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Anaerobic Metabolism of the Herbicide, Butachlor in soil

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토양중에 있어서 제초제 Butachlor의 혐기적 대사

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抄 錄

均一하게 環標識된 ^{14}C-butachlor를 二種의 한국토양중에서 三個月間 혐기적으로 배양시 생성된 $^{14}CO_2$와 휘발성 물질의 量은 근소하였다. 토양A에서는 고압살균한 경우와 살균하지 않은 경우 처리된 방사능의 77.52%와 45.36%가 각각 MeOH로 추출되었고 나머지는 토양중에 흡착되었다. 반면 토양B에서는 58.85%와 37.23%가 각각 추출되었고 나머지는 토양중에 잔류하였다. ^{14}C-butachlor의 흡착은 토양의 특성에 좌우되었다. 또한 GLC-MS에 의하여 2, 6-diethyl-N-(butoxymethyl)-acetanilide가 주된 대사산물로 밝혀졌고 2, 6-diethyl aniline과 2, 6-diethyl acetanilide는 소량 생성되었다.

INTRODUCTION

Butachlor, 2-chloro-2′, 6′-diethyl-N-(butoxymethyl) acetanilide, has now been in wide use as a preemergence herbicide for the main crops, including rice plant in Korea. In this connection, the elucidation of its degradation products is believed to be very important in terms of environmental safety. Degradation of an analogous herbicide, alachlor by a common soil fungus, *Chaetomium globosum* produced chloride and four organic metabolites: 2-chloro-2′, 6′-diethyl acetanilide, 2, 6-diethyl-N-(methoxymethyl) aniline, 2, 6-diethylaniline, and 1-chloroacetyl-2, 3-dihydro-7-ethylindole[1)]. Similar metabolic studies were also conducted with Antor (H-22234) and Dual (Metolachlor) herbicide, using *Chaetomium globosum* in pure culture[2)].

Environmental factors affecting the detoxication and subsequent degradtion of butachlor

were studied and three organic soluble and five aqueous soluble metabolites were separated. Two of the organic soluble compounds were tentatively identified as the N-dealklated and the α-hydroxy derivatives[3]. Several soil microorganisms were reported to degrade effectively butachlor in the 0.02M KH_2PO_4 buffer solution at pH 5.2 for fungi or pH 7.0 for bacteria.

Mucor sufui NTU-358 produced 2-chloro-2′, 6′-diethyl acetanilide, 2-hydroxy-2′, 6′-diethylacetanilide, 2, 6-diethylaniline, N-chloroacetyl-7-ethyl-2, 3-dihydroindole, 2, 6-diethylacetanilide and N-methyl-2-chloro-2′, 6′-diethylacetanilide as the metabolites[4]. Lee[5] also incubated *Chaetomium globosum* in a butachlor contained medium and characterized the metabolites obtained therefrom by GLC-MS. Three commercial α-chloroacetanilide herbicides, alachlor, butachlor, and propachlor, were surface-applied in aqueous solution on two soil types in the field to determine their soil half-lives. In this study, dissipation from sterilized soil was 50 times slower than that from viable soil, indicating that microbial decomposition played a major role in herbicide degradation[6].

Nevertheless, no attempt has been made of the elucidation of the anaerobic metabolites of butachlor. In the present investigation, uniformly ring-labeled ^{14}C-butachlor was applied to two types of soils and incubated anaerobically for three months. The identities of the resulting metabolites were elucidated.

Table 1. Physico-chemical properties of the soils used

Soils \ Characteristics	Total sand (%)	Silt (%)	Clay (%)	Textural class	pH (1 : 1)		
					H_2O	1N-KCl	0.01N-$CaCl_2$
Chong Ju Soil (Soil A)	43.1	41.4	15.4	Silty loam	4.83	3.82	4.7
Chung Ju Soil (Soil B)	30.5	53.1	16.5	Clayey loam	6.43	6.04	6.3

C (%)	OM (%)	Available P_2O_5(ppm)	T-N (%)	Exchangeable (me/100g)				C.E.C. (me/100g)
				K^+	Na^+	Ca^{++}	Mg^{++}	
1.01	2.45	35	0.12	0.08	0.08	2.33	1.23	7.7
1.79	2.9	44	0.14	0.05	0.09	10.5	1.3	14.14

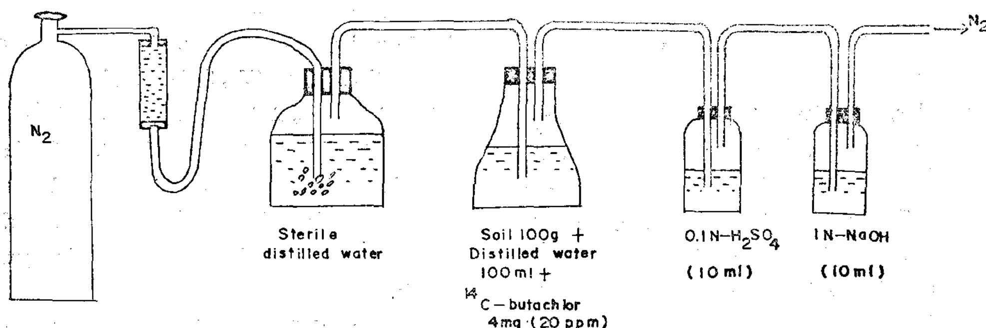

Fig. 1. The apparatus for an anaerobic incubation of ^{14}C-butachlor in soil

Materials and Methods

Chemicals: Butachlor of analytical grade and uniformly ring-labeled ^{14}C-butachlor were obtained from Monsanto Agricultural Products Co., St. Louis, Mo., U.S.A. The ^{14}C-butachlor has a chemical and radiochemical purity of 98%. The sample amounts to 50μCi and its specific activity was 11.31mCi/mM.

Soils used: The physico-chemical properties of the soils which were used for the anaerobic incubation are shown in Table 1.

Anaerobic incubation: 100g of soil A and soil B(*ChongJu* soil), each, was put in 500m*l* Erlenmeyer flasks and 100m*l* of distilled water added to them. The flasks were thoroughly shaken to give homogeneous mixtures. ^{14}C-butachlor solutions dissolved in ethanol were added to each flask to give a concentration of 20ppm. The initial radioactivity of ^{14}C-butachlor added to each incubation flask amounts to 413, 417 dpm. As the controls, 100g of esoil A and soil B, respectively, in 500m*l* erlenmeyer flasks was sterilized by autoclaving at 121°C for 30min. This procedure was repeated 5 times to ensure complete sterilization. 100m*l* of sterile distilled water was added to each flask in like manner. For the sterile control soil A and soil B, two replicates were placed and three replicates for viable soil A and soil B, respectively. The surfaces of soils were flooded with water of about 0.5cm thickness. Further, to provide an anaerobic condition, nitrogen gas passing through the cotton filter was flushed into the flask continuously throughout the incubation period. The incubation was kept at 18~20°C for three months. 0.1 N-H_2SO_4 and 1 N-NaOH solutions were used to trap the volatile products and $^{14}CO_2$ release from the ^{14}C-butachlor in soils, respectively, as shown in Fig. 1.

Measurement of radioactivity: The radioactivities trapped in the 0.1 N-H_2SO_4 and 1 N-NaOH solutions were measured with an Isocap/300 Liquid Scintillation System, Nuclear Chicago and Beckman Model LS 8000 Scintillation Counter with an LS 9000 Data Reduction System, Beckman Instrument Corp., Fullerton, California. Each sample, 0.5m*l*, was added to the scintillation vials containing 10m*l* of Bray's solution. The Bray's solution consists of 60g of naphthalene, 100m*l* of methanol, and 8g of Omnifluor dissolved in 1 liter of dioxane. Omnifluor® is the trademark of Pilot Chemicals, Inc., which is a blend of 98% PPO and 2% Bis-MSB.

Extraction of soils treated with ^{14}C-butachlor after anaerobic incubation.: After the anaerobic incubation of three months, the supernatants were separated from the soils. The soils were air-dried by leaving them at room temperature. About 10g of each sample was set aside for extraction experiments. The rest 90g of each sample was extracted with four 100ml portions of methanol by shaking for 40min on a shaker of 180rpm. The samples were centrifuged at 3,500rpm for 5min. in every extraction. The methanol extract which was collected after filtration and combined was concentrated on a rotary evaporator to dryness to remove the moisture. The residue was then redissolved in methanol.

Distribustion of radioactivity between aqueous and organic phases in the extraction of the supernatants: For the measurement of total radioactivity of the supernatant separated from the soil after incubation, 0.5m*l* of each supernatant was added to 10m*l* of the scintillation cocktail and the radioactivity was measured. For the radioactivity of aqueous and organic phases, 3m*l* of each supernatant and 3m*l* of chloroform were put in screw-capped test tubes, and shaken vigorously. After allowing them to stand for a while, the radioactivities of each phase were measured by taking 0.5m*l* as in the case of the total radioactivity.

Measurement of radioactivities of soils separated from supernatants.: Radioactivity was measured with a Packard Tri-Carb Sample Oxidizer (Tritium-Carbon Oxidizer). Each

weighed soil sample was combusted under oxygen flushing. The resulting $^{14}CO_2$ was trapped with ethanolamine and the radioactivity was measured in scintillation cocktail. $^{14}CO_2$ trapped with ethanolamine was rinsed 6 times with methanol from the apparatus.

Gas-liquid chromatography: The analyses were performed with a Finnigan 9500 gas chromatograph equipped with a flame ionization detector. The column was a pyrex glass of 6mm (OD)×6 ft packed with 5% SE-30 on Chromosorb W-HP. Operating parameters were as follows: helium carrier flow 30ml/min; oxygen 20ml/min; hydrogen 100ml/min; air 40ml/min; injection temp. 240°C; detector temp. 250°C. All analyses were made by temp-programming from 100°C to 250°C at the rate of 10°C/min.

Mass Spectrometry.: Mass spectra were obtained by a combination of GLC-MS using a Finnigan 3200 Gas Chromatogaph-Mass Spectrometer. The electron ionization potential was 70 eV. A Finnigan MS Data System 6000 was used for the recordings of the mass spectra.

Radioactivity scanning: In order to locate the radioactivity on TLC plates, TLC-Scanner LB 2760 (berthold) was used.

Thin-layer chromatography: It was accomplished on precoated analytical plates of silica gel HF-254 with fluorescent indicator (layer thickness 0.25mm, EM Laboratories Inc., Associate of E. Merck, Darmstadt, Germany) using a solvent system, benzene-methanol (85 : 15, v/v). The separated substances were detected under a UV lamp (254nm wavelength).

Results and Discussion

Volatile products from ^{14}C-butachlor in an anaerobic soil condition.: Table 2 presents the radioactivities of the volatile products from ^{14}C-butachlor in an anaerobic soil condition in the course of three months' incubation. As can be seen in this table, the amounts of volatilization are so small throughout the incubation period that only parts of the results are presented. In both soil A and B, the viable soils produced more volatile products than the sterile

Table 2. Volatile products from ^{14}C-butachlor in an anaerobic soil condition

Treatments	Replicates	Initial radioactivity (dpm)	Radioactivities at each incubation period(weeks) (dpm)		
			5	7	8
Soil A	1	413,417	65.8	69	83.8
sterile	2	〃	39.4	39.4	39.4
control	Mean	〃	52.6(0.01%)	54.2(0.01%)	61.6(0.01%)
Soil A	3	〃	98.4	201.2	300.6
viable	4	〃	148	329.8	480.4
	5	〃	31.8	197.2	403.2
	Mean	〃	92.7(0.02%)	242.7(0.06)	394.7(0.1%)
Soil B	6	〃	43	75.4	153.8
sterile	7	〃	29.8	63.8	108.2
control	Mean	〃	36.4(0.009%)	69.6(0.02%)	131(0.03%)
Soil B	8	〃	183.4	358.8	567.8
viable	9	〃	210.8	356.2	510.4
	10	〃	176.2	286.8	534.2
	Mean	〃	190.1(0.05%)	333.9(0.98%)	537.5(0.1%)

control soils, which is somewhat suggestive of the microbial activity. However, no significant differences were recognized between soil A and B.

$^{14}CO_2$ evolution: Table 3 shows the evolution of $^{14}CO_2$ from ^{14}C-butachlor as a function of incubation periods in an anaerobic soil condition. As seen in table3, in both soils no great differences were recognized between the sterile control and viable soils. There are no differences between soil A and B, either. At the 8th week of incubation, only an average of 0.03 and 0.05% of the initial radioactivity in viable soil A and B, respectively, was evolved as $^{14}CO_2$. This result indicates that in an anaerobic soil condition, the degradation of ^{14}C-butachlor to $^{14}CO_2$ resulting from the fission of the benzene ring is very small. Presumably, this is partly due to the relatively low temperature (18~20°C) and the anaerobic flooded condition, as pointed out by Bowman *et al.*[7], that decomposition was markedly diminished in wet soil.

Distribution of radioactivity between aqueous and organic phases in the solvent extraction of the supernatants separated from the soils: After three months of anaerobic incubation, each soil sample was centrifuged to separate the supernatant from the soil. the total radioactivities of the supernatants and their distribution between aqueous and chloroform phase in the extraction with chloroform are shown in Table 4. As can seen in Table 4, in both soil A and B, the radioactivities of the supernatants in sterile control soils were higher than those in viable soils. In other words, less ^{14}C-butachlor was adsorbed on the autoclaved soils. This result will presumably be due to the fact that the organic matter and clay in the soils which are responsible for the pesticide adsorption were destroyed during autoclaving. In addition, the possibility of microbial adsorption in the viable soils can not be ruled out relative to the sterile control soils. The radioactivity distribution between aqueous and chloroform phases is suggestive of the involvement of microorganisms in the degradation of ^{14}C-butachlor. In the case of the sterile control soil A, the radioactivity of chloroform phase is

Table 3. $^{14}CO_2$ evolution from ^{14}C-butachlor in an anaerobic soil condition

Treatments	Replicates	Initial radioactivity (dpm)	Radioactivities at each incubation period (weeks) (dpm)		
			5	7	8
Soil A	1	413,417	21.2	22	22
sterile	2	413,417	32.4	85.2	108.4
control	Mean	413,417	26.8(0.006%)	53.6(0.01%)	65.2(0.02%)
Soil A	3	413,417	81.2	170.2	170.2
viable	4	413,417	104.6	119.4	119.4
	5	413,417	70	70	70
	Mean	413,417	85.3(0.02%)	119.9(0.03%)	119.9(0.03%)
Soil B	6	413,417	33.2	51.8	108.6
sterile	7	314,417	24.6	25.4	161.8
control	Mean	413,417	28.9(0.007%)	38.6(0.009%)	135.2(0.03%)
Soil B	8	413,417	68	68	137.6
viable	9	413,417	90	231.8	330.4
	10	413,417	113,8	135.6	146.2
	Mean	413,417	90.6(0.02%)	145.1(0.04%)	204.7(0.05%)

Table 4. Distribution of radioactivity between aqueous and organic phases in the solvent extraction of supernatants separated from the soils after anaerobic incubation

Treatments	Replicates	Percentage to the initial radioactivity		
		Supernatant (total)	Aqueous phase	Chloroform phase
Soil A sterile control	1	9.7	1.6	8.1
	2	9.9	1.6	8.3
	Mean	9.8	1.6	8.2
Soil A viable	3	5.9	1.4	4.5
	4	5.9	1.3	4.6
	5	6.3	1.4	4.9
	Mean	6.0	1.4	4.7
Soil B sterile control	6	5.6	1.7	3.9
	7	6.2	2.2	4.0
	Mean	5.9	2.0	4.0
Soil B viable	8	3.0	1.3	1.7
	9	3.3	1.4	1.9
	10	2.7	1.3	1.7
	Mean	3.0	1.3	1.7

5-fold greater than that of aqueous phase, whereas in the viable soil A, the radioactivity of chloroform phase was only 3-fold greater than that of aqueous phase. These results indicate that in the viable soil A, more polar metabolites were formed by soil microorganisms during incubation. Meanwhile, in the sterile control soil B, the radioactivity of chloroform phase was 2-fold greater than that of aqueous phase, whereas in the viable soil B, the radioactivity of chloroform phase was almost the same as that of aqueous phase. The big differences between soil A and B will be due to the different physicochemical characteristics: soil B has higher pH (6.43), organic matter (2.9 %), and Ca^{++} content (10.5me/100g) compared with soil A. Accordingly, it is believed that anaerobic microorganisms are more active in degrading ^{14}C-butachlor in soil B than in soil A.

Radioactivities of the soils separated from the supernatants: The radioactivities (dpm/g soil) of the soils separated from the supernatants after an anaerobic incubation are presented in Table 5. As can be seen in this table, after extration of the sterile control soil A with methanol, an average of 22.48% of the radioactivity of the soil before extraction was adsorbed in soil and 77.62% extracted with methanol. Meanwhile, in the viable soil A, the radioactivities were 54.64 and 54.36%, respectively. On the other hand, in the sterile control soil B the radioactivities were 21.15 and 58.85%, respectively; whereas in the viable soil B, 62.77 and 37.23%, respectively. As discussed in the supernatants, more ^{14}C-butachlor was adsorbed in soil B which is characterized by the textural class of clay loam, higher organic matter content, and higher C.E.C. (14.14me/100g) compared with soil A. This result is in good agreement with the report by Chen and Chen[8] that Tainan silt loam with lower clay and organic matter content showed a tendency to adsorb less butachlor than Hsinchu silty clay loam.

Table 5. Radiocativities of the soils separated from the supernatants after incubation

Treatments	Replicates	Radioactivity (dpm/g soil)		
		Before extraction	After extraction	Methanol extract
Soil A	1	7575.75	1747.65(23.07%)	5828. 1(76.83%)
sterile	2	8037.7	1758.39(21.88%)	6279.31(78.12%)
control	Mean	7806.73	1753.02(22.48%)	6053.71(77.52%)
Soil A	3	7791.73	4078.25(52.34%)	3713.48(47.66%)
viable	4	7720.68	4615.66(59.78%)	3105.02(40.22%)
	5	8171.06	4233.73(51.81%)	3937.33(48.19%)
	Mean	7894.49	4309.21(54.64%)	3585.28(54.36%)
Soil B	6	9363.53	3578.99(38.22%)	5784.54(61.78%)
sterile	7	9074.05	4000.28(44.08%)	5073.77(55.92%)
control	Mean	9218.79	3789.64(41.15%)	5429.16(58.85%)
Soil B	8	8931.94	6027.22(67.48%)	2904.72(32.52%)
viable	9	9273.99	5608.48(60.48%)	3665.51(39.52%)
	10	9052.12	5463.35(60.35%)	3588.77(39.65%)
	Mean	9086.02	5699.68(62.77%)	3386.33(37.23%)

Metabolites of ^{14}C-butachlor: For the metabolic study of ^{14}C-butachlor in an anaerobic soil condition, the concentrated methanol extract was applied on a TLC plate and developed in the solvent system. The radioactivity of each band on the TLC plate was confirmed with a scanner (Fig. 2). For the elucidation of the metabolites, the methanol extract was analyzed by GLC-MS. Fig. 3 shows the gas-liquid chromatogram of the metabolites of ^{14}C-butachlor in an anaerobic incubation in soil B. Since the methanol extract of soil A showed the similar metabolites, it was not presented here. In Fig. 3, quite a few peaks stemming from different origins were not able to be identified. Only three metabolites could be elucidated based on the GLC-MS data at this time.

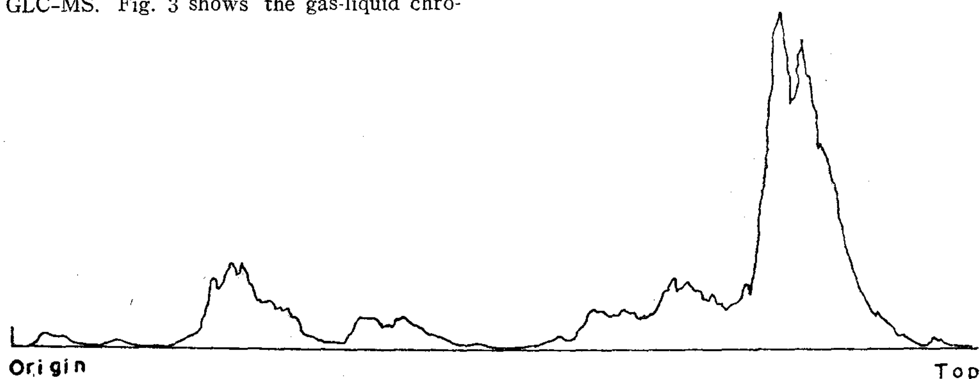

Fig. 2. Radioactivity scanning of the metabolites of ^{14}C-butachlor in soil B
Radioactivity applied: 10,000ppm
TLC developing solvent: Benzene-Methanol (85 : 15, v/v)

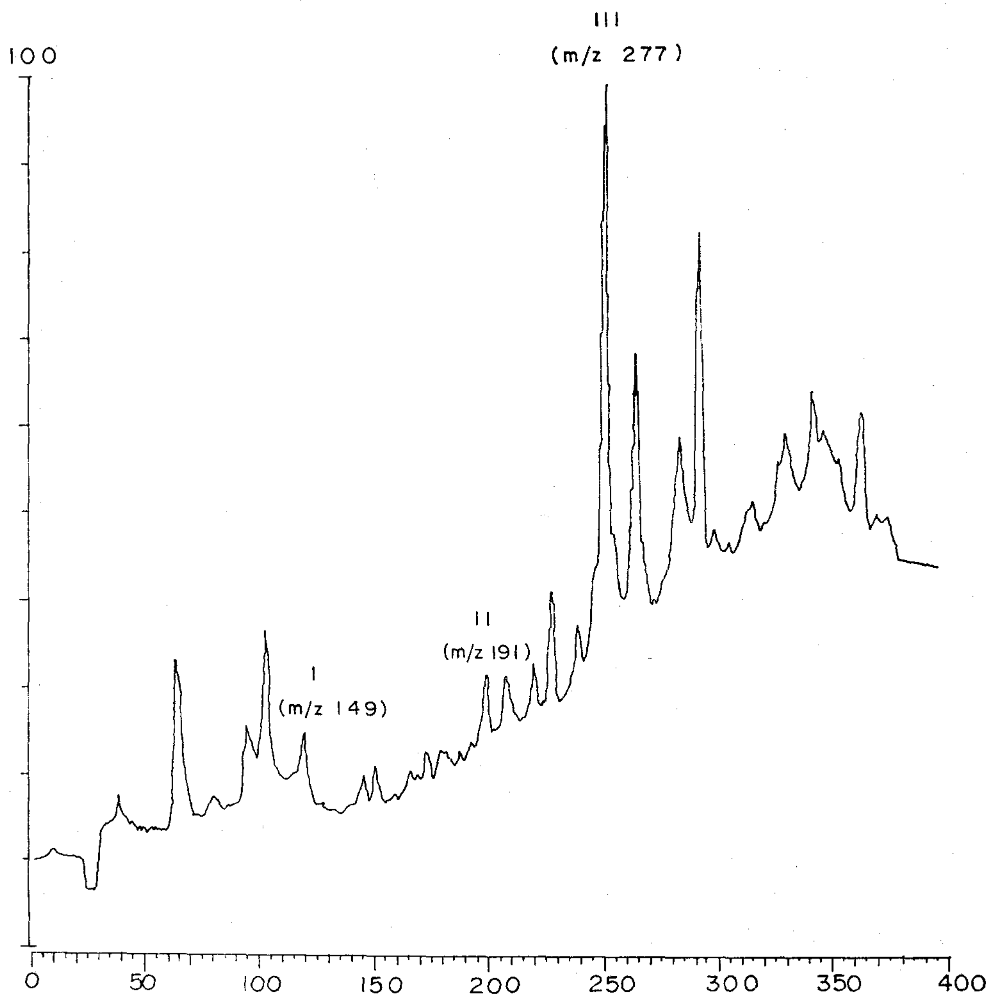

Fig. 3. Gas-liquid chromatogram of the metabolites of ^{14}C-butachlor in an anaerobic incubation in soil B for 3 months.

Metabolite Ⅰ : Fig. 4 shows the mass spectrum of metabolite Ⅰ. In this spectrum, the molecular ion occurs at m/z 149. The base peak at m/z 134 corresponds to the $(M—CH_3)^+$ ion. The fragment peak at m/z 119 corresponds to the $(M—CH_3, CH_2)^+$ion. The presence of the m/z 91 peak indicates the possible formation of tropylium ion in fragmentation. The fragment peak at m/z 77 will be the $C_6H_5^+$ ion. Based on this fragmentation pattern, metabolite Ⅰ must be 2, 6-diethylaniline.

Metabolite Ⅱ : *Fig. 5 shows the mass spectrum of metabolite* Ⅱ. Here the molecular ion appears at m/z 191. The base peak at m/z 43 *is in good agreement with the presence of an* acetylgroup in the structure. The fragment peaks at m/z 176 and 162 correspond to the $(M—CH_3)^+$ and $(M—CH_2CH_3)^+$, respectively. The fragment peak at m/z 149 is believed to *derive from the loss of a molecule of ketene* from the molecular ion, which is characteristic of the acetanilides. The intense peaks at m/z 148 and 134 are thought to correspond to the $(M—CH_3CO)^+$ and $(M—O=C=CH_2, CH_3)^+$ ions, *respectively. In consequence, metabolite* Ⅱ is believed to be 2, 6-diethylacetanilide.

Metabolite Ⅲ.: Fig. 6 shows the mass spectrum of metabolite Ⅲ. In this spectrum, the molecular ion appears at m/z 277. The base peak at m/z 57 indicates the presence of C_4H_9 moiety in the structure. The intense peak at m/z 43 is also suggestive of the presence of acetyl group. The fragment peak at m/z 220 will correspond to the $(M-C_4H_9)^+$ and/or $(M-O=C=CH_2, CH_3)_+$ ion. The fragement peaks at m/z 190, 178, 161, and 146 are thought to be the $(M-CH_2OC_4H_9)^+$, $(M-C_4H_9, O=C=CH_2)^+$, $(M-OC_4H_9, CH_3CO)^+$, and $(M-OC_4H_9, CH_3CO, CH_2)^+$ ions, respectively. Accordingly, metabolite Ⅲ

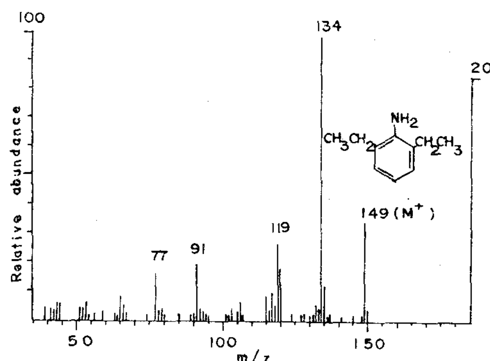

Fig. 4. Mass spectrum of metabolite Ⅰ, 2,6-diethyl aniline

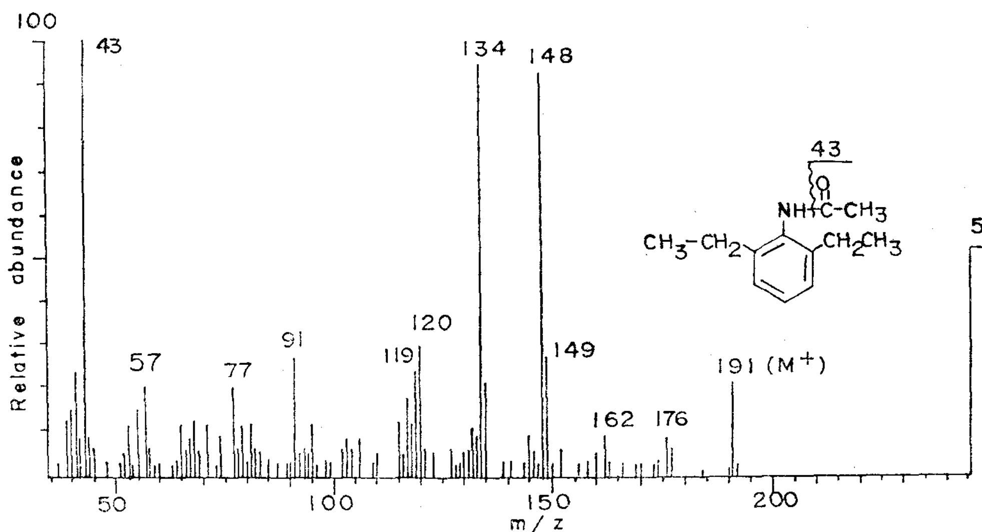

Fig. 5. Mass spectrum of metabolite Ⅱ, 2,6-diethyl acetanilide

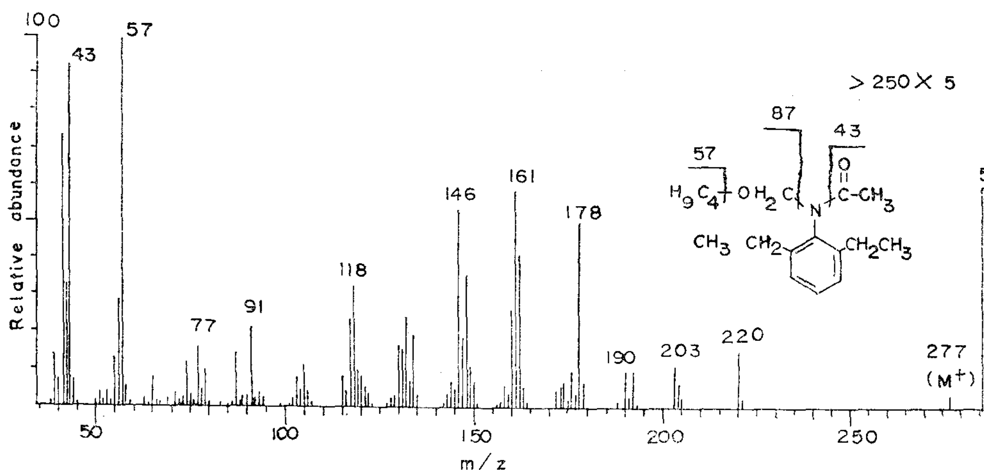

Fig. 6. Mass spectrum of metabolite Ⅲ, 2,6-diethyl-N-(butoxymethyl) acetanilide

is believed to be 2,6-diethyl-N-(butoxymethyl) acetanilide.

Of the metabolites obtained in the present investigation, 2,6-diethylaniline was also identified in pure cultures of *Chaetomium globosum* incubated with an analogous compound, alachlor[1] and of *Mucor sufui* NTU-358 with butachlor[4]. The formation of 2,6-diethylacetanilide was also reported by Chen and Wu[4]. Nevertheless, the metabolite Ⅲ, 2,6-diethyl-N-(butoxymethyl) acetanilide has not been previously reported. According to Baird[3] $^{14}CO_2$ evolution from carbonyl- and ring-labeled butachlor in Ray silt loam soil was the most significant at high temperature (32°C), and inhibited by flooding in comparison to nonflooded moisture levels of 15%. Additionally, he identified the N-dealkylated and α-hydroxy derivatives as the organic soluble metabolites of butachlor. Instead, in the present anaerobic soil condition, butachlor seems to undergo dechlorination very readily, since no chlorine-containing metabolites could be detected on GLC-MS. N-dealkylation also occurred to from 2,6-diethylaniline and 2,6-diethylacetanilide. Furthermore, it is noteworthy that the chlorine atom in the structure of butachlor was replaced by a hydrogen atom, not by a hydroxyl group which is thought to be common in soil and pure cultures of microorganisms[3,4]. The reaction mechanisms involved in the metabolism should be mainly biological, but the possibility of chemical degradation can not be ruled out, considering that the soil temperature was relatively low during incubation.

Acknowledgment

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Abstract

In an anaerobic incubation of uniformly ring-labeled ^{14}C-butachlor in two *Korean* soils for 3 months, very little $^{14}CO_2$ and volatile products were measured. In soil A, 77.52 and 45.36% of the sterile and viable soil radioactivity, respectively, were methanol-extractable and the rest were adsorbed in soil; whereas in soil B, 58.85 and 37.23%, respectively, were methanol-extractable and the rest remained in soil. The adsorption of ^{14}C-butachlor depends on the characteristics of the soils. The major metabolite was 2,6-diethyl-N-(butoxymethyl) acetanilide. 2,6-Diethylaniline and 2,6-diethylacetanilide turned out to be the minor metabolites on GLC-MS.

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