METABOLISM OF TWO NEW ACYLANILIDE HERBICIDES, ANTOR™ [2 - CHLORO - N - (2', 6' - DIETHYLPHENYL) - N - METHYL (ETHYL - CARBOXYLATE) ACETAMIDE] AND DUAL™ [2 - CHLORO - N - (2' - ETHYL - 6' - METHYLPHENYL) - N - (2 - METHOXY - 1 - METHYLETHYL) ACETAMIDE], BY THE SOIL FUNGUS, CHAETOMIUM GLOBOSUM

Thesis for the Degree of M. S.
MICHIGAN STATE UNIVERSITY
LINDA LEIGH McGAHEN
1977

ABSTRACT

METABOLISM OF TWO NEW ACYLANILIDE HERBICIDES, ANTOR^{TM} [2-CHLORO-*N*-(2',6'-DIETHYLPHENYL)-*N*-METHYL(ETHYLCARBOXYLATE)ACETAMIDE] AND DUAL^{TM} [2-CHLORO-*N*-(2'-ETHYL-6'-METHYLPHENYL)-*N*-(2-METHOXY-1-METHYLETHYL)ACETAMIDE], BY THE SOIL FUNGUS, *CHAETOMIUM GLOBOSUM*

By

Linda Leigh McGahen

Acylanilide herbicides are among the most popular chemicals used for weed control in major agricultural crops yet their metabolites have not been identified. I studied the metabolism of Antor^{TM} [2-chloro-*N*-(2',6'-diethylphenyl)-*N*-methyl(ethylcarboxylate)acetamide] and Dual^{TM} [2-chloro-*N*-(2'-ethyl-6'-methylphenyl)-*N*-(2-methoxy-1-methylethyl)-acetamide] by resting cells of *C. globosum*. Disappearance of the herbicides and production of metabolites were followed by gas-liquid chromatographic analysis of the organic extract. At least ten new products were observed with Antor as the substrate, and at least eight with Dual. The metabolites of Antor, identified by gas chromatograph-mass spectrometry using electron and chemical ionization were: [2-chloro-*N*-(2',6'-diethylphenyl)acetamide, 2-hydroxy-*N*-(2'-ethyl-6'-vinylphenyl)-*N*-methyl(ethylcarboxylate)acetamide, *N*-methyl(ethylcarboxylate)-2,3-dihydro-7-ethylindole, 2-chloro-*N*-(2'-ethyl-6'-vinylphenyl)-*N*-methyl-

---

[TM] Antor is a trademark of Hercules, Inc., Dual (metolachlor) is a trademark of Ciba-Geigy Corp., and Lasso (alachlor) is a trademark of Monsanto Agricultural Products Co.

(ethylcarboxylate)acetamide, and N-(2',6'-diethylphenyl)-α-(ethylcarboxylate)imine. Compounds produced from Dual were: 2-chloro-N-(2'-ethyl-6'-methylphenyl)acetamide, and 2-chloro-N-(2'-ethyl-6'-methylphenyl)-N-(2-hydroxy-1-methylethyl)acetamide, Generally, C. globosum removes one or both of the R-groups from the nitrogen, dehydrogenates the ethyl substituent, and in some cases forms an indoline ring. It may also remove the chloro, methoxy or ethoxy substituent from the R-groups with subsequent hydroxylation at that position.

METABOLISM OF TWO NEW ACYLANILIDE HERBICIDES, ANTOR^{TM} [2-CHLORO-N-(2',6'-DIETHYLPHENYL)-N-METHYL(ETHYLCARBOXYLATE)ACETAMIDE] AND DUAL^{TM} [2-CHLORO-N-(2'-ETHYL-6'-METHYLPHENYL)-N-(2-METHOXY-1-METHYLETHYL)ACETAMIDE], BY THE SOIL FUNGUS, CHAETOMIUM GLOBOSUM

By

Linda Leigh McGahen

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Crop and Soil Sciences

1977

To My Parents

ACKNOWLEDGEMENTS

I am very grateful to Dr. J. M. Tiedje for this opportunity and for his support, understanding, guidance and encouragement.

I am also quite thankful to Dr. R. Minard of the Department of Chemistry, Pennsylvania State University, for his counsel and for obtaining the electron and chemical ionization mass spectral data on his Finnegan gas chromatograph-mass spectrometer (GC-MS), and for teaching me to use the computor system which was coupled to the GC-MS.

Dr. J.-M. Bollag, also from Penn State, Department of Agronomy, is due very special thanks for the opportunity I had to work and learn under his guidance, for the opportunity I had to attend the National American Society for Microbiology meetings, and for his encouragement.

I would also like to extend a warm thanks to Dr. L. M. Jackman of the department of Chemistry, Penn State, for his advice and help. Lloyd, his wife Marie, and their sons Richard, Donald, and Andrew, also deserve special thanks for their friendship and encouragement.

I thank Dr. M. J. Zabik and Dr. D. Penner for their advice, and Dr. C. C. Sweeley for the use of his GC-MS.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figure Page

INTRODUCTION

Antor^{TM} [2-chloro-N-(2',6'-diethylphenyl)-N-methyl(ethylcarboxylate)-acetamide] and Dual^{TM} [2-chloro-N-(2'-ethyl-6'-methylphenyl)-N-(2-methoxy-1-methylethyl)acetamide] are selective pre-emergence herbicides with activity against many annual grasses and some broadleaved weeds. Hercules, Inc., is attempting to obtain registration for use of Antor on sugar beets, cotton, soybeans, corn, wheat and some vegetable crops. Dual was approved by the Environmental Protection Agency (December, 1976) for use on corn grown for grain, and it may eventually be approved for use on soybeans. Dual will henceforth be referred to by its common name, metolachlor.

Antor and metolachlor are acylanilide herbicides and structurally similar to a previous popular pre-emergence herbicide, alachlor (Lasso^{TM}). Alachlor was previously shown to be biodegraded in soil (Beestman and Deming, 1974; Chou, 1973) and by a soil fungus, *Chaetomium globosum* (Taylor, 1972). Chou (1973) and Taylor (1972) found that very little of the ring-labeled alachlor was released as $^{14}CO_2$ by soils or *C. globosum*, respectively. Subsequent studies by Tiedje and Hagedorn (1975) showed that *C. globosum* degraded alachlor to six extractable metabolites and released chloride ion. Four of the metabolites were identified as:

---

^{TM}Antor is a trademark of Hercules Inc., Dual is a trademark of Ciba-Geigy Corp., Agricultural Division, and Lasso is a trademark of Monsanto Agricultural Products Co. Use of trade names is for the purpose of identification and does not constitute endorsement by Michigan State University, Dept. of Crop and Soil Sciences.

2-chloro-N-(2',6'-diethylphenyl) acetamide, 2,6-diethylaniline, N-(2-chloracetyl)-2,3-dihydro-7-ethylindole, and 2',6'-diethyl-N-(methoxymethyl)aniline.

In this report, metabolites of Antor and metolachlor produced by *C. globosum* were identified and a plausible pathway of breakdown presented. Comparison among the metabolites of the three herbicides revealed similarities in metabolic attack on the herbicides and their metabolites. This document also contains a summary of my attempts to find an enzyme(s) of *C. globosum* responsible for the initial steps in degradation of alachlor (Appendix B).

MATERIALS AND METHODS

Instrumental Analysis. Gas-liquid chromatographic (GLC) analysis was done on a Perkin-Elmer 900 gas chromatograph with a flame-ionization detector. Operating temperatures were: detector and injector, 220°; oven, $135\text{-}210^{\circ}$ (3°/min) for programmed runs or at 210° for isothermal analysis of both compounds. Carrier gas flow rate was 30 ml/min. The column used was 2 m x 2 mm (ID) glass column, packed with 3% SP2100 on 100/120 mesh Supelcoport (Supelco, Inc., Bellefonte, Pa.). Prior to packing, the column was washed with chloroform, thoroughly dried, and silanized 2 times with Sylon-CT. After each silanization the column was rinsed sequentially with toluene and anhydrous methanol, and thoroughly dried. GLC data were collected on an Autolab System I programmable integrator.

Chemical and electron ionization mass spectra of the metabolites were obtained with Finnegan gas chromatograph-mass spectrometers (GC-MS), each equipped with quadrapole mass analyzers. Either methane or isobutane was used for chemical ionization mass analysis (CI), while electron ionization (EI) was done with an ionizing voltage of 70 eV. A column comparable to the one described above was also used in the GC-MS units. The oven was programmed $125\text{-}250^{\circ}$.

Radioactivity was measured on a Packard Tri-Carb Scintillation Spectrometer, Model 3310. All samples were counted in Bray's solution

(Bray, 1960), and were corrected for quenching by external standardization. The organic extracts were gently taken to dryness under a stream of dry air. The residue was redissolved in ethanol and counted in Bray's solution due to the high quenching caused by the extractant, Freon 113.

Reagents. Antor was obtained as formulation from Hercules, Inc., Wilmington, Delaware, and purified according to the method described for alachlor by Tiedje and Hagedorn (1975). The antor was recovered as white needle-shaped crystals after concentrating the hexane extract in a flash evaporator and then freezing it. This procedure was repeated several times until the Antor was 99.9% pure by GLC analysis. ^{14}C ring- and carbonyl-labeled Antor were also provided by Hercules, Inc. The Antor was uniformly labeled in the aromatic ring, while the carbonyl label was located in the carbonyl of the chloroacetyl moiety.

Metolachlor, a clear liquid, was provided as formulation by Ciba-Geigy Corp., Agricultural Division, Greensboro, N.C. It could only be partially purified using the above extraction procedure. However, purification was achieved by eluting the partially purified metolachlor or metolachlor formulation through florisil columns. In preparation for use, the florisil (60-100 mesh) was cleaned by refluxing with pure methylene chloride. Following drying and activation at 130°, 10.5-12 g of the florisil was packed into a glass column. Several columns were prepared and run simultaneously. Columns were kept at 130° until used. The reagent sequence for column elution was: 1) the column was activated with 50 ml of 1% methanol in benzene followed by 50 ml petroleum ether; 2) 3 to 5 ml of the impure metolachlor sample was added; 3) the sample was eluted with 50 ml petroleum ether followed by 200 ml benzene-methylene chloride-acetonitrile (60:30:1). The impurities remained on the column or were largely eluted with the petroleum ether fraction while the last solvent system eluted the metolachlor. The metolachlor fraction was concentrated in a flash evaporator. This purification procedure was repeated several times to remove most of the impurities. The florisil-cleaned metolachlor from the last elution step was again concentrated in a flash evaporator, after which the remaining solvent was removed, leaving the liquid metolachlor. The metolachlor was 95% pure by GLC analysis.

Procedures. A pure culture of *C. globosum*, which was maintained by asceptic transfer on potatoe dextrose agar, was used for all experiments. This strain has been deposited in the American Type Culture Collection. Mycelial pellets for resting cell experiments were obtained by growing the fungus in 500 ml flasks containing 250 ml potatoe dextrose broth, incubated at 28°, on a rotary shaker. The pellets were harvested asceptically by suction filtration and washed two times with 125 ml of sterile, deionized water. The mycelium was then resuspended in a 500 ml flask containing 300 ml of 0.02 M phosphate buffer, pH 7.2, in which either 0.32 mM Antor or 0.35 mM metolachlor was dissolved. The herbicide solution was sterilized by filtration through 0.22 µ Millipore filters prior to addition of the mycelium. Following suspension of the mycelium in the herbicide solution, the resting cells were returned to the rotary shaker and incubated at 28°.

Each resting cell experiment was composed of three flasks containing an herbicide solution with mycelium and two flasks containing only the herbicide solution as a control for non-biological degradation. Another set of three flasks contained only mycelium suspended in a 0.02 M phosphate buffer, pH 7.2; this served as a control for non-herbicide derived metabolites.

Analysis. In Antor experiments using radioactive Antor, 6 ml samples were periodically removed for the following determinations: 1 ml was analyzed for total ^{14}C content while the remaining 5 ml were reserved for GLC analysis. Samples were frozen until all had been collected before proceeding with extraction and GLC analysis. The 5 ml portion was extracted two times with 2.5 ml Freon 113 which contained an internal standard for GLC analysis. Antor was used as an internal standard for metolachlor experiments, while aldrin was used as an internal standard for Antor experiments. In experiments containing radioactive Antor, 1 ml each of the Freon and aqueous phases were assayed for ^{14}C content. The remaining portion of the 5 ml Freon extract was analyzed by GLC for disappearance of the Antor. In non-radioactive experiments only 5 ml aliquots were taken.

The fungus was removed from the remaining solution by suction filtration at the conclusion of the experiment, and the combined solutions from the replicates were extracted three times with Freon 113. Each Freon aliquot was one-third the volume of the solution being extracted. The extract was concentrated in a flash-evaporator and analyzed by GLC and GC-MS.

The chromatograms showing the Antor and metolachlor metabolites were drawn from the total ion current observed following chromatographic separation of the components in the GC-MS unit. These peaks are identified by numbers which correspond to the numbers under the proposed structures in the subsequent figures. In the text the structures are also referred to by these numbers but are preceded by an "A" or "M" prefix to identify Antor and metolachlor metabolites, respectively. The GC-MS data are presented as *m/e* followed in parentheses by the intensity of that fragment relative to the base peak (% of base peak). Background was subtracted from all metabolite data by using the data from scans of areas adjacent to the peaks. Structures were derived from the mass spectral data in accordance with what could be expected from the original herbicide.

RESULTS AND DISCUSSION

Metabolism of Antor. There was almost no loss of radioactivity from the resting cell experiments using ^{14}C carbonyl- or ring-labeled Antor, indicating that very little label was released as CO_2. This agrees with the previous results with this fungus metabolizing ring-labeled alachlor where no $^{14}CO_2$ could be detected (Tiedje and Hagedorn, 1975; Taylor, 1972).

The loss of label from the organic phase and the subsequent increase in label in the aqueous phase is shown in Figure 1. Nearly identical results were obtained with ring- and carbonyl-labeled Antor. After 144 hours incubation, the organic phases had lost 40% of the ring and 37% of the carbonyl labels, while the aqueous phases had gained 54% and 52% respectively. GLC results from both ring- and carbonyl- labeled experiments indicated that the parent compound had disappeared after 48 hours incubation. Metabolites were detected by GLC within 24 hours. No metabolites were noted in concentrated Freon extracts from control flasks containing Antor only, and no peaks appeared in chromatograms of concentrated extracts of controls where cells had been incubated in buffer without herbicide.

Initial GLC analysis of concentrated extracts of resting cell solutions containing Antor indicated that there were as many as 10 extractable metabolites (Figure 2). Separation of the later-eluting peaks was not adequate to obtain good GC-MS data, so the compound structures were not determined. However, seven of the metabolites could be fairly securely identified.

Figure 1. Degradation of ^{14}C ring- and ^{14}C carbonyl-labeled Antor by *C. globosum* in resting cell experiments. Antor degradation was also monitored by GLC analysis of the organic extract.

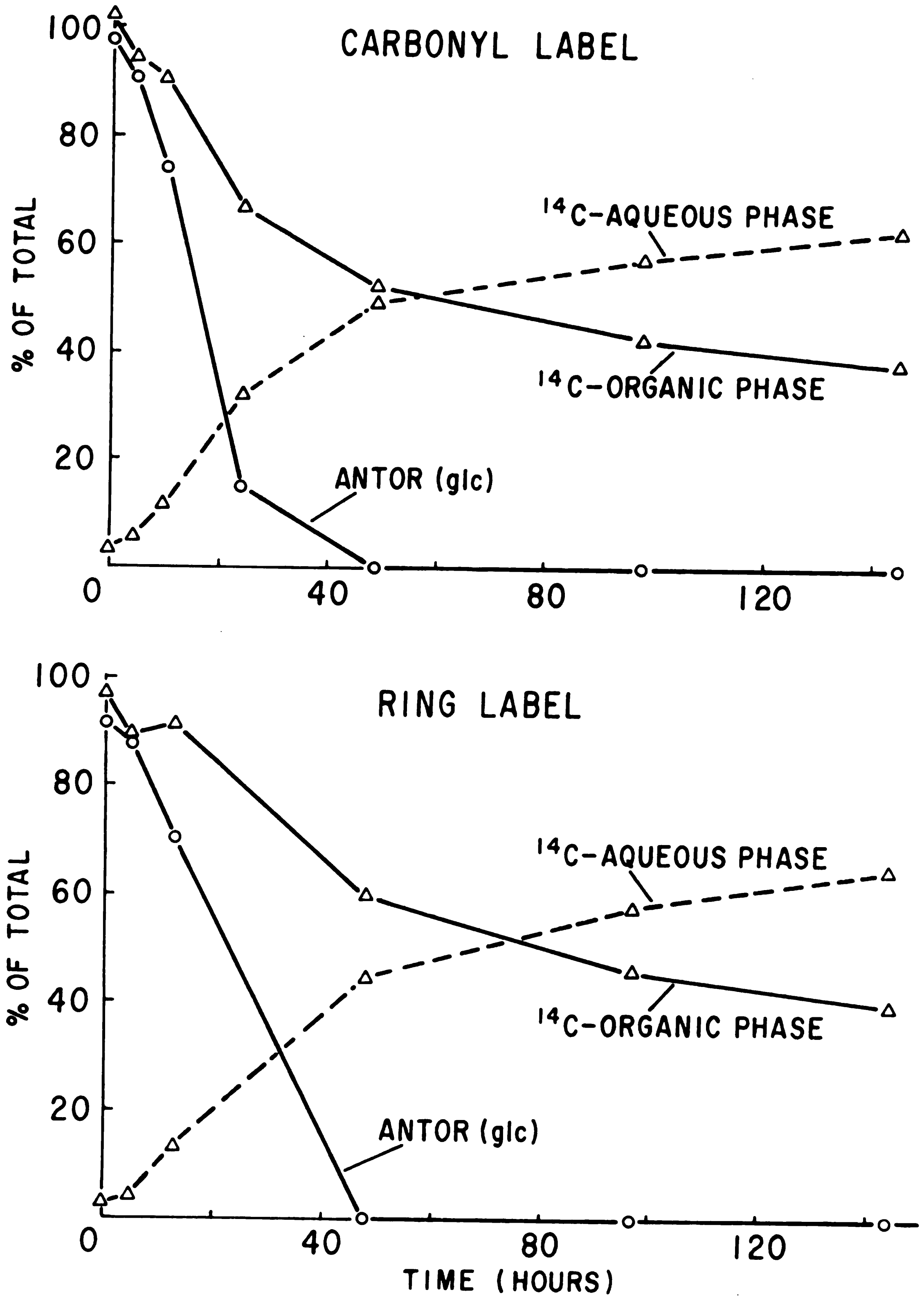
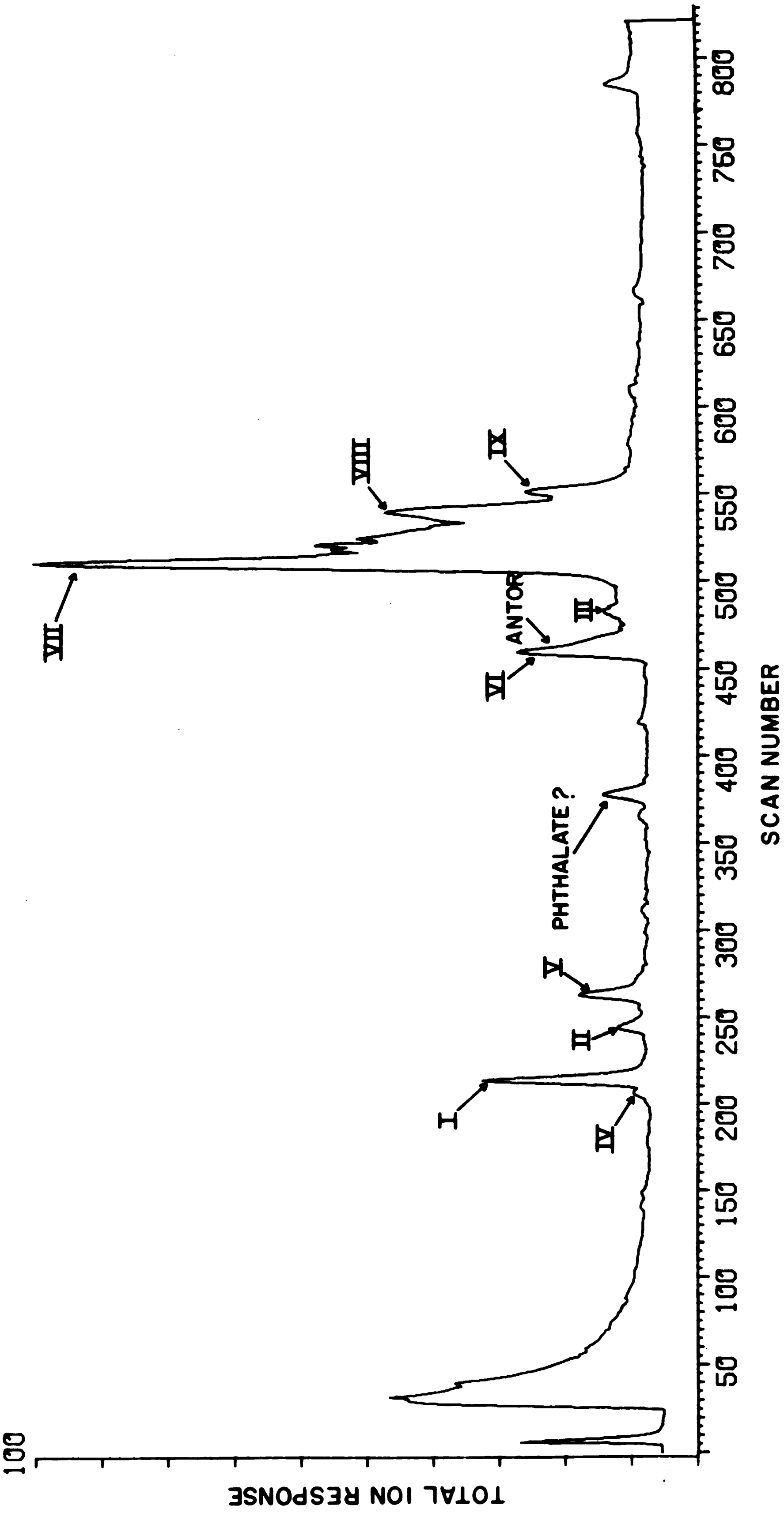

CARBONYL LABEL
100
80
60
40
20
0
% OF TOTAL
^{14}C-AQUEOUS PHASE
^{14}C-ORGANIC PHASE
ANTOR (glc)
0
40
80
120
RING LABEL
100
80
60
40
20
% OF TOTAL
^{14}C-AQUEOUS PHASE
^{14}C-ORGANIC PHASE
ANTOR (glc)
0
40
80
120
TIME (HOURS)

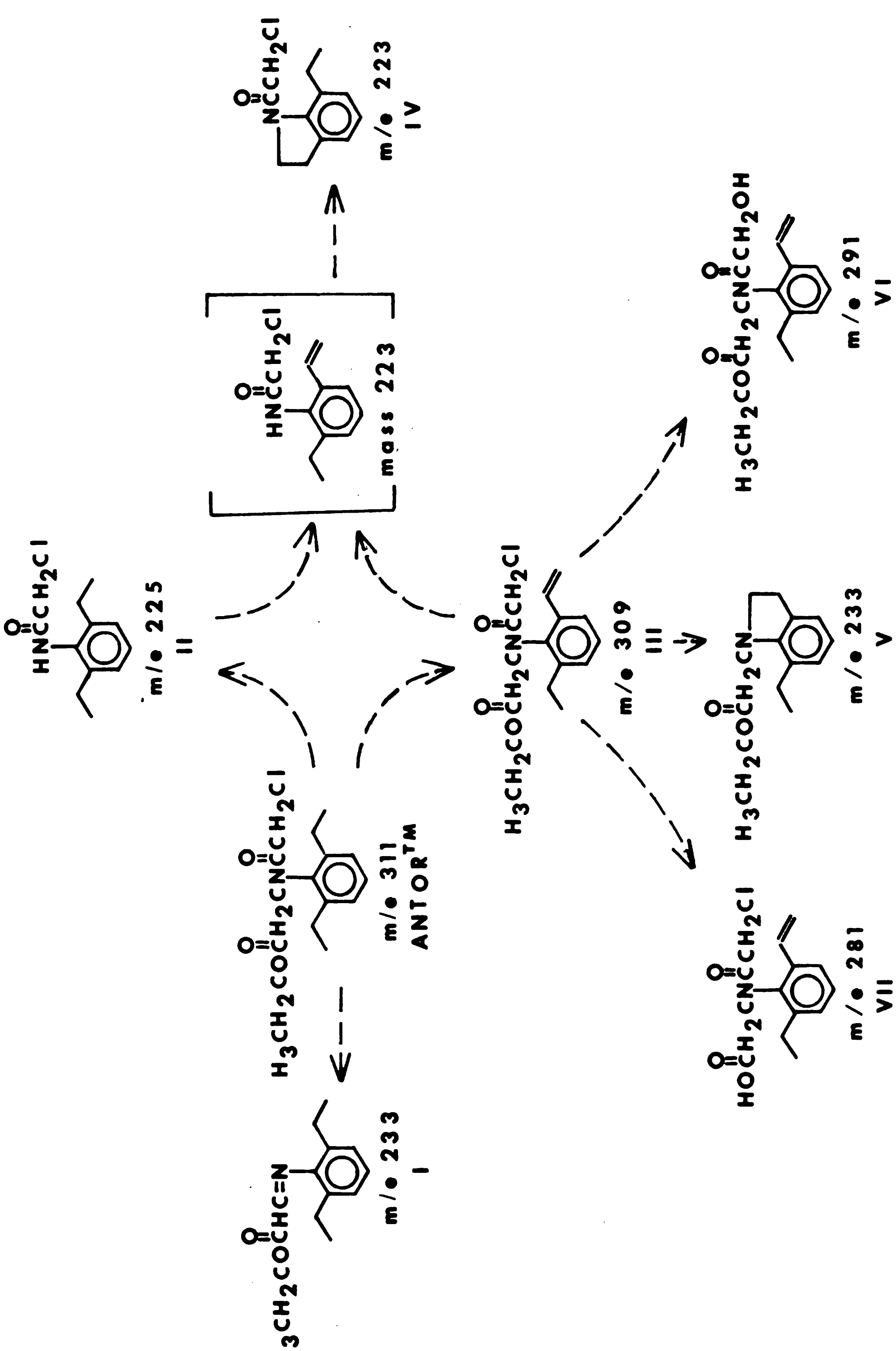
Figure 2. Gas-liquid chromatogram of the Antor metabolites as they eluted through the GC-MS. Each peak is labeled with the number corresponding to the metabolite (Figure 3) which was identified from the mass spectral data taken at that scan number.


TOTAL ION RESPONSE
100
SCAN NUMBER
50
100
150
200
250
300
350
400
450
500
550
600
650
700
750
800
IV
I
II
V
PHTHALATE ?
VI
ANTOR
III
VII
VIII
IX

Metabolites AI and AV both had a parent ion at m/e 233, by CI and EI, with no chlorine. Mass spectral data for AI were: 233(11), 160(100), 144(7), 132(49), 118(11), 105(28). Mass spectral data for AV were: 233(20), 160(100), 146(80), 144(22), 132(64), 130(66), 118(29). At least six different isomers or structures derived from Antor could yield this parent mass. Three were definitely eliminated because their expected fragmentation patterns could not fit this fragmentation data. The most reasonable structures derived from this data were: *N*-(2',6'-diethylphenyl)-α-(ethylcarboxylate)imine ($C_{14}H_{19}O_2N$), and *N*-methyl(ethylcarboxylate)-2,3-dihydro-7-ethylindole ($C_{14}H_{19}O_2N$). These are considered tentative identifications because the fragmentation evidence does not discount a third possibility, *N*-(2'-ethyl-6'-vinylphenyl)-*N*-methyl(ethylcarboxylate)aniline. This latter structure would be a logical product from Antor directly or from structure AVI. The first two structures, the imine and the indoline, were reported to be the correct identification by Hercules chemists based on their GC-MS analysis of these metabolites which I provided them, and synthesis of the imine (Black, 1977). They had reported finding the imine (AI) as a photodegradation product. However, in this experiment the imine was not found as a major component when Antor was incubated without cells. As all flasks were incubated in the dark, metabolite AI (whether it is the indoline or the aniline) appears to be a biological product.

The parent peak for AII was m/e 225 and it had a P+2 peak at m/e 227, indicating the presence of chlorine. This was confirmed by CI data. Mass spectral data were: 227(3), 225(9), 176(100), 160(5), 147(22), 132(12). This metabolite was identified as 2-chloro-*N*-(2',6'-diethylphenyl)acetamide ($C_{12}H_{16}ONCl$), which is also a major

Figure 3. Structures suggested for Antor metabolites arranged in a plausible scheme of degradation.


H$_3$CH$_2$COCH$_2$CNCCH$_2$Cl
m/e 311
ANTOR^{TM}
H$_3$CH$_2$COCHC=N
m/e 233
I
HNCCH$_2$Cl
m/e 225
II
HNCCH$_2$Cl
mass 223
NCCH$_2$Cl
m/e 223
IV
H$_3$CH$_2$COCH$_2$CNCCH$_2$Cl
m/e 309
III
H$_3$CH$_2$COCH$_2$CN
m/e 233
V
H$_3$CH$_2$COCH$_2$CNCCH$_2$OH
m/e 291
VI
HOCH$_2$CNCCH$_2$Cl
m/e 281
VII

metabolite in the degradation of alachlor (Tiedje and Hagedorn, 1975), where it was termed demethoxymethylalchlor (DMM). AII had the same retention time by GLC as did the DMM derived from alachlor.

The EI data obtained for peak AIII contained some background, though the major fragments could be discerned. The parent mass was confirmed by CI as *m/e* 309; the compound contains chlorine. Mass spectral data were: 309(3), 273(9), 232(7), 188(16), 186(16), 160(100), 158(45). The compound appears to be 2-chloro-*N*-(2'-ethyl-6'-vinylphenyl)-*N*-methyl(ethylcarboxylate)acetamide.

Metabolite AIV had a parent peak at *m/e* 223, determined by CI and EI, and definitely contained chlorine as shown by the large P+2 peak at *m/e* 225. Mass spectral data were: 225(8), 223(26), 210(8), 208(25), 174(43), 146(100). This fragmentation pattern is identical to that of the indoline formed by *C. globosum* during degradation of alachlor (Tiedje and Hagedorn, 1975), which was: 225(21), 223(66), 210(15), 208(45), 174(60), 146(100). The retention time of this compound was identical to that of the indoline from alachlor. Thus this indoline, *N*-chloroacetyl-2,3-dihydro-7-ethylindole ($C_{12}H_{14}ONCl$), is also a metabolite of Antor.

The fungus derived metabolite AVI from dechlorination with subsequent hydroxylation of Antor. The parent peak was *m/e* 291 by CI and EI data, and the compound does not contain chlorine. Mass spectral data were: 291(1), 273(11), 248(11), 218(14), 188(14), 176(34), 174(46), 160(100). The only suitable structure found whose mass corresponded to the parent mass and which could produce this fragmentation pattern was 2-hydroxy-*N*-(2'-ethyl-6'-vinylphenyl)-*N*-methyl(ethylcarboxylate)acetamide ($C_{16}H_{21}O_4N$). Although masses 248, 188, 176, and 174 do not fit possible fragmentation schemes for this compound, they could be background from the Antor peak and perhaps metabolite peak AIII, as these peaks are not well separated (Figure 2).

An interesting metabolite, AVII, was apparently formed by the cleavage of the ethyl moiety at the ester linkage, forming an acid, and dehydrogenation of the 6'-ethyl group. This compound has a mass of 281, determined by CI and EI, and has chlorine. Mass spectral data were: 283(1), 281(4), 232(3), 204(5), 202(12), 188(26), 186(19), 160(100). The structure derived from this data was N-(2-chloroacetyl)-N-(2'-ethyl-6'-vinylphenyl)glycine ($C_{15}H_{20}O_2NCl$). This was the most abundant metabolite detected from Antor.

There appear to be two unresolved peaks eluting after metabolite AVII (m/e 281) which could not be interpreted. Peak AVIII may have a parent ion of m/e 309, while peak AV may have a parent ion of m/e 325. Both peaks contain chlorine. CI data for both peaks also contained m/e 282 which was probably due to tailing from peak AVII.

CI data and coinjection of Antor with the metabolite peaks showed that Antor elutes approximately at scan number 467 (Figure 2), but is not resolved from peak AVI.

Metabolism of metolachlor. As shown in Figure 4, GLC analysis showed disappearance of metolachlor when incubated with resting cells but no loss from the control which contained no mycelium. After 144 hours incubation with cells, only 55% of the metolachlor remained. GLC of the concentrated extract from the resting cell experiment showed that at least eight extractable metabolites were resolved.

Metolachlor metabolite MI had a parent mass of 211, and a strong P+2 peak, indicating that chlorine was present. Mass spectral data

Figure 4. Degradation of metolachlor by resting cells of *C. globosum*. Metolachlor degradation was monitored by GLC analysis of the organic extract.

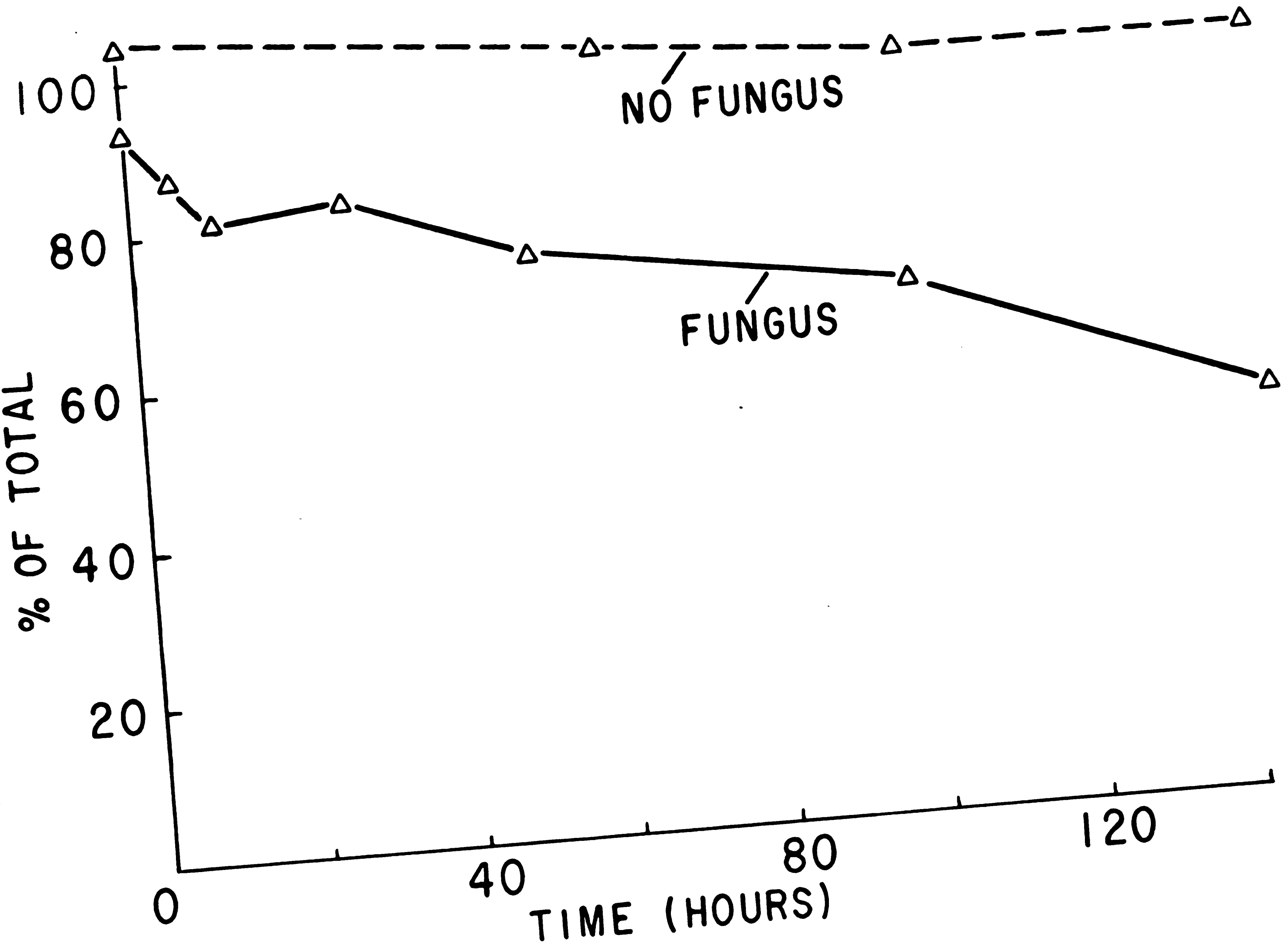
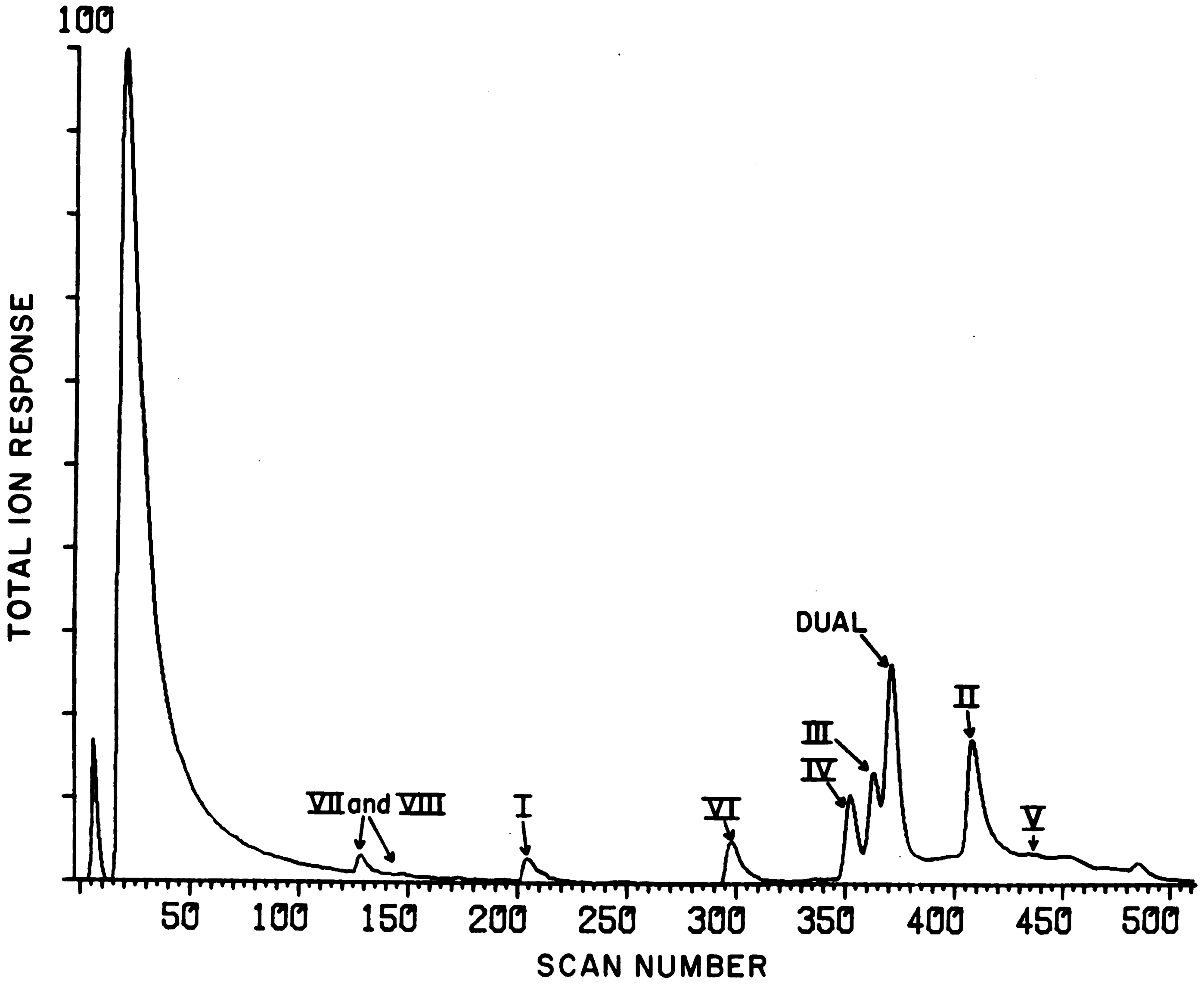

NO FUNGUS
FUNGUS
% OF TOTAL
100
80
60
40
20
0
40
80
120
TIME (HOURS)

Figure 5. Gas-liquid chromatogram of the metolachlor metabolites as they eluted through the GC-MS. Each peak is labeled with the number corresponding to the metabolite (Figure 6) which was identified from the mass spectral data taken at that scan number.


100
TOTAL ION RESPONSE
VII and VIII
I
VI
IV
III
DUAL
II
V
50
100
150
200
250
300
350
400
450
500
SCAN NUMBER

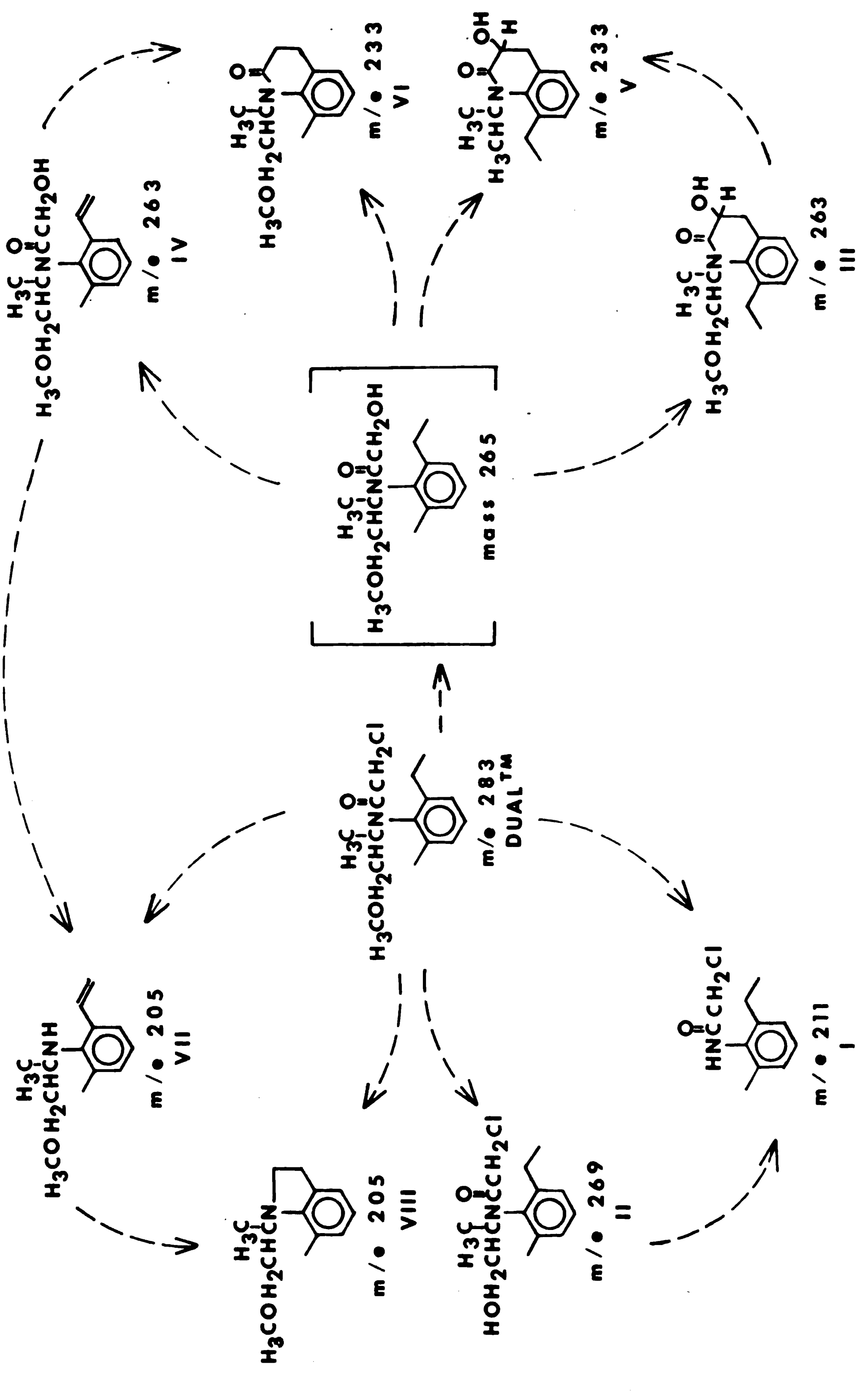
were: 213(3), 211(8), 162(100), 147(9), 134(20), 120(25). This compound was identified as 2-chloro-N-(2'-ethyl-6'-methylphenyl)acetamide, and corresponds to the demethoxymethylalachlor formed from both alachlor and Antor.

MII had a parent mass at m/e 269 (present by CI only) and contained chlorine. Mass spectral data were: 240(9), 238(26), 211(2), 162(100). The only structure derived from metolachlor which could fit this data is 2-chloro-N-(2'-ethyl-6'-methylphenyl)-N-(2-hydroxy-1-methylethyl)-acetamide.

MIII and MIV had identical parent peaks determined by CI only as m/e 263; neither compound had a chlorine moiety. Mass spectral data for MIII were: 218(20), 191(22), 160(100). The structure which best fits this data is 8-ethyl-3-hydroxy-N-(2-methoxy-1-methylethyl)-2-oxo-1,2,3,4-tetrahydroquinoline ($C_{15}H_{21}O_3N$). Mass spectral data for MIV were: 218(19), 191(11), 174(10), 160(100). The structure which best fits this data is 2-hydroxy-N-(2'-methyl-6'-vinylphenyl)-N-(2-methoxy-1-methylethyl)acetamide ($C_{15}H_{21}O_3N$). These two structures can be concluded from the data but it is less certain as to which of the two peaks each structure corresponds.

MVI shows a parent peak at m/e 233, with no indication of a chlorine moiety. This was confirmed by CI data. Mass spectral data were: 233(42), 218(11), 204(12), 188(38), 161(95), 160(60), 146(100). The peak for MV also has a parent ion at m/e 233, but there is no confirming CI data. Mass spectral data for MV were: 233(4), 218(18), 191(17), 160(100). A structure whose fragmentation and mass correspond to the data for MVI is 8-methyl-N-(2-methoxy-1-methylethyl)-2-oxo-1,2,3,4-tetrahydroquinoline ($C_{14}H_{19}O_2N$), while the structure which best fits the

Figure 6. Structures suggested for metolachlor metabolites arranged in a plausible scheme of degradation.


H3C O
H3COH2CHCNCCH2Cl
m/e 283
DUAL™
H3C O
H3COH2CHCNCCH2OH
mass 265
H3C O
H3COH2CHCNCCH2OH
m/e 263
IV
H3C O
H3COH2CHCN
m/e 233
VI
H3C O OH H
H3CHCN
m/e 233
V
H3C O OH H
H3COH2CHCN
m/e 263
III
H3C
H3COH2CHCNH
m/e 205
VII
H3C
H3COH2CHCN
m/e 205
VIII
H3C O
HOH2CHCNCCH2Cl
m/e 269
II
O
HNCCH2Cl
m/e 211
I

data for MV is 8-ethyl-3-hydroxy-*N*-isopropyl-2-oxo-1,2,3,4-tetrahydroquinoline.

Peaks MVII and MVIII both had parent peaks of *m/e* 205. Mass spectral data for MVII were: 205(10), 175(5), 160(100), 148(28), 144(12), 132(11), 130(6); for MVIII they were: 205(5), 160(100), 145(36), 144(28), 132(27), 130(18). Of several possible structures, two compounds appear to be likely choices: *N*-(2-methoxy-1-methylethyl)-*N*-(2'-methyl-6'-vinyl)aniline ($C_{13}H_{19}ON$), and *N*-(2-methoxy-1-methylethyl)-7-methyl-2,3-dihydroindole ($C_{13}H_{19}ON$). A third possibility is 8-methyl-*N*-(1-hydroxyethyl)-2-oxo-1,2,3,4-tetrahydroquinoline ($C_{12}H_{15}O_2N$), although the expected fragmentation for this compound would not yield a *m/e* 145 component, a fragment which was found in both scans. The quinoline could be formed from further breakdown of metabolite MVI.

Metolachlor was eluted at scan number 372. The parent peak for metolachlor is visible only by CI. Mass spectral data were: 240(9), 238(28), 213(1), 211(5), 162(100).

Conclusion. I believe the Antor metabolites having masses 225 (AII), 309 (AIII), 291 (AVI), and 281 (AVIII) are firmly identified. Considering that the compounds had to be derived from Antor, there were, in these cases, no other possible structures which could fit the fragmentation data. The structure of AIV, the indoline, could be confirmed because it was also formed during the breakdown of alachlor and a comparison of these showed identical retention times and fragmentation patterns.

Antor metabolites I and V are a bit more tentatively identified as the imine and the indoline on the basis of my data alone, although

Hercules concluded that the identity of AI and AV as shown in Figure 5 is correct. In this case the imine, discovered by Hercules as a product of photodegradation, may also be a biodegradation product.

Metolachlor metabolites having masses 211 (MI), 269 (MII), and 263 (MIII and MIV) I consider firmly identified. The structures corresponding to the metabolites having mass 233 (MV and MVI, Figure 6) were decided on because their expected fragmentation pattern fit the data exactly. Also, MV and MVI appear to be logically derived from compounds III and IV, respectively. The alternatives would be N-(2-methoxy-1-methylethyl)-6-ethyl-2-oxindole and 8-methyl-N-(2-methoxy-1-methylethyl)-2-methyl-1,2,3,4-tetrahydroquinoline. The former compound (oxindole) is similar to MVIII, except that it has a ketone group on the two position, beside the nitrogen. Its fragmentation may fit the data, but it seems more likely to expect a m/e 147 rather than m/e 146, which was found, if this compound were fragmented. In the degradation scheme (Figure 6), this oxindole would have to be derived from the compound labeled mass 265 by removal of the hydroxymethyl and coupling to the methyl on the ring. This also seems rather unlikely as most R-N-C(O)-R bonds (i.e., peptides) are broken or formed between the nitrogen and carbonyl moieties, not between the carbonyl and the "R" group.

Formation of the latter compound, another quinoline, would also require an unlikely metabolic reaction. The structure resembles MVI (Figure 6) except that the ketone is replaced by a methyl and a hydrogen. The fungus must remove the entire chloracetyl group and add an ethyl or remove the methylchloride and the ketone oxygen and add a methyl; neither possibility seems plausible biochemically.

Scan numbers (SN) 129 and 148 (peaks MVII and MVIII, Figure 5) are very similar except that SN 148 has more background; it is unclear as to whether *m/e* 91 (tropilium ion) is real or is background. Both scans have a parent peak at *m/e* 205. This is important in that an alternative structure having mass 205, *N*-(2-methoxy-1-methylethyl)-2-oxindole, seems incapable of producing the tropilium, considering the expected fragmentation pattern. This compound would be an extremely unusual product for the fungus to produce as it lacks the 6'-methyl on the phenyl. Considering the known metabolites which were extracted from the media, the fungus has apparently not yet removed any group from the ring, although it has dehydrogenated the ethyl moiety to form a vinyl on both the metolachlor and Anthor rings. This is also a proposed reaction for alachlor in route to the indoline. This particular indole, then, is considered to be a very unlikely candidate as a metolachlor metabolite. It does not easily fit into the degradation scheme without several manipulative reactions. The last alternative structure having mass 205 which could produce the appropriate fragmentation is *N*-(2'-ethyl-6'-methylphenyl)-*N*-(2-methoxy-1-methylethyl)imine, similar in structure to Antor metabolite AI (Figure 3). This seems unlikely as a biologically formed metabolite, and apparently is not formed via photodegradation as there were no peaks in metolachlor control flasks incubated without mycelium.

Summary. The Antor and metolachlor metabolites are arranged in plausible schemes of degradation (Figures 3 and 6). The arrows between the compounds indicate possible routes of degradation. Hypothetical intermediates which were not recovered are in brackets. A general pattern of metabolism of these two new herbicides and alachlor by _C_. _globosum_ has emerged. The general transformations which occur are:

1. Dehalogenation with hydroxylation: MIV, AVI; postulated but not observed for alachlor.
2. Dehydrogenation of the 6'-ethyl: MIV, MVII, AIII, AVI, AVII; also postulated but not observed for alachlor.
3. Dealkylation:
    a. from the nitrogen - MI, MVII, AI, AII; AII is also formed from alachlor (demethoxymethylalachlor, or "DDM"), as well as formation of 2',6'-diethylaniline.
    b. demethoxylation, leaving hydroxyl - MII.
    c. deethoxylation, leaving hydroxyl (acid) - AVII.
4. Dealkylation and ring formation:
    a. indoline formation - MVIII, AIV, AV; AIV is also formed from alachlor.
    b. oxoquinoline formation - MIII, MV, MVI.

With respect to known metabolites, Antor is unique from alachlor and metolachlor in the formation of metabolite AVII where the ethoxy moiety is removed and an acid is formed. Similarly, formation of the three quinolines, MIII, MV, and MVI, from metolachlor is a reaction unique to this herbicide. MIII is formed from the coupling of the hydroxyacetyl group with the 6'-methyl on the ring to form the oxoquinoline. Further paring of the 2-methoxy-1-methylethyl group forms the isopropyl moiety, thus MV. MVI could be formed from MIV by removal of the hydroxyl and subsequent ring formation.

Formation of an imidazole has also been observed in the degradation of isopropalin (2,6-dinitro-_N_,_N_-dipropylcumidine) in field soil (Golab and Althaus, 1975). This imidazole is structurally similar to the indoline but contains two nitrogens in the one and three positions. To the knowledge of this author, formation of the oxoquinoline is a previously undiscovered transformation.

Since approximately 50% of the Antor and alachlor metabolites and probably some of the metolachlor metabolites remain in the aqueous fraction after extraction, it would be of interest to determine what further types of transformations occur to cause these compounds to become water soluble. This is especially important since water soluble products would more likely leach through soil. Also, work of S.-F. Chou (Michigan State University, unpublished data) indicates that many of the metabolites found in soil, for example from Antor, are quite polar and can only be extracted with sodium pyrophosphate.

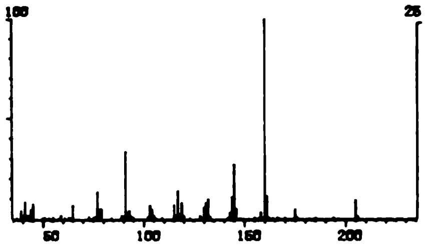
Appendix A

Appendix A

This appendix contains the original mass spectral data which were used to determine the structures of Antor and metolachlor (Dual) metabolites. The structures are presented in the results section of this thesis. The mass spectral data of the Antor and metolachlor metabolites has been organized according to the scan numbers, located in the left corner of each scan. The scan numbers correspond to the peaks in the GLC traces in Figures 2 and 4. The number of the structure (from Figures 3 and 6) corresponding to each scan is written as a Roman Numeral in the left corner of each scan.

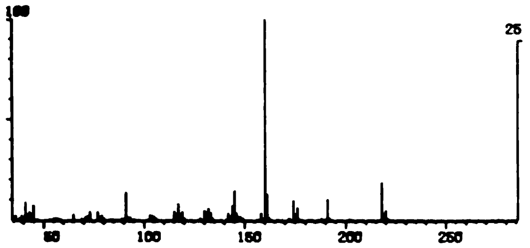
DUAL VII OR VIII

• 129



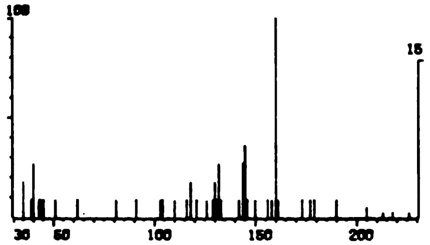
DUAL IV OR III

• 383



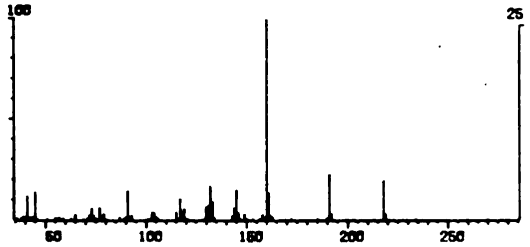
DUAL VII OR VIII

• 140



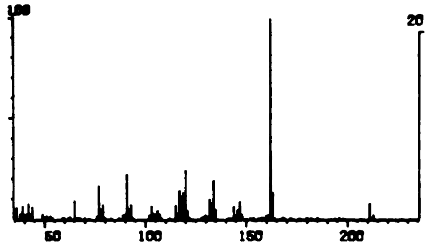
DUAL III OR IV

• 384



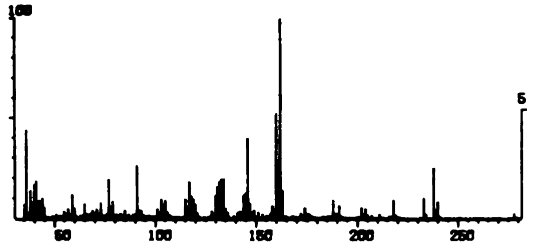
DUAL I

• 208



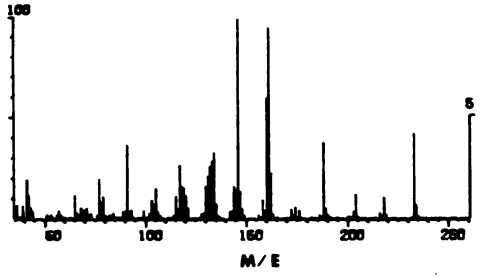
DUAL II

• 408



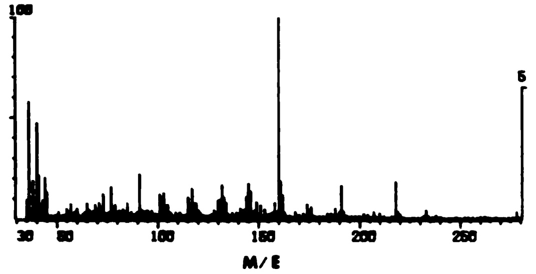
DUAL VI

• 230



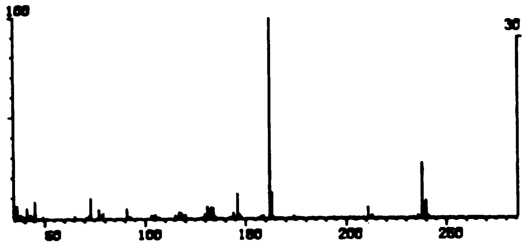
DUAL V

• 437



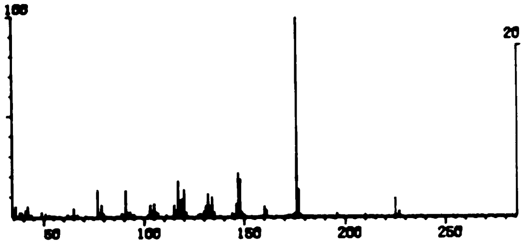
DUAL

• 372



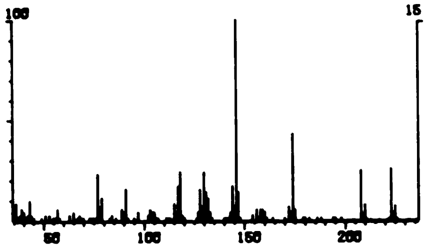
ANTOR II

• 244



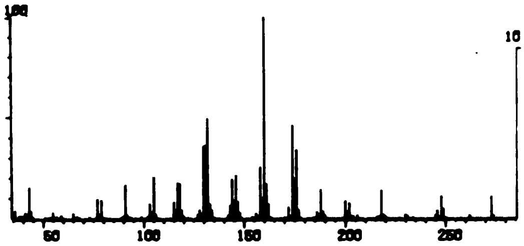
ANTOR IV

• 206



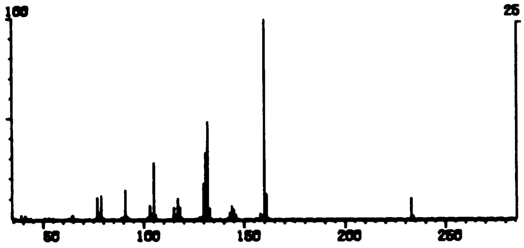
ANTOR VI

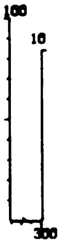
• 460



ANTOR I

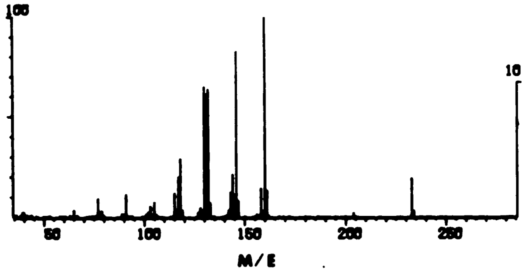
• 214





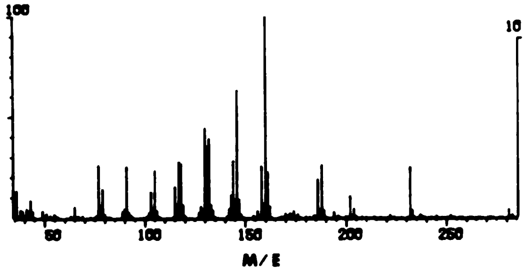
ANTOR V

• 283

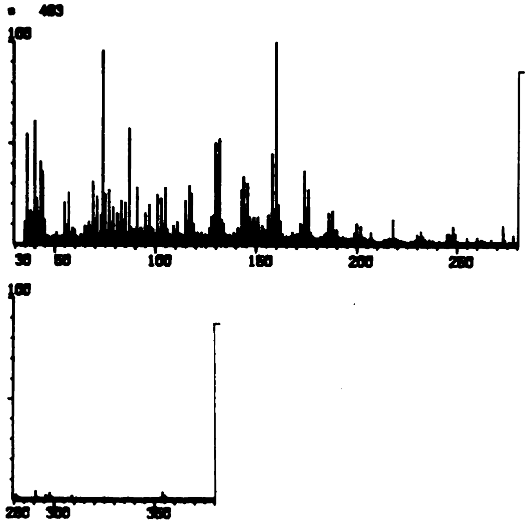


ANTOR VII

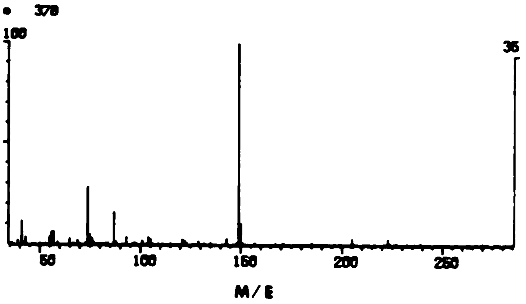
• 611



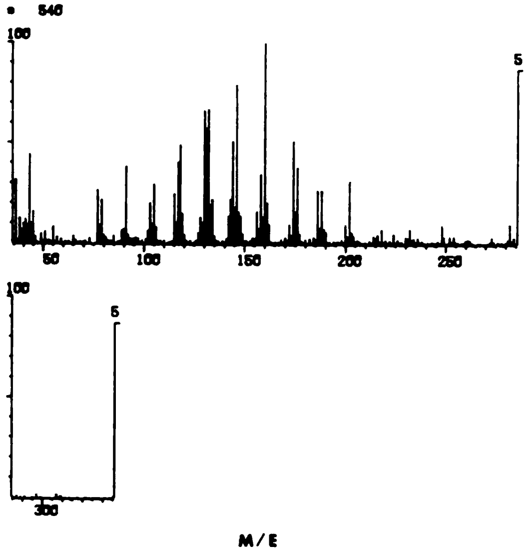
ANTOR III



ANTOR; PHTHALATE?



ANTOR VIII



Appendix B

Appendix B

Appendix B contains a brief presentation and discussion of work done in an attempt to find an enzyme(s) of _C_. _globosum_ responsible for the initial steps in the degradation of alachlor.

MATERIALS AND METHODS

Instrumental Analysis and Reagents. Gas-liquid chromatographic analysis was done on the same instruments and under identical conditions as those used for analysis of Antor, as their retention times are similar. However, a 1.95% OV-17-1.5% QF-1 liquid phase on Chromosorb W solid support (Supelco, Inc., Bellefonte, Pa.) was used. This was packed in a 2 m x 2 mm stainless steel column. The source and purification of alachlor are presented by Tiedje and Hagedorn (1975).

Procedures. Cells were grown for use in preparing enzyme solutions as described in the text of this thesis. I made several attempts to find enzyme activity on alachlor after breakage of the fungal cells. Following growth of the fungal cells, they were harvested, and then suspended in an alachlor solution (resting cells), usually for 18-25 hours, to promote production of the enzyme (if it is an inducible enzyme). A comparison of induced and non-induced cells in resting cell experiments showed that there was no enhancement of alachlor degrading activity in induced cells, thus indicating that the enzyme was constitutive.

In experiments testing for an inducible exoenzyme, a "zero time" sample was removed when the resting cell experiment was begun. After incubating 12-24 hours, the cells were harvested and the media sterilized by filtration through an 0.22 μ Millipore filter. The filtrate was incubated with alachlor for 65-96 hours, after which the alachlor was quantitated in all samples (e.g. 0, 24, and 96 hours) to determine if it had been degraded further after removal of the fungus.

In experiments testing for an enzyme in the cell extract, the cells were harvested by filtration, weighed, washed, and ground in a mortar and pestle. Grinding of the cells was performed with the presence of very fine glass beads and 0.02 M phosphate buffer on a 1:1 (w/w) and 1:1 (w/v) basis, respectively. This cell slurry was then homogenized in a glass homogenizer, and finally passed through a French press to ensure more extensive breakage. All the cell breakage steps were performed in ice or with equipment which had been cooled in the freezer. Occasionally extra buffer was added. The buffer frequently contained various reagents for stabilizing enzyme activity such as dithiothreitol, phenylmethylsulfonylfluoride (PMSF), thioglycolic acid, sodium salt (Na-thioglycolate), or cysteine (all from Sigma Chemical Co., St. Louis, Mo.) as described in the results (Table 1).

After cell breakage the extract was centrifuged in a refrigerated centrifuge at 13,000 rpm for 15-20 min. Generally, the enzyme assay was begun by adding 1 ml of the cell extract to 3 ml of alachlor solution. This mixture was then incubated at 28° in a water bath. Further details of the variations in each experiment are reported in Table 1.

The anaerobic enzyme experiment was done in Thunberg tubes which were evacuated to 30 psi, then flushed with argon to equalize the pressure. Na-thioglycolate was added to preserve reductive conditions. For the dialysis experiment, the cell extract was dialyzed against 3 liter 0.02 M phosphate buffer, pH 7.2, for 6 hours 20 minutes; the buffer was changed, and dialysis continued for 10 more hours before the assay was begun. Most of the assay incubations were for 20-25 hours.

The 4 ml enzyme assay was stopped by precipitation of the protein with 10% trichloroacetic acid (final concentration). It was then extracted and analyzed by the same methods as those used for the antor and metholachlor experiments.

RESULTS AND DISCUSSION

Enzyme Activity on Alachlor. The induced exoenzyme experiments showed that no exoenzyme was produced by *C. globosum* since there was no alteration of the alachlor molecule. Even though some of the enzyme could have adhered to the filter, the incubation times were sufficient (65-96 hours) that if any enzyme were present a detectable change in the amount of alachlor should have occurred. Finally, further investigations using cell extract did not produce an active enzyme preparation capable of transforming alachlor or demethoxymethylalachlor (Table 1).

A number of variations in enzyme treatment and assay conditions were attempted in an effort to obtain a cell-free extract active on alachlor. These include various buffers, reductants, dialysis, protease inhibitors, cofactors, and ATP and NADPH generating systems. These variations are more completely described in Table 1. In no case was any enzyme activity observed. There is no obvious explanation for the complete loss of activity after cell breakage; however, it is of note that a similar circumstance has plagued those studying lignin metabolism by fungi (Kirk, 1976).

Table 1. Variations in treatment of cell extract after breakage and additions to the assay in an attempt to obtain activity on alachlor.

Purpose	Buffer[2] (pH)	Additions[3] to buffer	Cofactors, generating systems	Cell extract -- c filtrate - f whole cell extract[1] - w	Substrate[6] (ppm)	Incubation time (hours)	Enzyme activity
"Simple" Assay	A(7.6)	dtt (2 mM)		c[5]	A(100)	25	-
Cofactors; uncentrifuged cell extract[1]; filtrate	A(7.6)	dtt (1 mM)	NADH or NADPH$_2$ 10 μM/tube	c, w	A(75)	25	-
Activity on alachlor metabolite (DMM)	A(7.6)			c	B(75)	24	-
Induced exoenzyme	B(7.2)			f	A(100)	65	-
Induced exoenzyme	B(7.2)			f	A(100)	96	-
Protease inhibitor; chelator; pH	B(8.0) B(5.2)	PMSF (4 mM) EDTA (1 mM) dtt (1 mM)		c	A(75)	25	-
Anaerobic conditions	B(7.0)	dtt (1 mM) NaTG (2 g/l)		c	A(75)	25	-
Dialysis of cell extract	B(7.0)	dtt (2 mM)		c	A(75)	40	-
Cofactors; diluted (1:100) vs undiluted cell extract	B(7.5)	Cyst (2 mM)	a. NADH, ATP b. NADPH$_2$, ATP c. NADH, ATP	c	A(75)	40	-
ATP & NADPH$_2$ generating systems	C(7.6)		reference[4]	c	A(60)	20	-

TABLE 1

[1] Uncentrifuged cell extract was used to check for membrane-bound enzyme activity.

[2] Buffer A: 0.05 M tris(hydroxymethyl)aminomethane-hydrochloride (TRIS) buffer; Buffer B: 0.02 M phsophate buffer; Buffer C: 0.05 M Tris-maleate buffer

[3] dtt - dithiothreitol
PMSF - phenylmethylsulfonylfluoride
EDTA - ethylenediamine tetraacetic acid [(ethylene dinitrilo)-tetraacetic acid], disodium salt
NaTG - thioglycolic acid, sodium salt
Cyst - cysteine
All concentrations refer to the concentration of the "addition" in the buffer before adding it to the cells which were being broken

[4] Procedures from Worthington loose-leaf notebook, *Enzymes, Enzyme Reagents* (Worthington Biochemical Corp.), page 4-68, 2.7.3.2 (ATP generating system), and page 4-69, 1.1.1.49 (NADPH generating system).

[5] In all cases, except this one, the cells were induced in a resting cell experiment prior to extracting.

[6] Substrate A is alachlor; substrate B is demethoxymethylalachlor (DMM).

[7] No activity as determined by no change in substrate concentrations as detected by GLC analysis after incubation.

List of References

LIST OF REFERENCES

1. Beestman, G. B., and J. M. Deming. 1974. Dissipation of acetanilide herbicides from soils. Agron, J. 66:308-311.

2. Black, D. 1977. Personnal communication. (Hercules, Inc., Wilmington, De.)

3. Bray, G. A. 1960. A simple efficient liquid scintillator for counting aqneous solution in a liquid scintillation counter. Anal. Biochem. 1:279-285.

4. Chou,S.-F. J. 1974. Biodegradation of alachlor [2-chloro-2',6'-diethyl-N-(methoxymethyl)arentanile-6] in soils. M. S. Thesis, Michigan State University. 28 pp.

5. Golab, T., and W. A. Althaus. 1975. Transformation of isopropalin in soil and plants. Weed Sci. 23:165-171.

6. Kirk, K. 1976. Personal communication

7. Taylor, R. W. 1972. Degradation of alachlor [2-chloro-2',6'-diethyl-N-(methoxymethyl)acctomide] in soils and by microorganisms. M. S. Thesis, Michigan State University. 25 pp.

8. Tiedje, J. M., and M. L. Hagedorn. 1975. Degradation of alachlor by a soil fungus, Chaetomium globosum. J. Agr. Food Chem. 23:77-81.