Microbial Degradation of ¹⁴C-2, 6-Diethylaniline in Soil and in Pure Culture

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¹⁴C-2, 6-Diethylaniline의 토양미생물에 의한 분해

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抄 錄

¹⁴C-2, 6-diethylaniline 을 토양중에서 호기적으로 21주간 배양시 발생된 ¹⁴CO₂ 는 살균하지 않은 A토양(clay loam)과 B토양(coarse sandy loam)에서 각각 6.5%와 10.1%이었다. Methanol 로 토양을 추출시 A토양에서는 3.1%, B토양에서는 13.5%의 토양방사능이 추출되었다. 토양중에서의 분해산물은 2, 6-diethylacetanilide 이었으며 *Chaetomium globosum* 은 순수배양시 분해산물로 2, 6-diethyl-p-benzoquinone 을 생성하였고 이의 생성경로는 2, 6-DEA 의 p-hydroxylation, quinoneimine 의 형성 그리고 암모니아 발생을 수반하는 가수분해 등으로 제안하였다.

Introduction

Quite a few pesticides containing a substituted aniline moiety produce the aniline as one of their main biodegradation products in soil and in pure culture of microorganisms. Particularly, 2, 6-diethylaniline (2, 6-DEA) was reported to be formed by the microbial degradation of butachlor[1] and alachlor[2] which have now been in wide use as preemergence herbicides in Korea. Usually, the anilines are very difficult to extract with organic solvents or to release

by acid and alkaline hydrolysis from soil[3,4]. In soil and/or in pure cultures of isolated microorganisms, halogenated anilines have been known to undergo condensation to form azobenzenes[5] or diphenylamine[6], acylation[7,8], oxidation of the amine to a nitro group[9], and phenoxazinone formation[10,11,12]. In the biological effects on humus-bound 3, 4-dichloroaniline, the evolution of ¹⁴CO₂ under aerobic conditions was found to be at the rate of 1% per week[13]. Bollag et al.[14] treated 4-chloroaniline, 4-chloro-o-toluidine, 4-isopropylaniline, and 2, 6-dimethylaniline in autoclaved and non-autoclaved

soils and incubated for six weeks. The release of volatile products and $^{14}CO_2$ was measured for each aniline.

In the present investigation, ^{14}C-2, 6-DEA was applied to two different Korean soils and incubated under aerobic conditions for 21 weeks, since very little research has been done on this chemical. The amounts of $^{14}CO_2$ and volatile products liberated from soil were determined during the incubation period and the degradation products were followed. In addition, several soil microorganisms were tested for their possible degradability of 2, 6-DEA and the de-

gradation products were elucidated.

Materials and Methods

^{14}C-2, 6-DEA.

The uniformly ring-labeled ^{14}C-2, 6-DEA-HCl was purchased from New England Nuclear Corp., Boston, Mass., U.S.A. It had a radiochemical purity of 98% and a specific activity of 11.4 mCi/mmole.

Soils and incubation.

The physico-chemical properties of the soils which were used for the aerobic incubation are presented in Table 1. One hundred grams of

Table 1. Physico-chemical properties of the soils used

Characteristics / Soils	Total sand (%)	Silt (%)	Clay (%)	Textural class	pH(1 : 1)		
					H$_2$O	1N-KCl	0.01N-CaCl$_2$
A	30.4	53.1	16.5	CL	6.43	6.04	6.3
B	71.0	17.7	11.3	CoSL	4.4	3.3	3.65

C (%)	OM (%)	Available P$_2$O$_5$(ppm)	T-N (%)	Exchangeable(me/100g)				C.E.C. (me/100g)
				K$^+$	Na$^+$	Ca$^{+ +}$	Mg^{++}	
1.79	2.9	44	0.14	0.05	0.09	10.5	1.3	14.14
0.88	1.51	15.2	0.21	0.23	0.02	0.75	0.92	10.50

each of soil A and B were put in 500ml Erlenmeyer flasks and a ^{14}C-2, 6-DEA solution dissolved in 1ml of ethanol was added to each flask to give 692, 285 dpm and the two were mixed thoroughly. Forty milliliters of distilled water were then added to each flask to provide 60~70% of the water holding capacity of the soils. The final concentration of ^{14}C-2, 6-DEA in the moist soils amounts to 21 ppm. As controls, 100 g of soil A and B in 500 ml Erlenmeyer flasks were sterilized by autoclaving at 121°C for 30 min; this procedure was repeated 4 times to ensure complete sterilization and 40ml of a sterilized 0.1% sodium azide solution was added to each flask. For soil A and B, two replicates were placed as sterile controls and three replicates as non-sterile soils. To provide aerobic conditions, air was continuously flushed through the flasks during the incubation period of 21

weeks at a temperature of 20~30°C. 0.1 N H$_2$SO$_4$ and 1 N NaOH solutions were used to trap volatile products and $^{14}CO_2$, respectively, from the soils treated with ^{14}C-2, 6-DEA (Fig. 1).
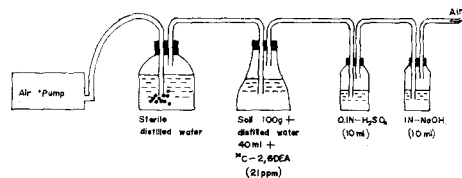

Fig. 1. The apparatus for an aerobic incubation of ^{14}C-2, 6-diethylaniline in soil

Measurement of radioactivity.

Radioactivity was measured with a Beckman Model LS-100C Liquid Scintillation Counter (Beckman Instruments Inc., Irvine, California). Samples of 1 ml were added to vials containing

10 ml of a scintillation solution consisting of 60 g of naphthalene, 100 ml of methanol, and 8 g of Omnifluor (New England Nuclear Corp., Boston, Mass.) which is a blend of 98% PPO (2, 5-diphenyloxazole) and 2% Bis-MSB (p-bis [o-methylstyryl]benzene) dissolved in one liter of dioxane. The radioactivity countings of volatile products and $^{14}CO_2$ were made every week up to the 21st week. After 21 weeks of aerobic incubation, the soils were air-dried at room temperature. About 10 g of each soil sample was set aside for the radioactivity measurement before extraction with methanol. The remaining 90 g of each sample was extracted with five 50 ml portions of methanol by shaking for 20 min on a shaker at 180 rpm. The samples were then centrifuged at 5, 000×g for 5 min. The methanol extract was collected and the combined extracts were concentrated on a rotary evaporator to dryness. The residue was redissolved in chloroform. Radioactivity of soil samples was measured after dry combustion with a Packard Tri-Carb Sample Oxidizer (Tritium-Carbon Oxidizer). The resulting $^{14}CO_2$ was trapped with ethanolamine, washed several times with methanol and analyzed for radioactivity. The volatile products trapped in 0.1N H_2SO_4 were neutralized with NaOH, extracted with chloroform, dried over anhydrous sodium sulfate, and concentrated to a small amount for elucidation.

Thin-layer chromatography.

TLC was performed on precoated analytical plates of silica gel HF-254 with a fluorescent indicator (Art. 5554, DC-Alufolien Kieselgel 60 F_{254}, 20×20 cm, Schichtdicke 0.2 mm, E. Merck, Darmstadt) using solvent systems consisting of (A) xylene-chloroform-acetone (40 : 25 : 35, v/v) and (B) chloroform-ethanol (90 : 10, v/v). The separated substances were visualized under a UV lamp (254 nm wavelength).

Autoradiography.

The radioactive spots on the plates were detected by autoradiography using an X-ray film (FUJI RX, Safety, 4). The X-ray film was exposed to the developed TLC plates for 15 days.

Gas-liquid chromatography.

The analyses were performed with a GC-4C Shimadzu Gas Chromatograph, equipped with a flame ionization detector. The column was 6 ft×1/8 in, 5% SE-30 on 60~80 mesh Shimalite W(AW-DMCS) 201 D. Operating parameters were as follows: helium carrier flow, 60 ml/min; hydrogen, 0.7 kg/cm²; air, 0.8 kg/cm²; injection port temp, 190°C; detector temp, 210°C. All analyses were made by temperature-programming from 100 to 240°C at a rate of 8°C/min.

Mass spectrometry.

Mass spectra were obtained by a combination of GC-MS and the direct insertion probe using a Finnigan 3200 Gas Chromatograph-Mass Spectrometer. The electron ionization potential was 70 eV. A Finnigan MS Data System 6000 was used for the recordings of the mass spectra.

High performance liquid chromatography.

A Waters Liquid Chromatograph Model 450 (Variable wavelength detector) was used with acetonitrile-water (60 : 40, v/v) as the solvent.

Media used and incubation of microorganisms in pure culture.

For the incubation of bacteria, Bacto Nutrient Broth (Difco Laboratories, Detroit I, Michigan, U.S.A.) was used. To rehydrate the medium, 8 g was dissolved in 1, 000 ml of distilled water and the pH was adjusted to 7.0. The medium was autoclaved at 121°C for 15 min. As the medium for fungi, potato dextrose broth was used[15]. Microorganisms tested for the degradability of 2,6-DEA include *Pseudomonas putida*, *Pseudomonas denitrificans*, *Bacillus megaterium*, and two isolates from the soil incubated with 2,6-DEA for 21 weeks as bacteria. *Chaetomium globosum* and *Rhizoctonia solani* were assayed as fungi. Each bacterium was incubated for 4 days in 100 ml of nutrient broth medium. To each pre-incubated flask, 2 mg of 2,6-DEA dissolved in ethanol was added and

incubated for 10 days. After completion of the incubation, the mixtures were extracted with two 30 ml portions of chloroform.

The combined extracts were concentrated on a rotary evaporator to a small amount for GLC. The fungi were incubated for 8 days in 500 ml of potato dextrose broth at 28°C, respectively. At the end of 8 day incubation, 10 mg (20 ppm) of 2-6-DEA was added to the medium and further incubated for 21 days. The extraction of the degradation products was done in a like manner.

Synthesis of authentic 2, 6-diethyl-p-benzoquinone.

2, 6-Diethyl-p-benzoquinone was prepared by a commercial method of p-benzoquinone by the oxidation of aniline[15]. A mixture of 2, 6-DEA, manganese dioxide, and dilute sulfuric acid (3 N) was allowed to react overnight at 5°C and steam-distilled. The red brown 2, 6-diethyl-p-benzoquinone in the distillate was extracted with chloroform. The extract was then concentrated by flushing air through the test tube. The material obtained was further purified twice on preparative TLC plates using the solvent B as the developing solvent for mass spectrometry (direct insertion probe).

Results and Discussion

Volatile products and [14]CO$_2$ from ^{14}C-2, 6-DEA-treated soils under aerobic conditions.

As can be seen in Table 2 and Fig. 2, volatile products were formed at an average rate of 0.1% per week throughout the 21 weeks of incubation in both soil A and B, regardless of the sterile or non-sterile soil. Meanwhile, the evolution of [14]CO$_2$ varies considerably with soil types. In the case of the sterile control, an average of 0.1% per week was measured in both soil A and B all alike.

In the case of non-sterile soils, however, an average of 0.31% and 0.48% was evolved from soil A and B, respectively. In other words, in the 21st week of incubation, 6.5% and 10.1% of [14]CO$_2$ were formed from soil A and B, respectively. These results prove that microorganisms were involved in the degradation of 2, 6-DEA in soil. Among other things, comparing the physical properties of the two soils used, soil B is a textural class of coarse sandy loam with 71% of total sand. Accordingly, soil B will be far better for the growth of the aerobic microorganisms responsible for the degradation of 2, 6-DEA than soil A which is a textural class of clay loam. The chemical properties of the soils do not seem to affect the degradation of this aniline compound, since there is not much difference between soil A and B. Considering the pH 4.4 of soil B, it seems probable that fungi rather than bacteria would have been involved in this degradation.

Adsorption of 2, 6-DEA on soils.

Table 3 shows the radioactivity of the methanol extracts from the soils incubated with ^{14}C-2, 6-DEA. In the sterile controls, 13.2% and
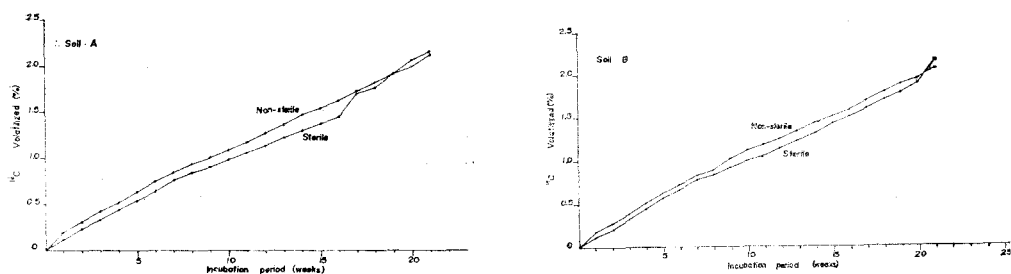

Fig. 2. Volatile products trapped in 0.1N H$_2$SO$_4$ from ^{14}C-2, 6-DEA during incubation in sterile and non-sterile soil A and B. The ^{14}C trapped is presented as percent of the total radioactivity applied to the soils.

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Table 2. Release(%) of $^{14}CO_2$ and volatile ^{14}C-labeled products from ^{14}C-2, 6-DEA in sterile and non-sterile soils incubated under aerobic conditions (Initial radioactivity: 692, 285 dpm/100 g soil)

Soils	Treatments	Weeks / Products	1st	2nd	3rd	4th	5th	6th	7th	8th	9th	10th	11th
Soil A	Sterile	Volatile products	0.12	0.23	0.34	0.45	0.54	0.65	0.76	0.84	0.90	0.99	1.07
		$^{14}CO_2$	0.14	0.28	0.41	0.53	0.65	0.77	0.84	0.93	1.06	1.15	1.25
	Non-sterile	Volatile products	0.19	0.31	0.42	0.52	0.63	0.75	0.84	0.93	1.01	1.09	1.18
		$^{14}CO_2$	1.48	1.66	2.37	2.62	3.08	3.34	3.72	4.09	4.43	4.73	4.86
Soil B	Sterile	Volatile products	0.08	0.19	0.31	0.45	0.57	0.67	0.77	0.86	0.92	1.00	1.06
		$^{14}CO_2$	0.12	0.23	0.35	0.47	0.62	0.73	0.82	0.95	1.08	1.17	1.24
	Non--sterile	Volatile products	0.12	0.26	0.37	0.52	0.63	0.73	0.82	0.89	1.02	1.11	1.18
		$^{14}CO_2$	1.05	2.20	3.75	4.86	5.65	6.12	6.61	7.16	7.87	8.28	8.59

Soils	Treatments	Weeks / Products	12th	13th	14th	15th	16th	17th	18th	19th	20th	21st	Average/week
Soil A	Sterile	Volatile products	1.14	1.22	1.30	1.38	1.45	1.70	1.77	1.91	2.06	2.15	0.10
		$^{14}CO_2$	1.34	1.40	1.48	1.57	1.71	1.79	1.88	1.98	2.14	2.25	0.11
	Non-sterile	Volatile products	1.28	1.37	1.47	1.55	1.63	1.71	1.82	1.91	2.00	2.13	0.10
		$^{14}CO_2$	5.02	5.12	5.23	5.33	5.47	5.67	5.88	6.05	6.26	6.47	0.31
Soil B	Sterile	Volatile products	1.15	1.23	1.31	1.42	1.50	1.59	1.70	1.77	1.94	2.17	0.10
		$^{14}CO_2$	1.37	1.47	1.57	1.66	1.77	1.86	1.99	2.05	2.25	2.41	0.11
	Non-sterile	Volatile products	1.26	1.35	1.43	1.50	1.58	1.69	1.78	1.87	1.97	2.08	0.10
		$^{14}CO_2$	8.75	8.87	9.00	9.12	9.22	9.45	9.68	9.76	9.88	10.08	0.48

Table 3. Radioactivity of the soils after an aerobic incubation of 21 weeks with ^{14}C-2, 6-DEA

		Replicates	Radioactivity (dpm/g soil)		
			Before extraction	After extraction	Methanol extract
Soil A	Sterile	1	3810	3157	653(17.1 %)
		2	4433	4019	414(9.3 %)
		Average	4122	3588	534(13.2 %)
	Non-sterile	3	4725	4478	247(5.2 %)
		4	6227	6127	100(1.6 %)
		5	5648	5510	138(2.4 %)
		Average	5533	5372	162(3.1 %)
Soil B	Sterile	6	8950	7420	1530(17.1 %)
		7	7583	6016	1567(20.7 %)
		Average	8267	6718	1549(18.9 %)
	Non-sterile	8	8465	7648	817(9.7 %)
		9	8137	7175	962(11.8 %)
		10	6819	5515	1304(19.1 %)
		Average	7807	6779	1028(13.5 %)

18.9 % of the radioactivity of soil A and B, respectively, were extractable.

Meanwhile, in the non-sterile soils, 3.1% and 13.5% of the radioactivity of soil A and B were extracted. As can be expected from the physico-chemical properties of the soils (Table 1), a much smaller amount of radioactivity could be extractable from soil A which has a higher content of organic matter (2.9%) than from soil B with a lower content (1.5%). Besides this characteristic, soil A is clay loam in textural class, whereas soil B is coarse sandy loam. Hsu and Bartha[17] suggested that

anilines are bound to humus or soil organic matter rather than to clay particles. Nevertheless, it appears that soil textural class affects much the extraction of the radioactivity from the soils with organic solvents. In an attempt to clarify the identity of the volatile products, the chloroform extract from the 0.1N H_2SO_4 solution which was thought to trap the volatile products was subjected to HPLC, GLC, and TLC, followed by autoradiography. Even if it showed a few peaks in HPLC and GLC, no radioactivity could be detected on the autoradiogram as can be seen in Fig. 4. Therefore, the identification
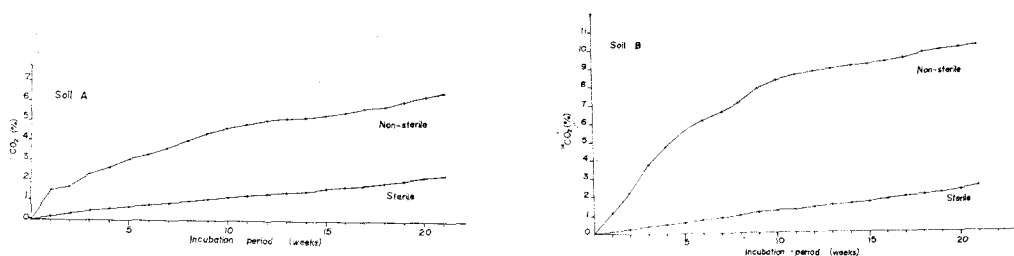

Fig. 3. $^{14}CO_2$ evolution from soil A and B treated with ^{14}C-2, 6-DEA as a function of incubation period. $^{14}CO_2$ is presented as percent of the total radioactivity applied to the soils. Negligible $^{14}CO_2$ formation was found in the sterile soils.

was not able to be made.

Degradation products of 2, 6-DEA in soil.

The methanol extracts from the soils incubated with ^{14}C-2, 6-DEA were subjected to GLC. Small amounts of 2, 6-diethylacetanilide (Retention time: 7.95 min) were detected in both the sterile and non-sterile samples of soil A and B all alike. The fact that 2, 6-diethylacetanilide was formed in the sterile as well as the non-sterile soils might be due to the prolonged incubation period of 21 weeks, which could provide a chance of inoculation. In the case of 3, 4-dichloroaniline, the formation of 3, 4-dichloroacetanilide has been shown in soil, but not in sterile soil[18]. In general, acylation is thought to be a detoxification mechanism in various microorganisms.

The gas-liquid chromatograms of the methanol extracts showed quite a few peaks. Therefore, in order to check the radioactivity of the peaks, an autoradiogram was prepared (Fig. 4). As
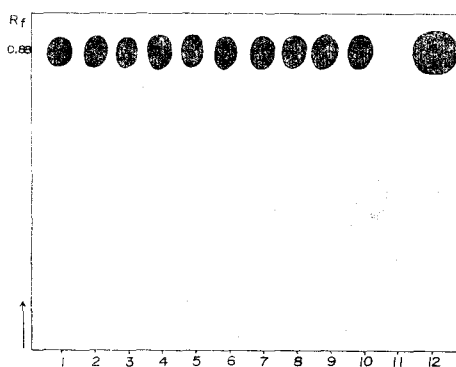

Fig. 4. Autoradiogram of methanol extracts from soils treated with ^{14}C-2, 6-DEA and incubated aerobically for 21 weeks
Solvent : chloroform-ethanol
   (90 : 10, v/v)
1, 2 : Sterile soil A
3, 4, 5 : Non-sterile soil A
6, 7 : Sterile soil B
8, 9, 10 : Non-sterile soil B
11 : 0.1 N H_2SO_4-absorbed material
   (Volatile products)
12 : Authentic ^{14}C-2, 6-DEA

seen in this autoradiogram, only the spots corresponding to undegraded ^{14}C-2, 6-DEA (Rf: 0. 88) were detected in all the extracts. And hence, further GC-MS analyses were not made of these soil extracts. Concerning HPLC, as the separation of the degradation products was not satisfactory, GLC was adopted for the subsequent analyses.

Degradation products of 2, 6-DEA in pure cultures of selected bacteria.

The chloroform extracts of the media which were incubated with the respective bacteria in the presence of 2, 6-DEA were subjected to GLC. The gas-liquid chromatograms did not show any degradation products, compared with the control media which were incubated with only the bacteria without 2, 6-DEA in a like manner.

Degradation products by *Chaetomium globosum* in pure culture.

Since *Rhizoctonia solani* did not produce any degradation products from 2, 6-DEA, *Chaetomium globosum* was followed for the degradation. The chloroform extract of the medium in which *Chaetomium globosum* was incubated with 2, 6-DEA was also subjected to GLC (Fig. 5). The
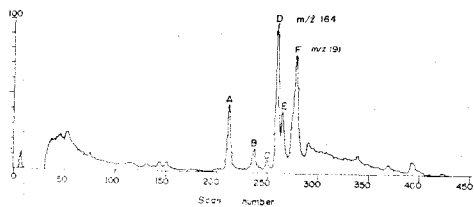
Fig. 5. Gas-liquid chromatogram of the degradation products of 2, 6-DEA by *Chaetomium globosum*

mass spectra of all the peaks on the gas-liquid chromatogram were taken. Of the mass spectra, however, only two could be elucidated. One of these two products was 2, 6-diethylacetanilide which is known to be usually formed. Therefore, its mass spectrum is not presented here. Fig. 6 shows the mass spectrum of the other degradation product of 2, 6-DEA which has not
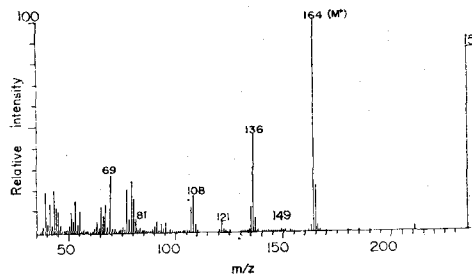
Fig. 6. Mass spectrum of the degradation product of 2, 6-DEA (m/z 164) by *Chaetomium globosum*

been reported as yet. In this spectrum, the molecular ion peak occurs at m/z 164, which is also the base peak. The M-15 peak at m/z 149 corresponding to M-CH_3 should occur. In this case, however, this fragment ion is not prominent as expected. The M-28 fragment ion at m/z 136 corresponds to M-CO. The fragment ion at m/z 121 is thought to be due to M-CO, CH_3. The fragment ion peak at m/z 108 derives from the elimination of two molecules of carbon monoxide from the molecular ion. In general, most benzoquinones give intense molecular ion peaks, and a common feature of their spectra is the occurrence of ions corresponding to the loss of one and of two molecules of carbon monoxide. Based on all this fragmentation pattern, the mass spectrum of the degradation product is in good agreement with that of the authentic 2, 6-diethyl-p-benzoquinone. For further verification, the mass spectrum of authentic 2, 6-diethyl-p-benzoquinone was obtained by the direct insertion probe (Fig. 7). Comparing these two spectra (Fig. 6 and 7), all the main fragmentation pattern is almost identical with minor differences in intensity of the fragments. It might be due to the different methods of getting mass spectra by GC-MS and direct insertion probe using different instruments. For a qualitative test for quinones, the chloroform extract obtained from the incubation medium of *Chaetomium globosum* was developed on a TLC plate using the solvent A. The spot corresponding to the
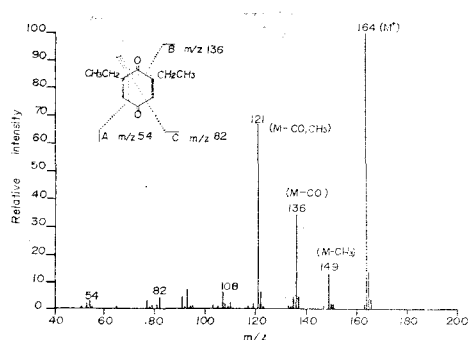
Fig. 7. Mass spectrum (direct insertion probe) of authentic 2,6-diethyl-p-benzoquinone

authentic 2,6-diethyl-p-benzoquinone (Rf: 0.93) was visualized as violet with the spraying reagent, o-dianisidine solution saturated in acetic acid which is usually used for the detection of aldehydes and ketones[19].

Finally, a possible pathway for the formation of the degradation product, 2,6-diethyl-p-benzoquinone, is proposed in Fig.8. In this scheme, the formation of II which was not detected in this incubation is quite probable. Fletcher and Kaufman[20] reported the hydroxylated products of 2-, 3-, and 4-chloroanilines in the cultures of *Fusarium oxysporum* Schlecht. The evidence for the ring hydroxylation in substituted anilines can also be found in other papers[9,10,21].

Sloane et al[22]. reported the formation of aniline from p-aminobenzoic acid and the oxidation of aniline to p-aminophenol by *Mycobacterium smegmatis*. They suggested a long preoxidative lag phase in the oxidation of aniline. In this respect, at least, the relatively long incubation period of 21 weeks in our study will be sufficient for the hydroxylation of 2,6-DEA. III can be formed by dehydrogenation from II as in the case of p-aminophenol[22]. The degradation product, IV, will be readily formed by hydrolysis of III removing ammonia. A similar mechanism can be found in the metabolism of 3-chloro-4-methoxyaniline and some N-acyl derivatives in soil[6].

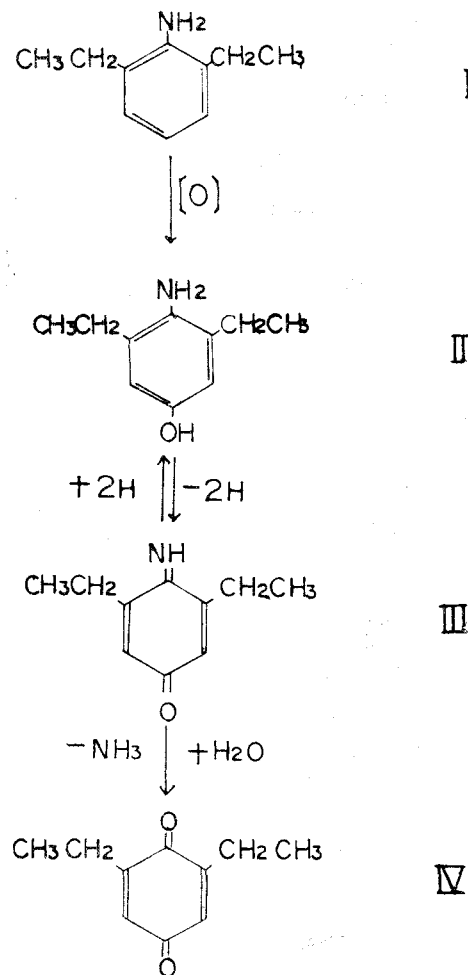Meanwhile, in the study on aniline utilization

Fig. 8. Possible pathway for the formation of the degradation product, 2,6-diethyl-p-benzoquinone from 2,6-DEA in *Chaetomium globosum* culture

by a soil bacterium, Briggs and Walker[10] postulated that the early stages of aniline metabolism involved oxidation to catechol with liberation of ammonia. Udod et al[23]. showed the possibility of hydrolytic deamination in the decomposition of p-nitroaniline. In our scheme, however, p-hydroxylation of 2,6-DEA, formation of quinoneimine, and the subsequent hydrolysis with the release of ammonia are more favored than the other alternative pathways.

Abstract

When ^{14}C-2,6-diethylaniline (2,6-DEA) was incubated aerobically in soil, $^{14}CO_2$ evolved from non-sterile soil A and B was 6.5 and 10.1%, respectively, in the 21st week. Methanol could extract 3.1 and 13.5% of the radioactivity from soil A and B, respectively. 2,6-Diethylacetanilide was detected as a degradation product in soil. *Chaetomium globosum* produced 2,6-diethyl-p-benzoquinone as a degradation product in pure culture. A possible pathway was proposed to include p-hydroxylation of 2,6-DEA, formation of quinoneimine, and the subsequent hydrolysis with the release of ammonia.

Acknowledgment

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