Degradation of Alachlor in Chironomid Larvae (Diptera: Chironomidae)

Lester Y. Wei

Sandoz Crop Protection Corporation, 1300 East Touhy Avenue, Des Plaines, Illinois 60018

Charles R. Vossbrinck*

Office of Agricultural Entomology, University of Illinois, 147 PABL, 1201 West Gregory Drive, Urbana, Illinois 61801

Field-collected chironomid larvae were placed in 2-mL sterile water solutions containing 4.5 ppm unlabeled or ^{14}C-labeled alachlor. Within 1 h, the alachlor was assimilated into the larvae to a 7–10-fold concentration. The rate of disappearance of alachlor from aqueous solution was measured with gas chromatography. Within 17 h, 83% of the alachlor was gone from the water. Alachlor metabolites were isolated by high-performance liquid chromatography or thin-layer chromatography and were identified by thin-layer chromatography or mass spectroscopy. The major metabolite returning to the water was tentatively identified as the O-demethylated derivative of alachlor. The metabolites CDEPA and DEA were also identified. An additional unidentified metabolite was found in the larvae at high levels. Differences in the metabolites found in the water versus the insect tissue may be important when considering the actual levels of alachlor in the aquatic ecosystem and the ultimate fate of alachlor in the environment.

INTRODUCTION

Alachlor is used as an herbicide throughout the midwestern and southern United States for weed control in corn, soybeans, and cotton. Approximately 65 million pounds of this herbicide are used each year (Brown et al., 1988). Although metabolic studies on alachlor in plants, mice, rats, and mammalian liver homogenates, as well as in mixed and model ecosystems, have been reported (Sharp, 1988; Yu et al., 1975; Bollag et al., 1986; Novick and Alexander, 1985), we know of no such studies in insects.

Because of the amount of herbicide runoff from soil into water, understanding the fate of alachlor in the aquatic ecosystem and the breakdown of this compound by aquatic organisms, including insects, is important. This paper reports the major pathways of alachlor degradation in aquatic chironomid larvae.

MATERIALS AND METHODS

Chemicals. Analytical standards of alachlor and ring-labeled ^{14}C alachlor (specific activity = 13.74 mg/mCi, radiochemical purity = 95%) were obtained from Monsanto Co. Diethylaniline (DEA, 99.5% purity) was purchased from Aldrich Chemical Co. Chloro-*N*-(diethylphenyl)acetamide (CDEPA) was synthesized by the reaction of DEA with chloroacetyl chloride (98% purity, Aldrich Chemical Co.) (Berntsson et al., 1976).

Gas Chromatography (GC). A Varian 3400 gas chromatograph equipped with a nitrogen phosphorus specific detector was used for the quantitative analysis of alachlor. Chromatography was performed with a glass column (2 mm × 90 cm) packed with 5% Apiezon and 0.125% DEGS maintained isothermally at 190 °C. The detector and injector were held at 250 °C, and the nitrogen flow rate was 20 mL/min.

Mass Spectroscopy (MS). Metabolites purified by thin-layer chromatography (TLC) or high-performance liquid chromatography (HPLC) were analyzed in electron impact (EI) mode by the direct probe method on a Varian MAT CH-7 mass spectrometer. MS operating conditions were as follows: electron energy, 70 eV; emission current, 0.3 mA; ion source temperature, 200 °C. The mass spectral data were acquired with an SS-100 MAT computer.

* Author to whom correspondence should be addressed.

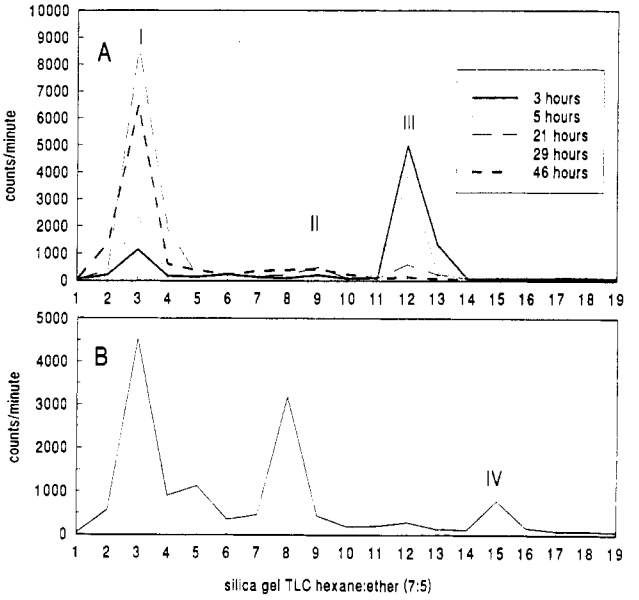

Figure 1. Silica gel TLC analysis of the degradation of alachlor by chironomid larvae over 46 h. (A) Analysis of the alachlor solution. Peak I represents a polar compound believed to be the O-demethylated derivative of alachlor, peak II CDEPA, and peak III alachlor. (B) Analysis of the larval extracts at 46 h. Peak IV cochromatographed with DEA.

Table I. Disappearance of Alachlor from 2 mL of a 4.5 ppm Alachlor Solution Containing Five Chironomid Larvae, Measured by GC

	% alachlor remaining			% alachlor remaining	
time, h	expt 1	expt 2	time, h	expt 1	expt 2
0.5	86.0	98.0	4	51.0	48.0
1	62.3	68.2	7	44.5	40.1
2	69.8	64.9	17	17.9	17.0

High-Performance Liquid Chromatography. A C-18 column was used for HPLC (Beckman 4270) isolation of an unknown. An aqueous solution containing alachlor and metabolites was run through a C18 Sep-pak cartridge (Waters Associates, Inc.). The compounds were eluted with ethyl ether and concentrated.

0021-8561/92/1440-1695$03.00/0

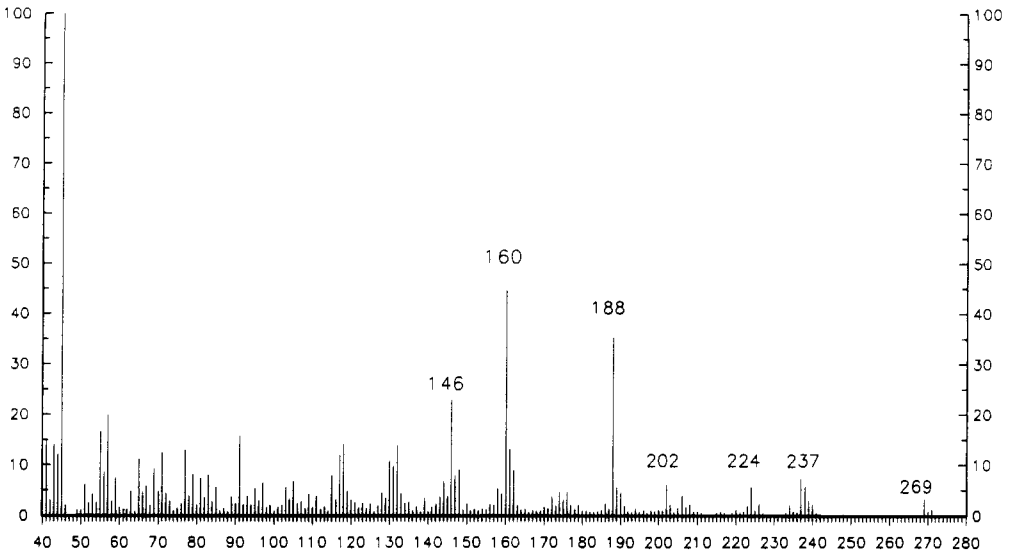

Figure 2. EI mass spectrum of peak III, Figure 1A, identified as alachlor.

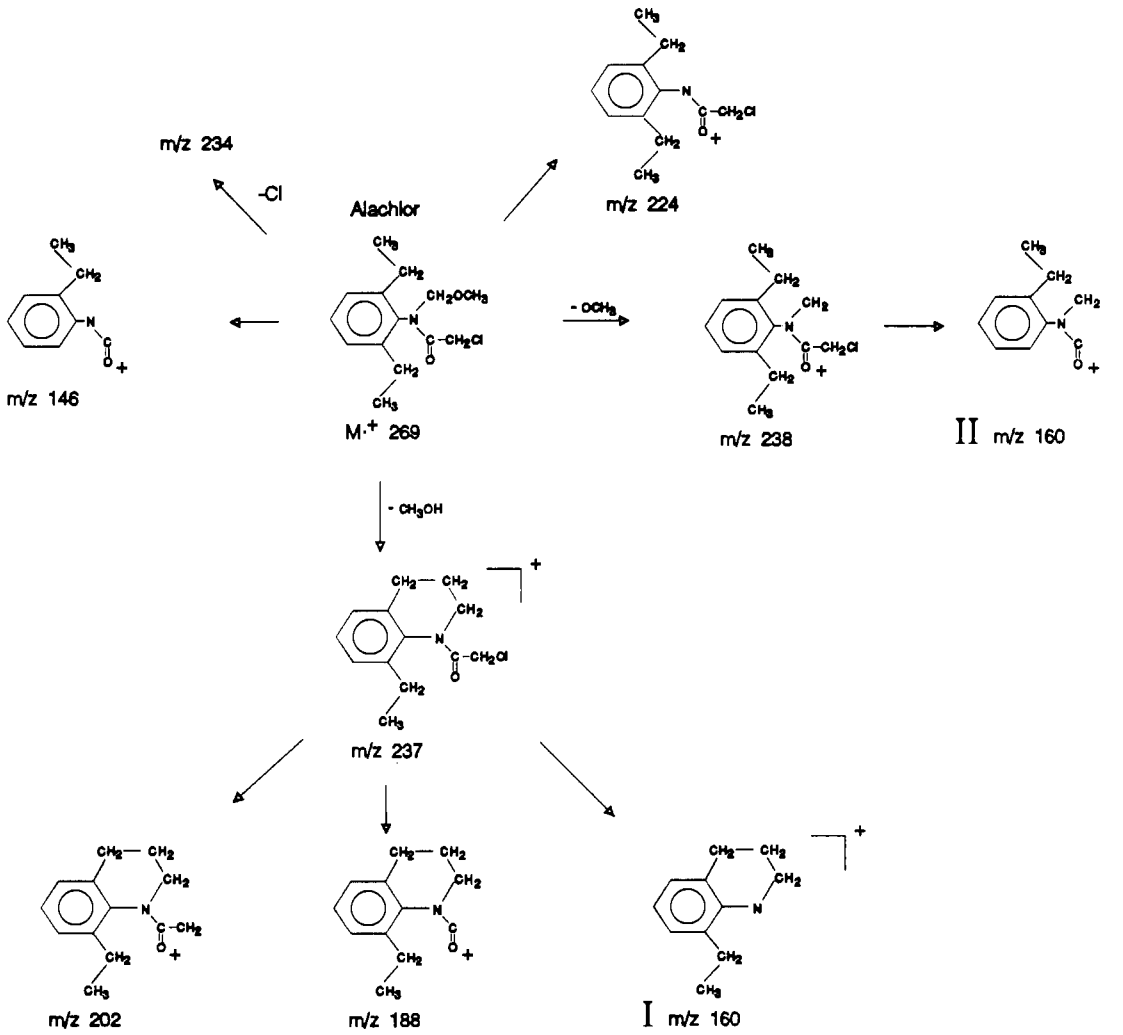

Figure 3. Proposed fragmentation pattern of alachlor.

This solution was loaded onto the HPLC column. The solvent system was 45% acetonitrile in acetate buffer (5 mM, pH 4.5) with a flow rate of 1 mL/min. The effluent was analyzed by an ultraviolet (UV) monitor at 254 nm (Beckman 163 variable-wavelength detector). The unknown was concentrated and analyzed by direct probe MS.

Thin-Layer Chromatography. TLC was performed on 20- × 20-cm precoated plastic silica gel sheets or Whatman KC-18 reverse phase plates. The solvent system consisted of hexane–ethyl ether (7:5) and ethanol–water (4:1) for silica gel and KC-18 reverse phase, respectively. Alachlor and metabolites were located by cutting individual lanes into 0.9-cm strips and reading the strips in a liquid scintillation counter (Packard 1900 TR).

Sample Preparation. Chironomid larvae were collected from a local pond (Champaign, IL). Groups of five larvae were weighed and placed together in a 20-mL vial containing 2 mL of a 4.5 ppm unlabeled or ^{14}C-labeled alachlor solution. Samples of the alachlor solution were collected for analysis at various time points.

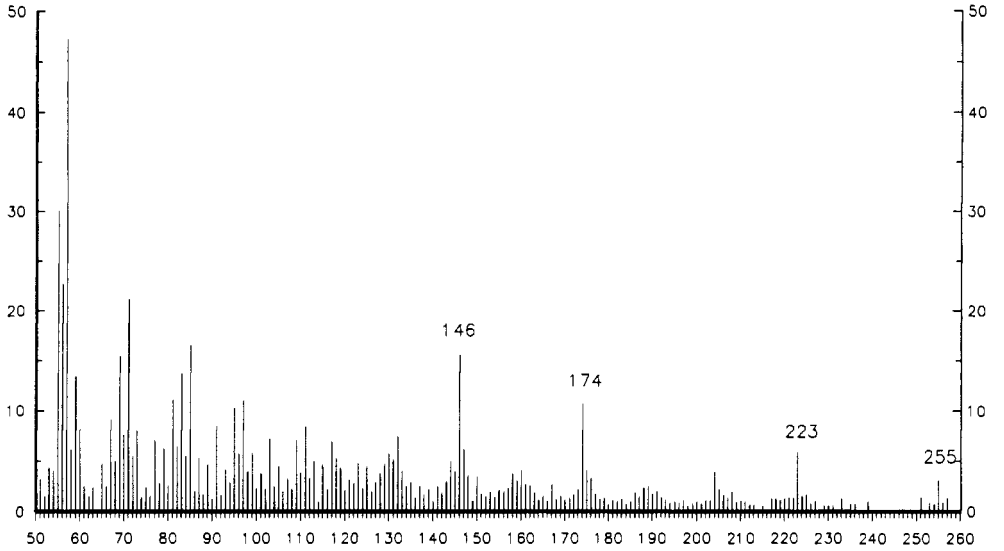

Figure 4. EI mass spectrum of peak I, Figure 1A, identified as the O-demethylated derivative of alachlor.

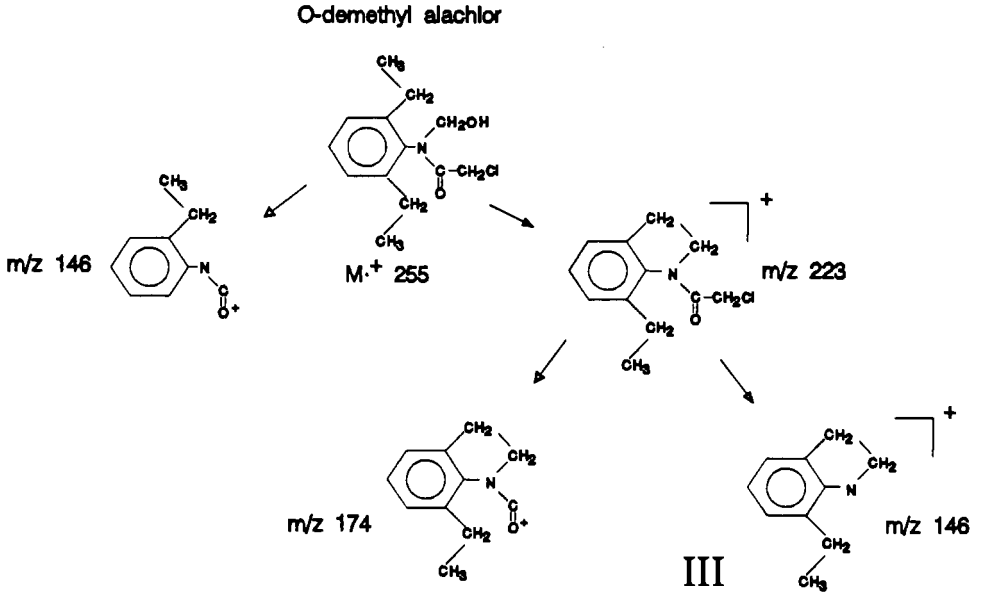

Figure 5. Proposed fragmentation pattern of the major metabolite, tentatively identified as the O-demethylated derivative of alachlor.

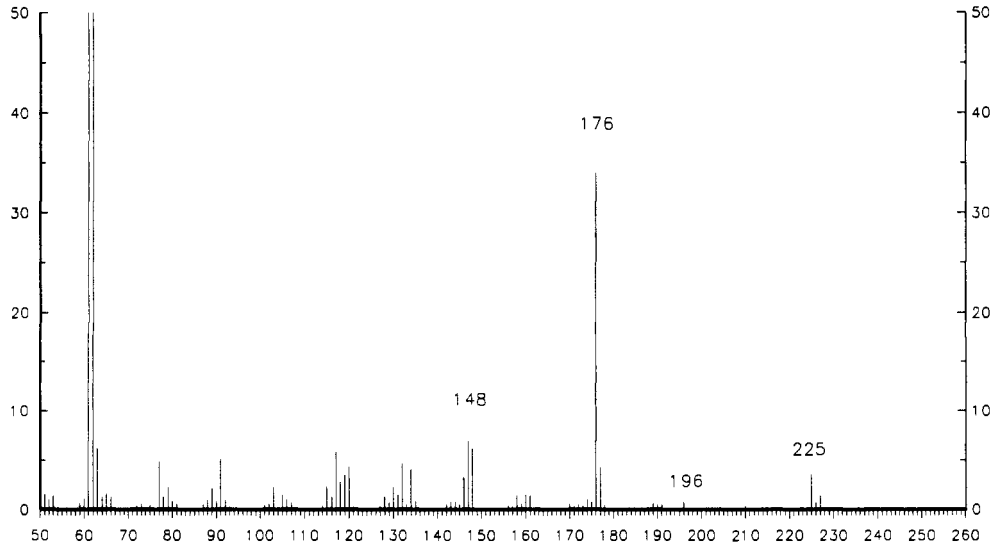

Figure 6. EI mass spectrum of peak II, Figure 1A, identified as CDEPA.

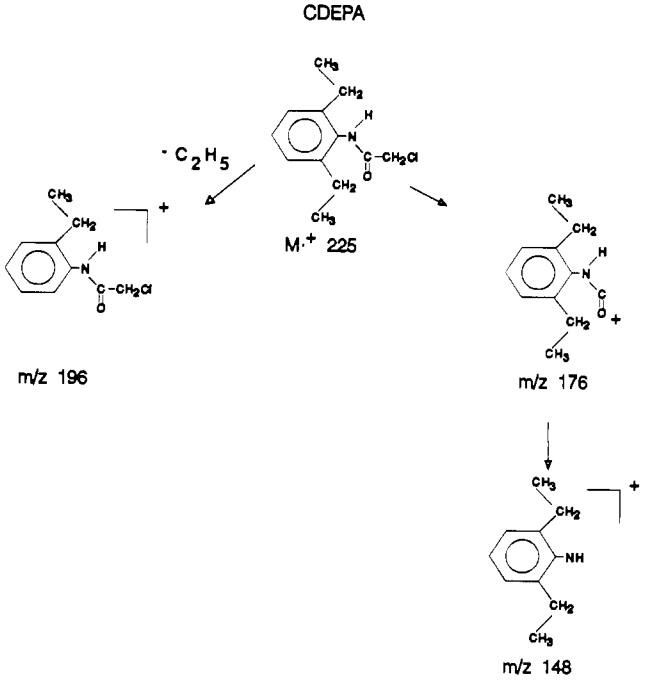

Figure 7. Proposed fragmentation pattern of CDEPA.

Samples to be assayed for the disappearance of alachlor were extracted three times with 2 volumes of ethyl acetate. The ethyl acetate fractions were combined and concentrated for GC analysis.

Samples to be assayed for alachlor metabolites were spotted (10 μL) directly from the vial containing the larvae onto TLC plates. After development of the TLC plate, lanes were cut into strips as described above. The strips from the ^{14}C-labeled lanes were read in a liquid scintillation counter. For those strips which showed ^{14}C activity, the adjacent strips from the nonlabeled lanes were scraped from the TLC plate and analyzed by MS.

Larvae to be assayed directly for incorporation of ^{14}C-labeled alachlor were ground in a tissue grinder, in a small amount of scintillation fluid, and then counted in the scintillation counter.

Larvae used for TLC analysis were ground in ethyl acetate in a tissue grinder and spun briefly in a Beckman microfuge E to pellet the cell debris. Ten-microliter aliquots of the ethyl acetate solution were spotted onto the TLC plate.

RESULTS AND DISCUSSION

Of initial concern was whether the alachlor entered the body of the chironomid larvae. Six larvae were put in 2 mL of a 4.5 ppm ^{14}C-labeled alachlor solution. At 1 h they were removed from the alachlor solution, weighed individually, and read individually in the scintillation counter. We found a 7.32-fold concentration of alachlor (standard deviation of 1.90) in the larvae to 32.94 ppm. Two additional experiments with five larvae in 2 mL of 4.5 ppm alachlor solution, in which the larvae were weighed and counted together, showed similar (8–10-fold) concentrations of alachlor in the larvae at 1 h.

The rate of disappearance of alachlor from aqueous solution is shown in Table I. Each data point represents five larvae (with a combined weight of approximately 39 mg) in 2 mL of a 4.5 ppm alachlor solution. A 1-mL aliquot of the alachlor solution was taken from each vial at the appropriate time point, extracted with ethyl acetate, and analyzed by GC for change in alachlor levels. The rate of change in alachlor levels was quite rapid, with only 17% of the alachlor remaining in solution at 17 h. In the absence of larvae, alachlor in aqueous solution is quite stable. We detected no significant loss of alachlor from aqueous solution over 48 h (data not shown) and have stored alachlor in aqueous solution for more than 2 weeks without appreciable change in concentration.

Figure 1A illustrates the relatively rapid breakdown of alachlor by five larvae in 2 mL of a 4.5 ppm ^{14}C-labeled alachlor solution. Ten-microliter aliquots of the aqueous solution were taken at various time points and analyzed by TLC. A major peak appeared in fraction 3 (peak I) as the alachlor, fractions 11–12 (peak III), disappeared. Peak I reached a maximum at 21 h and then began to decrease. Within 21 h more than 90% of the alachlor was gone from the water. We saw a peak in fraction 9 (peak II), which comigrated with CDEPA during TLC analysis and which was large enough, during some runs, to collect from the TLC plate and analyze by mass spectroscopy. In an effort to obtain a larger amount of peak II, to confirm the MS data, we incubated five larvae in 2 mL of a 4.5 ppm alachlor solution for 24 h and then ran the solution over an HPLC column (see Materials and Methods). The smallest peak to come off the column was collected and analyzed by MS. The mass spectrum of this peak was identical to that of peak II.

At the end of the experiment, at 46 h, the larvae were extracted and 10-μL aliquots were analyzed by TLC (Figure 1B). In addition to the peaks seen in Figure 1A, several new peaks can be seen in fractions 5, 8, and 15 of the larval extracts. Fraction 15 (peak IV, Figure 1B) comigrated with DEA on both silica gel and DC-18 TLC plates (R_f of 0.65 and 0.53, respectively).

The mass spectrum of peak III, Figure 1A, was identical in appearance to the mass spectrum of an authentic sample of alachlor (Figure 2). Alachlor gives the molecular ion of m/z 269 (3%). The major fragmentation pathways of alachlor are given in Figure 3. The fragment ion at m/z 237 results from the loss of methanol with hydrogen abstraction from the ortho ethyl on the ring (referred to as ortho effects (Hamming et al., 1972)) followed by cyclization. The intense peak at m/z 160 is a composite peak made up of two ions: the fragment ion resulting from the loss of a chloroacetyl radical and methanol (m/z 160, ion I) and the fragment ion resulting from the loss of methoxyl, ethyl, and chloromethyl from the molecular ion (m/z 160, ion II).

The mass spectrum of the major metabolite (peak I, Figure 1A), tentatively identified as the O-demethylated derivative of alachlor, is shown in Figure 4. The metabolite has a molecular ion of m/z 255 with a chlorine isotope peak at 257, implying an O-demethylation from either the

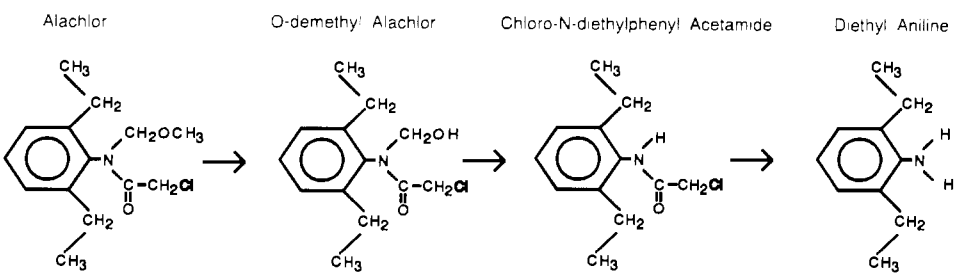

Figure 8. Proposed pathway for alachlor degradation in chironomid larvae.

methoxy group (resulting in an OH group) or from one of the ethyl side chains (resulting in a CH_3 group) of alachlor. The placement of the peak so near the origin of the silica gel plate implies a marked increase in polarity as would be expected from the addition of an OH group.

The O-demethylated metabolite and alachlor are homologs. Fragmentation pathways similar to those for alachlor can be envisaged to explain the characteristic fragment ions present in the spectrum of this metabolite (Figure 5). The abundance of ion m/z 146 is expected because it is a composite peak made up of two ions shown in Figure 5. The fragment ion m/z 146 III is analogous to fragment ion m/z 160 I in Figure 4.

In Figure 6, the mass spectral analysis of peak II, Figure 1A, identical to that of the HPLC-purified unknown, indicates a molecular weight of 225 with a chlorine isotope peak at 227. Fragment ions were observed at m/z 176 and 196. The peak at m/z 176 is due to the loss of a chloromethyl radical. Loss of an ethyl side chain produces the ion of m/z 196 (Figure 7).

Alachlor at 4.5 ppm in an aqueous solution is rapidly taken up by chironomid larvae and converted to a much more polar compound which we have tentatively identified as O-demethylated alachlor. We hypothesize that the O-demethylated derivative is further broken down to the less polar DEA metabolite and accumulates in the insect. Upon examination of the compounds we have identified (O-demethylated alachlor, CDEPA, and DEA), it appears that the pathway illustrated by Feng and Patanella (1989) and by Brown et al. (1988), involving alachlor → O-demethylated alachlor → CDEPA → DEA, may be an important pathway in chironomid larvae (Figure 8). As in most other alachlor degradation studies (Sharp, 1988), additional metabolites are found, as illustrated by the peaks in fractions 5 and 8 of Figure 1B.

The rate at which alachlor is converted to the O-demethylated derivative is quite rapid. Several studies on human hemoglobin have shown that hemoglobin can act as an enzyme, catalyzing hydroxylation and demethylation of aniline and various derivatives (Mieyal et al., 1985; Starke et al., 1984). Williams et al. (1984) have shown that *Chironomus riparius* larvae, which contain hemoglobin, have a high tolerance to pollutants. We speculate that hemoglobin, present in the chironomid larvae used in this study, may play a role in the O-demethylation of alachlor.

ABBREVIATIONS USED

CDEPA, chloro-*N*-(diethylphenyl)acetamide; DEA, diethylaniline; EI, electron impact; GC, gas chromatography; HPLC, high-performance liquid chromatography; ppm, parts per million; TLC, thin-layer chromatography; UV, ultraviolet.

LITERATURE CITED

Berntsson, P.; Brandstrom, A.; Junggren, U.; Palmer, L.; Sjestrand, S. E.; Wilander, B. Gastric Acid Secretion Inhibitors: Compounds Chemically Related to Lidocaine. *Acta Pharm. Suec.* **1976**, *13*, 385–390.

Bollag, J.; McGahen, L. L.; Minard, R. D.; Liu, S. Bioconversion of Alachlor in an Anaerobic Stream Sediment. *Chemosphere* **1986**, *15*, 153–162.

Brown, M. A.; Kimmel, E. C.; Casida, J. E. DNA Adduct Formation by Alachlor Metabolites. *Life Sci.* **1988**, *43*, 2087–2094.

Feng, P. C. C.; Patanella, J. E. In Vitro Oxidation of Alachlor by Liver Microsomal Enzymes From Rats, Mice, and Monkeys. *Pestic. Biochem. Physiol.* **1989**, *33*, 16–25.

Hamming, M. C.; Fester, N. G. *Interpretation of Mass Spectra of Organic Compounds*; Academic Press: New York, 1972; pp 303–309.

Mieyal, J. J.; Ackerman, R. S.; Blumer, J. L.; Freeman, L. S. Characterization of Enzyme-like Activity of Human Hemoglobin. *J. Biol. Chem.* **1985**, *251*, 3436–3441.

Novick, N. J.; Alexander, M. Cometabolism of Low Concentrations of Propachlor, Alachlor, and Cycloate in Sewage and Lake Water. *Appl. Environ. Microbiol.* **1985**, *49*, 737–743.

Sharp, D. B. Alachlor. In *Herbicides: Chemistry, Degradation and Mode of Action*; Kearney, P. C., Kaufman, D. D., Eds.; Dekker: New York, 1988; Vol. III, Chapter 6.

Starke, D. W.; Blisard, K. S.; Mieyal, J. J. Substrate Specificity of the Monooxygenase Activity of Hemoglobin. *Mol. Pharm.* **1984**, *25*, 467–475.

Williams, K.; Green, D.; Pascoe, D. Toxicity Testing with Freshwater Macroinvertebrates: Methods and Application in Environmental Management. In *Advances in Water Pollution Control: Freshwater Biological Monitoring*; Pascoe, D., Edwards, R. W., Eds.; Pergamon Press: Oxford, 1984; pp 81–91.

Yu, C.; Booth, G. M.; Hansen, D. J.; Larsen, J. R. Fate of Alachlor and Propachlor in a Model Ecosystem. *J. Agric. Food Chem.* **1975**, *23*, 877–879.

Received for review March 13, 1992. Accepted June 8, 1992.