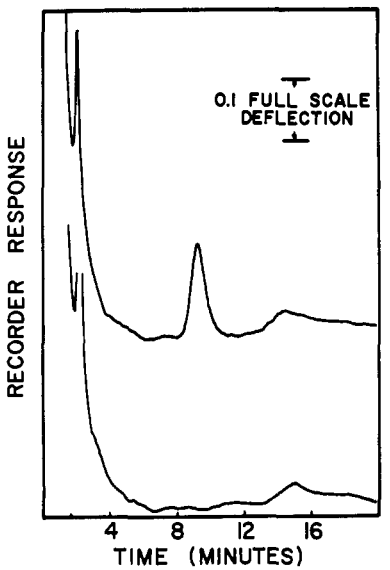

Figure 3. Gas chromatograms of control and 0.05 ppm recovery of Maretin from bovine fat tissue

again to ensure that the response falls within the linear portion of the response curve.

The fluorometric procedure was checked by adding known amounts of Maretin to the various tissues and milk at the blending step. Recoveries were run at the 0.1- and 0.05-ppm level for meat tissues and at the 0.005-ppm level for milk. Recoveries were generally in the 75–110% range. Representative values including controls are shown in Table I.

Naphthostyril can be analyzed directly by gas chromatography, but it does not contain any atom or functional groups which are specifically sensed by the various selective detectors available for use. Bromination converts the compound to a derivative to which the electron capture detector is quite sensitive. Mass spectrometry studies show that bromination adds two bromine atoms (mol wt 327), most probably in the para position on each of the two rings (Figure 1). Chromatography of this derivative on FFAP liquid phase yields a symmetrical peak with sensitivity down to the subnanogram range. The brominated derivative is quite stable and does not decompose even on standing for several weeks at room temperature in the presence of sample extracts.

Controls and fortified samples of each of the various tissues analyzed by the fluorometric procedure were further analyzed by the gas chromatographic confirmatory procedure. This procedure involved brominating the $^2/_3$ of the sample to which no increment was added and analyzing by electron capture gas chromatography. Resolution was obtained between the brominated naphthostyril peak and any peaks due to tissue extractives. Control values, recoveries, and sensitivity all compare favorably with the photofluorometric procedure, as shown in Table I. Representative control and recovery chromatograms are shown in Figure 3.

In order to check the specificity of the fluorometric method, an interference study was conducted. All of the 55 compounds registered for use on meat and milk as of May 1, 1970, were tested at or above their tolerances as listed in the Federal Register. Compounds with a zero tolerance were checked at 0.1- and 0.01-ppm for meat and milk, respectively. No interferences with the fluorometric method were noted above 0.05 ppm for meat or above 0.005 ppm for milk.

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Gas Chromatographic Method for Analysis of Chlorpyrifos and Endosulfan Insecticides in Topically Treated Houseflies

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Chlorpyrifos (Dursban) and endosulfan (Thiodan) can be determined in houseflies at the 10-pg level. Gas chromatographic methods and a high temperature Ni^{63} detector were used after *n*-hexane extraction without cleanup. Cuticle penetration was estimated along with rates of *in vivo* metabolism of these insecticides in the housefly. Rates of 50% disappearance of chlorpyrifos and endosulfan from the cuticle were 36 and 83 min, respectively. The 50% disappearance rates from the whole body were 67 and 176 min, respectively. Thoracic cholinesterase activity of flies surviving the LD_{50} dose treatment with chlorpyrifos declined to a plateau of 70% of the nontreated controls after 3 hr and remained unchanged for a 12-hr period. The enzyme activity of flies at "knock-down" averaged 36% regardless of knock-down time. Endosulfan did not inhibit thoracic cholinesterase.

Analysis of insecticides by gas chromatographic methods (glc) is generally limited to residue problems. There is a theoretical importance to the development of highly sensitive analytical methods for very low levels of insecticides in target insects. They are significant in the study of efficiency in the application of insecticides under field conditions. This is an area of increasing ecological importance. In the substantial absence of analytical methods for direct determination of dose–mortality relationships, the lethal dose is determined by application of measured amounts of insecticide (in microliter volumes) on individual insects. There is no known correlation between mortality from laboratory LD data and the methods of delivery which occur in typical field applications of insecticides.

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Efficiency in the application of insecticides is related to the optimum droplet size for sprays. It has been discussed by Himel (1969a,b), Lofgren (1970), and Mount (1970). Optimum droplet size research requires a method for measurement of the actual amount of insecticide delivered to target insects under field conditions. Analysis of dieldrin in mosquitoes has been reported by Pennell *et al.* (1964) using glc. They did not correlate their data with mortality. Weidhaas *et al.* (1970) applied malathion sprays to mosquitoes and analyzed the amount of the insecticide to obtain a limiting value for a 100% mortality (LD_{100}).

This paper is concerned with the development of gas chromatographic methods whereby as little as 10 picograms (pg) of chlorpyrifos and endosulfan could be detected in houseflies. As with many insecticides, chlorpyrifos and endosulfan are rapidly metabolized. For this reason, the study included the determination of rates of *in vivo* metabolism of the toxicants. In addition, the degree of cholinesterase inhibition by chlorpyrifos was correlated with time. The analytical methods reported herein have been used by Himel and Uk (1971) to determine the dose-related toxicity of chlorpyrifos and endosulfan when applied to houseflies by topical application of single microliter-size droplets, spray impingement of multiple droplets smaller than 50 μ diameter, and vapor. The structures of these chlorine-containing insecticides are given in Figure 1. They were used in this study as being representative of thionophosphate and cyclodiene-type insecticides.

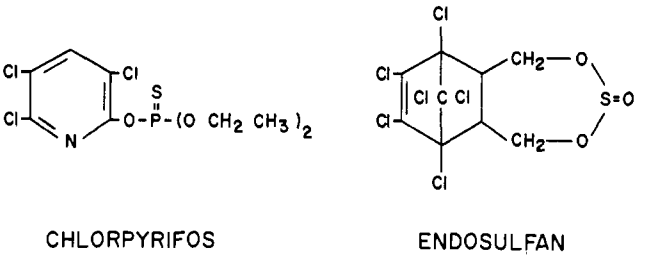

Figure 1. Structural formulas of the insecticides used

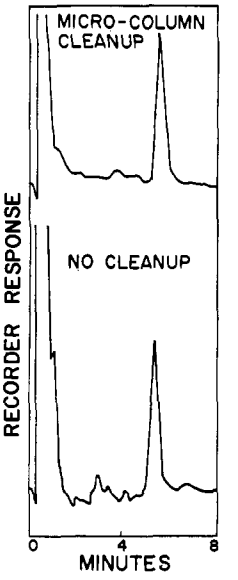

Figure 2. Gas chromatograms of chlorpyrifos extracts with silica gel microcolumn cleanup and without cleanup analyzed on the high temperature Ni^{63} ec detector

MATERIALS AND METHODS

Technical and analytical grades of chlorpyrifos were supplied by Dow Chemical Co., Midland, Mich., and those of endosulfan by Niagara Chemicals Co., Middleport, N.Y. Houseflies used were a wild strain collected in Athens, Ga., and bred in the laboratory on CSMA-medium (Ralston Purina Co., St. Louis, Mo.) at 21°C and 80% relative humidity. Topical LD_{50}'s on 4-day old female houseflies, previously estimated to be 40 ng/female for chlorpyrifos and 160 ng/female for endosulfan, were used as the basis for residue analysis as well as for disappearance rate studies.

Residue Analysis. APPARATUS. A MicroTek GC-2000 MF gas chromatograph equipped with a Ni^{63} electron capture detector (ECD) was used (Tracor Analytical Instruments, Inc., Austin, Tex.).

REAGENTS. All solvents were MCB pesticide grades and were used as received. Silica gel (Baker "Analyzed" no. 3405) and carbon–attaclay AOAC mixture (Kensington Scientific Corp., Oakland, Calif.) were used as received.

EXTRACTION. Twenty-five female houseflies (*ca.* 500 mg) were put in a ground glass tissue grinder containing 5 ml of acetone, along with a standard insecticide solution. The contents were homogenized for 2 min. The supernatant was filtered into a 50-ml Erlenmeyer flask. The debris was re-extracted with two additional grindings in 3-ml portions of acetone. The combined filtrates were kept in the refrigerator until cleanup by silica gel microcolumns.

For the analysis of extracts without cleanup, the flies were homogenized four times in 2-ml portions of *n*-hexane. The supernatant was filtered through glass wool over 1 g of anhydrous Na_2SO_4 into a graduated centrifuge tube. The combined filtrate was adjusted to 10 ml for direct glc analysis.

CLEANUP. Carbon–Attaclay Adsorption. After evaporation of the extract to about 1 ml, the insecticide residue was picked up in 5 ml of benzene. One gram of carbon–attaclay mixture was added to the benzene with continuous stirring for 5 min. The benzene was filtered and the carbon–attaclay washed with two additional 5-ml portions of benzene. The benzene fractions were combined and evaporated just to dryness. The residue was picked up in 5 ml of *n*-hexane for glc analysis.

Microcolumn Chromatography. The method of Kadoum (1967), as modified by Jennings (1970), was followed with minor rearrangements in apparatus setup. The support for microcolumns consisted of a $^3/_4 \times 6 \times 12$ in. board provided with a clamp attachment. Thirty $^3/_8$-in. holes 1 in. apart were drilled across the board in three rows. The disposable Pasteur pipettes used as microcolumns were supported across the board by O-rings made from small sections of a $^3/_8$-in. rubber tubing. One gram of silica gel was used in each column. The column was washed with 10 ml of *n*-hexane. The acetone extract of the insecticide which has been evaporated to 0.5 ml was diluted with 1 ml of benzene and put in the column. The holding flask was rinsed with an additional 1 ml of benzene which was then transferred to the column. When the benzene level reached that of the silica gel, the receiver was changed and elution was followed immediately. The eluting solvent consisted of a mixture of benzene–hexane [1/1, v/v, (Jennings, 1970)]. The elution was continued until 10 ml were collected in the graduated centrifuge tube. One to 5 μl of the eluate was injected into the glc without further concentration.

Gas Chromatography. The following glc conditions were used.

Column. U-shape glass, 6 ft $\times$ 4 mm i.d.

Table I. Recoveries of Chlorpyrifos and Endosulfan Insecticides from Housefly Grindings Fortified Just Prior to Extraction

	Insecticides			
	Chlorpyrifos[a]		Endosulfan[b]	
	No. rep	Recovery % ± SD	No. rep	Recovery % ± SD
Acetone extraction and silica gel cleanup	3	92 ± 4.9	5	81 ± 14
Acetone extraction and carbon–attaclay cleanup	4	108 ± 6	3	79 ± 7
Hexane extraction without cleanup	3	96 ± 5	4	94 ± 5

[a] Analyzed on QF-1 column which can separate chlorpyrifos from its oxygen analog. [b] Analyzed on SE-30 column.

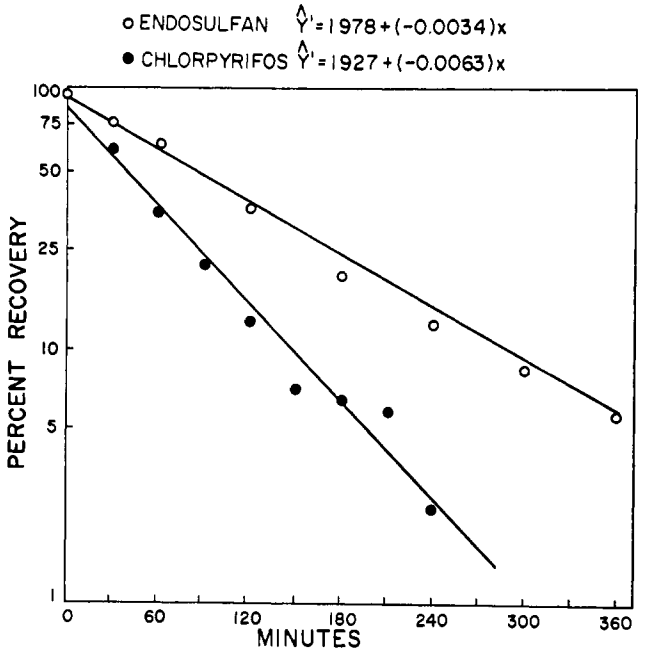

Figure 3. Recoveries in cuticle rinses of the LD_{50} dose of insecticides topically applied to the housefly

Packing. 3% SE-30 on Chromosorb W, AW-DMCS (80–100 mesh) and 3% QF-1 on Chromosorb G, AW-DMCS (80–100 mesh).

Carrier Gas. Prepurified extra high purity nitrogen at 70 cm^3/min through the column plus 30 cm^3/min through the detector purge line under 40 PSIG.

Temperatures. Columns, 190°C; injection port, 200°C; detector, 335°C.

in vivo **Disappearance Rates.** Disappearance from the Cuticle. One microliter of the insecticide–acetone solution containing the LD_{50} dose of either chlorpyrifos or endosulfan was applied to the pronotum of female houseflies by means of a microapplicator (ISCO Model M; Instrument Specialty Co., Lincoln, Neb.). At specified times after treatment, flies were knocked down with CO_2 and put in a 10-ml test tube. Twenty females were used in each treatment. The flies were washed by shaking lightly in *n*-hexane for 1 min and decanting it into a graduated centrifuge tube over 1 g of anhydrous Na_2SO_4. The washing was repeated five times with 2-ml portions of *n*-hexane. The combined washings were concentrated to 5 ml for glc quantitation.

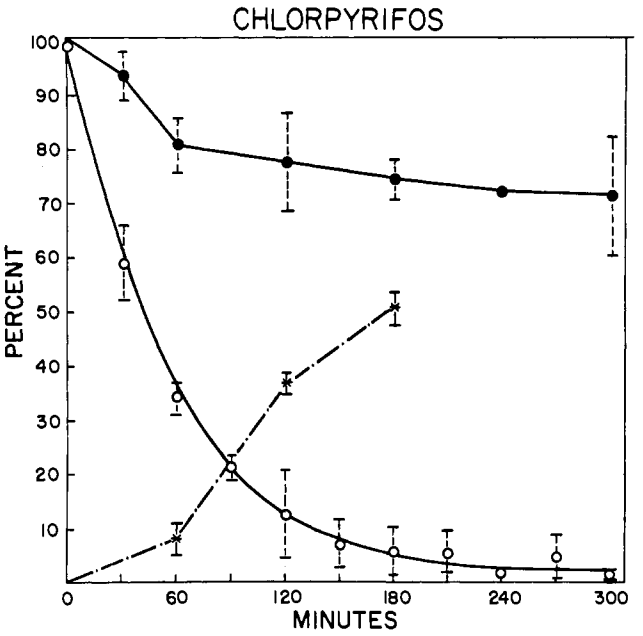

Figure 4. Recoveries in the cuticle wash ○, % individuals knocked down *, and ChE activity (% of control) ●, as a function of time after treatment

Total Disappearance Rates from the Whole Body. The flies were topically treated in groups of 20 as above. At specified time intervals they were put in test tubes containing 2 ml of *n*-hexane and kept frozen until extraction. When it was determined that exhaustive extraction of the residue in *n*-hexane without cleanup was the most efficient way for the glc analysis at the level of tissues and the insecticide used, that technique was adopted throughout the study.

The insecticide recoveries from the cuticle wash or from the total homogenization were reported as percent of the total amount applied after correction for extraction efficiency (90–95%).

Cholinesterase Inhibition Study. Cholinesterase tests were done by the pH-stat method using the substrate protection technique of Van Asperen (1960), as reported by Brady and Sternberg (1966) and Zettler and Brady (1969), with modification in the molarity of acetylcholine bromide (AchBr) used. Preliminary assays with DDVP as a cholinesterase inhibitor showed an optimum protection of the enzyme with 0.3 *M* AchBr. Therefore, homogenization of 12 fly thoraces in 1 ml of 0.3 *M* AchBr was followed. The monitoring of thoracic cholinesterase was chosen on the basis that coordinated locomotion in insects is largely controlled by thoracic nervous centers (Stegwee, 1960) and that decapitated flies respond to organophosphate insecticides in the same manner as the nondecapitated ones (Mengle and Casida, 1960). All homogenizations were carried out at 0°C for 1 min. The enzymatic activity was assayed by putting 0.5 ml of the homogenate in a 50-ml beaker containing 9.5 ml of 0.5 *M* NaCl after the pH of the latter had been adjusted to 7.5. This gave an equivalent of six thoraces per 10 ml of assay medium at an optimum substrate concentration of 10^{-3} *M* AchBr. Enzymatic hydrolysis of acetylcholine was followed at pH 7.5 by titrating the released acetic acid with 0.005 *N* NaOH. The cholinesterase activity was measured from both the knockdown and surviving flies and was recorded in percent of the activity in the nontreated controls. As the flies were knocked down they were immediately collected in Dry Ice and kept at −18°C until assay. Survivors were collected at various time intervals and kept in the same manner.

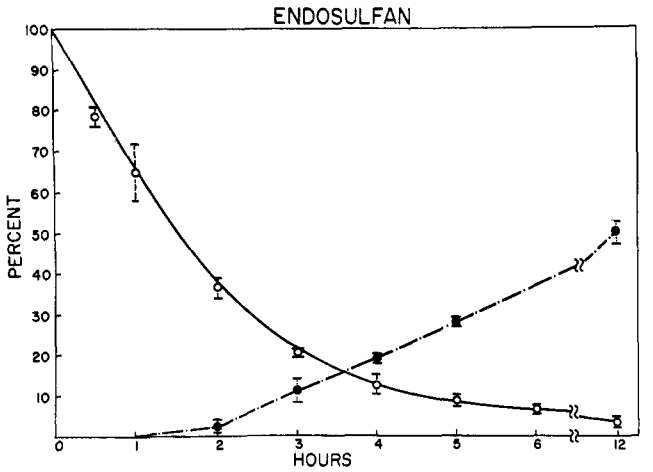

Figure 5. Recoveries in the cuticle wash ○, and % individuals knocked down ● as a function of time after treatment

RESULTS AND DISCUSSION

Residue Analysis. Recoveries of chlorpyrifos and endosulfan from fortified houseflies are presented in Table I. Chlorpyrifos disappears quite rapidly from the surface of metals, wood, glass, and paper in spite of its low vapor pressure of 118 × 10^{-5} mm at 25°C (Smith, 1966). The volatility of insecticides with low vapor pressure from various substrates has been reported by Lichtenstein and Schulz (1970). In our work it was necessary to avoid evaporation of the insecticide extracts to complete dryness, or to use a noninterfering "keeper" liquid such as white paraffin oil. The use of the new high temperature Ni^{63} ec detector operated at 335°C or higher appeared to eliminate need for the microcolumn cleanup methods (Figure 2). The amount of initial tissue used should not exceed 1 g/10 ml of final *n*-hexane extract. Four homogenizations with *n*-hexane was an efficient technique. The use of the high temperature Ni^{63} ec detector in the microanalysis of insecticide residue without cleanup has been discussed by Radomski and Rey (1970).

in vivo **Disappearance Rates.** The recoveries of insecticides from cuticle rinses are presented in Figure 3. The external disappearance rates for both chlorpyrifos and endosulfan follow the usual pattern of first-order kinetics of penetration (Olson and O'Brien, 1963; Buerger, 1966). Linear regression analysis of the percent recoveries (after log transformation) as a function of time after treatment shows a highly significant correlation. The elapsed time allowing 50% disappearance from the cuticle (arbitrarily designated as td_{50}) can be estimated graphically or calculated from the regression equation by taking $Y' = \log 50$. The calculated td_{50}'s were 36 min for chlorpyrifos and 83 min for endosulfan. These values were used as estimates of "relative penetration rates" for comparative purpose between chlorpyrifos and endosulfan. The faster "penetration rate" of chlorpyrifos could be further substantiated by the fact that it caused 50% knock-down of flies in 3 hr while endosulfan took up to 12 hr to produce similar response (Figures 4 and 5). Sun (1968) reported that, on a percentage basis, organophosphates penetrate into houseflies more readily than other insecticides and that their maximum rates are much higher and can be reached in a shorter period after topical application.

Estimation of cholinesterase levels in flies surviving the LD_{50} dose treatment of chlorpyrifos showed an initial rapid decline that appeared to reach a plateau of about 70% of the controls after 3 hr (Figure 4). The level remained unchanged throughout the 12-hr period observed (Table II). On the other hand, cholinesterase activity of the knocked-down flies showed a constant value of 36 ± 4% of that of the controls regardless of the time of knock-down (Table II). Similar observations have been made by Brady (1970). There is neither a recovery of cholinesterase levels of houseflies surviving a given organophosphate treatment nor a correlation between degrees of enzyme inhibition and the speed of knock-down. Thiodan showed no sign of anticholinesterase activity, as has been previously reported by Maier-Bode (1968).

Table II. Thoracic Cholinesterase Activity of Houseflies Topically Treated with LD_{50} Dose of Chlorpyrifos (Average of Three Replicates)

Time after treatment, min	ChE activity % of control ± SD	
	Surviving flies	Knocked-down flies
Control	100[a]	100[b]
30	93 ± 4	
60	81 ± 5	40 ± 0
90		35 ± 7
120	78 ± 9	41 ± 3
180	75 ± 4	31 ± 2
240	73	
300	72 ± 11	
360	60 ± 11	

[a] Average thoracic cholinesterase activity was 1.52 ± 0.30 μM AchBr hydrolyzed/thorax/hr. [b] Average thoracic cholinesterase activity was 1.354 ± 0.062 μM of AchBr hydrolyzed/thorax/hr.

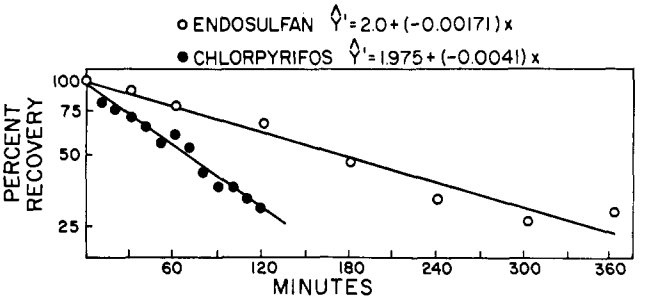

Figure 6. Total recoveries from whole body extract of the LD_{50} dose of insecticides topically applied to the housefly

The total recoveries of the LD_{50}-applied chlorpyrifos and endosulfan from treated houseflies are presented in Figure 6. Again, the observed disappearance rates for both insecticides appear exponential in nature. The 50% disappearance time ($t_{1/2}$) calculated from the regression equation $Y' = 50$ was 67 min for chlorpyrifos and 176 min for endosulfan. The rapid disappearance of chlorpyrifos was anticipated, for the compound would be quickly converted to its oxygen analog in the fly body. The minimum sensitivity of chlorpyrifos O analog to electron capture glc is about 1000-fold less than that of the parent chlorpyrifos and was not detected at the level of insecticide used in the study. This disadvantage of glc response of organophosphates, as compared to their thio analogs, has been reported by Bowman and Beroza (1966a,b, 1967). The measurements of disappearance of chlorpyrifos were thus based only on the parent compound.

Endosulfan was also rapidly converted into endosulfan sulfate by the housefly; detectable sulfate appeared about 30 min after topical treatment. The sulfate, however, is readily detected at picogram levels, and was included in the percent recoveries reported. Other metabolites such as endosulfan alcohol and endosulfan ether (Barnes and Ware, 1965) were not detected.

Excretion of the intact parent material was found to be negligible. Repeated washings of the holding container after 3 hr exposure yielded less than 2% recovery. Similar results were observed by Wilkinson (1967) in his study of a series of 1,3-benzodioxole derivatives. Burt and Lord (1968) reported a 9% recovery of the penetrated amount of diazoxon after 2 hr from cockroaches topically treated with LD_{90} dose.

Analysis of the amounts of a toxicant that has reached exposed insects by spray or vapor requires a recovery of at least 90%. The elapsed time when 90% recovery is feasible (arbitrarily called tr_{90}) can be graphically estimated or calculated from the regression equations in Figure 5 using log 90. Tr_{90} for chlorpyrifos was 5 min and for endosulfan was 27 min. These insecticides change their native structure very rapidly in houseflies. The quantitation of their residues must take into consideration the elapsed time between application and sampling. Houseflies kept in Dry Ice or organic solvents such as acetone or hexane showed minimal enzymatic loss of insecticide. The data on the disappearance rates were used as guidelines for sampling times in the study of the dose toxicity of chlorpyrifos and endosulfan insecticides (Himel and Uk, 1971).

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Photochemistry of Bioactive Compounds. Photolysis of *m*-(*N*,*N*-Dimethylformamidine)phenyl *N*-Methylcarbamate Hydrochloride in Water

George C. C. Su and Matthew J. Zabik*

The photolysis (λ >286 nm) of *m*-(*N*,*N*-dimethylformamidine)phenyl *N*-methylcarbamate hydrochloride (**I**) was carried out in water at pH 3.1 and 7.1 at a concentration of 250 ppm. The photoproducts, after they were isolated and purified by two-dimensional tlc, were identified as *m*-formamidophenol (**VI**) (~60%), *m*-aminophenyl *N*-methylcarbamate (**IV**) (~25%), *m*-formamidophenyl *N*-methylcarbamate (**III**) (~10%), and *m*-hydroxyphenyl *N*-methylcarbamate (**VIII**) (~5%) by comparison of their infrared and mass spectra with their respective authentic samples.

The new pesticide *m*-(*N*,*N*-dimethylformamidine)phenyl *N*-methylcarbamate hydrochloride (**I**) has a broad spectrum of activity against mites, and apparently will gain widespread large-scale application (Jenny, 1971). Carbamate insecticides and their photoproducts were reported to be potent cholinesterase inhibitors (Abdel-Wahab and Casida, 1967; Crosby *et al.*, 1965). Recent studies on the metabolism of **I** by orange seedlings revealed the conversion of **I** to *m*-(*N*-methylformamidine)phenyl *N*-methylcarbamate (**II**), *m*-formamidophenyl *N*-methylcarbamate (**III**), *m*-aminophenyl *N*-methylcarbamate (**IV**), *m*-(*N*,*N*-dimethylformamidine) phenol (**V**), *m*-formamidophenol (**VI**), *m*-aminophenol (**VII**), and possibly the glucosides of **I**, **III**, **VI**, and **VII** (Knowles, 1970). The metabolism of **I** by rats and rat liver homogenates (Knowles and Sen Gupta, 1970; Knowles, 1970) have also been reported. The major products were the same as those produced by orange seedlings.

Irradiation of **I** with uv (254 nm) and fluorescent light, and the "dark" reaction of **I** on silica gel chromatoplates all gave products identified as **I**, **II**, **III**, **IV**, **V**, **VI**, **VII** and unidentified products at the origin, after the chromatoplates were developed in methylene chloride–benzene–diethylamine (Knowles, 1971). In all cases, the major decomposition product was **VI**.

Hydrolysis of **I** at the carbamate and the amidine groups apparently proceeded easily in soil. At pH 9, 50% reaction was reached in 100 min (Jenny, 1971). Photocatalyzed hydrolysis of carbamates (Pape *et al.*, 1970) and amidines (Su and Zabik, 1972) were reported to occur readily.

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