactivity was further characterized by barium chloride precipitation. About 76% of the radioactivity in the sodium hydroxide trapping solution was precipitable and thus identified as $^{14}CO_2$.

Conclusion. Niclosamide degrades under aqueous photolytic conditions, but no loss of niclosamide was observed in the pH 7 or 9 dark controls. In neutral and basic solutions, niclosamide degradation is a result of photolysis only and is not affected by hydrolysis. Only in pH 5 solution did niclosamide show a 13% decline in a single analysis after 24 h of dark incubation. The hydrolysis half-life in pH 5 was 120 h with a rate constant of 5.8×10^{-3} h^{-1}, and the photolytic half-life was 7.2 h (k = 9.6

Table 4. Identification and Distribution of Unknown Degradates in the 360 h Sample from the pH 9 Photolysis Study

component	identity	label	relative area %[a]	% of 360 h[b]	% of T0[c]	μg/mL
360 h sample		A		100	93	2.36
		B		100	93	2.04
peak 1		A	6.3	4.1	3.8	0.10
		B	8.6	6.1	5.7	0.12
peak 2	oxalic acid	A	17	11	10	0.26
		B	22	15	14	0.31
peak 3	glyoxylic acid, glyoxal	A	4.5	3.0	2.8	0.07
		B	4.7	3.3	3.1	0.07
peak 4	maleic acid	A	15	9.6	9.0	0.23
		B	15	11	9.7	0.21
peak 5		A	13	8.2	7.7	0.19
		B	13	9.4	8.7	0.19
peak 6		A	25	17	15	0.39
		B	23	16	15	0.32
peak 7	CO_2	A	20	13	12	0.31
		B	14	9.8	9	0.20
volatiles	CO_2	A		34	32[d]	0.81
		B		30	28[d]	0.61
total		A	100	100	93	2.36
		B	100	100	93	2.04

[a] Relative HPLC area percent of 360 h sample after storage. [b] Percent of original 360 h sample assay proportioned to include volatilized CO_2. [c] Percent of initial radioactivity (time 0 sample) applied to the test system. [d] Trapped in solution.

Table 5. Distribution of Radioactivity in the pH 7 and 5 Irradiated Test Systems as Percent of Applied Radioactivity

	pH 7 photolysis			pH 5 photolysis		
sample interval (h)	test solution	volatiles[a]	total	test solution	volatiles	total
0	100	N/A[b]	100	100	N/A[b]	100
1		N/A		88.7	0.5	89.2
2	101	0.04	101	83.8	1.0	84.8
4	98.1	0.17	98.3	80.5	2.5	83.0
6	102	0.45	102	77.3	4.8	82.1
8	99.4	0.98	100		N/A	
10	98.3	1.5	99.8		N/A	
24	81.6	8.8	90.4	76.1	22.9	99.0
48	76.9	21.3	98.2		N/A	

[a] Volatiles accumulated throughout the study. [b] Not applicable.

Table 6. Photolytic Decline of Percent Niclosamide Concentrations[a] from pH 7 and 5 Buffered Solutions

sample interval (h)	pH 7		pH 5	
	irradiated	dark control	irradiated	dark control
0	96.5	100	104	100
1			66.5	n/d[b]
2	91.5	105	56.2	n/d
4	78.5	101	38.9	n/d
6	87.9	105	35.5	n/d
8	79.7	102		
10	63.1	102		
24	26.5	101	6.9	87.0
48	4.7	97.0		

[a] Percent of initial radioactivity (time 0 sample) applied to the test system. [b] Not determined.

$\times 10^{-3}$ h^{-1}). Our pH 7 and 9 observations are consistent with the hydrolysis findings of Schultz and Harman (*22*). These authors did not detect any niclosamide hydrolysis in pH 5 buffered solution after 56 days. Niclosamide is more soluble and stable in basic solution, as evidenced by more rapid photolysis in pH 5 and 7 buffer. In pH 9 solution, the end result is complete degradation of the benzene rings and formation of two-, three-, and four-carbon aliphatic acids (**Figure 8**). The only aromatic compound isolated in the study was 2-chloro-4-nitroaniline, which was observed in the label B 24 and 48 h photolysis samples but did not persist thereafter. 2-Chloro-4-nitroaniline is not simply a hydrolysis product because it was not formed in the dark controls. The formation of radiolabeled 2-chloro-4-nitroaniline in the label B test solution does indicate that the amide bond between the chlorosalicylic acid and nitroaniline moieties of niclosamide is susceptible to aqueous photolysis. The corresponding compound from label A, 5-chlorosalicylic acid, was not observed, likely due to its rapid transformation in basic solution. 5-Chlorosalicylic acid has been reported in the photolysis of niclosamide on silica gel plates but not identified from photolysis in solution (*22*). Muir and Yarechewski (*24*) observed 5-chlorosalicylic acid in sediment samples only after 40 h of water reflux. Their conclusion was that its presence in this extract may be due to hydrolysis of aminoniclosamide in hot water.

In summary, the first step in the photolysis of niclosamide is the cleavage of the amide bond to yield 5-chlorosalicylic acid and 2-chloro-4-nitroaniline (**Figure 8**). 5-Chlorosalicylic acid and 2-chloro-4-nitroaniline then further break down to yield maleic acid, oxalic acid, glyoxylic acid, and glyoxal. Hence, if there are functional groups on the aromatic ring, they will yield substituted maleic acid and other degradates. Therefore, there is a likelihood of the formation of nitromaleic acid, aminomaleic acid, and chloromaleic acid. Similar polar material was observed in both the aerobic and anaerobic aquatic metabolism studies (*25*).

Two other conclusions result from the present study. Volatile carbon dioxide is capable of being released from pH 9 solutions even when stored at <-20 °C. Carbon dioxide remaining in pH 9 samples is able to be identified and quantitated by HPLC comparison to radiolabeled sodium carbonate.

ABBREVIATIONS USED

HPLC, high-performance liquid chromatography; LSC, liquid scintillation counting; TLC, thin-layer chromatography.

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