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Studies on the Decomposition of Environmental Pollutants by Utilizing Microorganisms

— Ⅱ. Microbial Degradation of TOK (2,4-Dichloro-4′-nitro diphenyl ether) in Soil.—

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微生物을 利用한 環境汚染源의 分解에 關한 硏究

— Ⅱ. 土壤中에서 TOK (2,4-Dichloro-4′-nitro diphenyl ether)의 微生物學的 分解—

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ABSTRACT

1. When Chong Ju and Chung Ju soils possessing different physicochemical properties were treated with 500ppm of TOK and incubated in flooded anaerobic condition for 2, 4, and 6 months, respectively, they produced 4-Chloro-4′-amino diphenyl ether, 2,4-Dichloro-4′-amino diphenyl ether(amin-TOK), N-[4′-(4-Chlorophenoxy)] phenyl acetamide, and N-[4′-(4-Chlorophenoxy)] phenyl formamide as the metabolites. This result indicates that TOK undergoes the reduction of its NO_2 to NH_2 group, dechlorination, acetylation, and formylation under this condition. The cleavage of ether linkage does not occur. In addition, TOK degrades more readily in Chung Ju soil which is characterized by pH 6.43 and higher contents of Ca^{++} and C. E. C. than in Chong Ju soil which is lower in pH, Ca^{++}, and C.E.C.
2. In the aerobic incubation of TOK of 25 ppm in Chung Ju soil suspensions for 21 days, the ratio of the resulting metabolites, TOK: amino-TOK: 4-Chloro-4′-amino diphenyl ether was 100 : 130 : 76. Meanwhile, in the 42 day incubation, the ratio was 100 : 19 : 5, which indicates that TOK in aerobic condition does not necessarily degrade as a function of the incubation period.
3. The citrate buffer extract of Chung Ju soil has the capability of degrading TOK, which was verified to be due to the action of the microorganisms involved.
4. Twelve strains of soil bacteria were isolated from the TOK-treated soils. In the incubation of TOK in pure cultures of the respective isolates, the strain T-1-1 isolated from Chong Ju

脚註 : 本 硏究는 1981年度 文敎部 基礎科學 硏究費에 依한 것임.

soil had almost no degradability. whereas the strain T-2-3 was the most potent. The degradation of TOK by the isolates constituted mostly the reduction of the nitro group to amino group.

5. In a test for the degradability of TOK by some selected microorganisms, *Pseudomonas* species were more potent than fungi. Yet, Isolate B which had been isolated from Chung Ju soil suspension was the most potent.

INTRODUCTION

TOK(2, 4-Dichloro-4′-nitrodiphenyl ether) is one of the most important herbicides being used in flooded rice fields in Korea. Nevertheless, the environmental fate of the diphenyl ethers including TOK has received relatively little attention. In general, substituted diphenyl ether compounds have excellent herbicidal activity and have been reported to function essentially as inhibitors of chloroplast noncyclic electron transport. The main site of action appears to be associated with light reaction II and the oxygen evolution pathway(Moreland *et al.*, 1970).

Gutenmann, *et al.* (1967) reported that 2,4-Dichloro-4′-amino-diphenyl ether(Amino-TOK) was rapidly detected in rumen fluid as a metabolite of TOK in dairy cows. Crosby, *et, al.* (1971) demonstrated that TOK was quickly photolyzed in water to 2,4-Dichlorophenol and p-Nitrophenol, and the nitro group also undervent both reduction (formation of Amino-TOK) and nucleophilic displacement by hydroxide ions to give the corresponding phenol and nitrite ion.

Ichihashi, *et al.* (1971) compared the residual effects in soil of MO-338 (2, 4, 6-Trichloro-4′-nitrodiphenyl ether), TOK, and X-52 (2,4-Dichloro-3′-methoxy-4′-nitrodiphenyl ether) by bioassay with barnyard grass and other weeds. They found that in enriched soil solution, the reduced amino derivatives formed from all these diphenyl ethers.

MO-338, TOK, X-52, and MO-500 (2,4-Dichloro-6-fluoro-4′-nitro-diphenyl ether) were compared by Kuwatsuka (1971) as to their degradation in soils under flooded and upland conditions.

The fate of TOK in the foliage of some weeds was investigated by Hawton, *et al.*(1971) with the aid of C^{14}-TOK.

In the present investigation, as part of an effort to understand and elucidate what noxious materials can be produced in our environment from the xenobiotics, TOK was incubated in several ways in Korean rice paddy soils which are characterized by different physico-chemical properties, with special emphasis on microbial degradation.

The metabolites obtained from each incubation were identified by means of TLC, GLC, and MS.

MATERIALS AND METHODS

Soils used

The physico-chemical properties of the soils which were used in this investigation are presented in Table 1.

Culture media used

The composition of Medium A which was used for the soil suspension is presented in Table 2. Table 3 shows the composition of Medium B which was used for the pure culture of fungi. The composition of Medium C which was used for the culture of bacteria is shown in Table 4.

Table 1. Physico-chemical properties of the soils used

Characteristics / Soils	Total sand (%)	Silt (%)	Clay (%)	Textural class	pH(1 : 1) H_2O	pH(1 : 1) 1N-KCl	pH(1 : 1) 0.01N-$CaCl_2$	C (%)	OM (%)	Available P_2O_5(ppm)	T-N (%)	Exchangeable(me/100 g) K^+	Na^+	Ca^{++}	Mg^{++}	C.E.C. (me/100g)
Chong Ju Soil	43.1	41.4	15.4	Silty loam	4.83	3.82	4.7	1.01	2.45	35	0.12	0.08	0.08	2.33	1.23	7.7
Chung Ju Soil	30.5	53.1	16.5	Clay loam	6.43	6.04	6.3	1.79	2.9	44	0.14	0.05	0.09	10.5	1.3	14.14

Table 2. Composition of glucose-inorganic salt medium (Medium A)

K_2HPO_4	0.8 g
KH_2PO_4	0.2 g
$MgSO_4 \cdot 7H_2O$	0.2 g
$CaSO_4$	0.1 g
$(NH_4)_6Mo_7O_{24}4H_2O$	1 mg
$(NH_4)_2SO_4$	5.0 g
Glucose	10 g
Distilled water	1,000 m*l*
pH	7.0

Table 3. Composition of Czapeck-Dox medium (Medium B)

Sucrose	30 g
$NaNO_3$	2 g
K_2HPO_4	1 g
$MgSO_4 \cdot 7H_2O$	0.5 g
KCl	0.5 g
$FeSO_4 \cdot 7H_2O$	0.01 g
Distilled Water	1,000 m*l*
pH	6.0

Table 4. Composition of Nutrient Broth medium (Medium C)

Meat extract	10 g
Peptone	10 g
NaCl	5 g
Distilled Water	1,000 m*l*
pH	7.2

Preparation of Amino-TOK (Lee, 1974)

Four milligrams of TOK were put to a 50 m*l* round-bottomed flask, and 10 m*l* of 95% ethanol, 10 m*l* of distilled water, 2 m*l* of 5 N-hydrochloric acid, and 0.2 g of zinc dust were added to it. An air condenser was attached and the mixture boiled for 5 min. After cooling, the contents were transferred to a separatory funnel, made basic, and extracted with benzene. The benzene extract was evaporated to dryness on a rotary evaporator to get the Amino-TOK crystals. The purity was confirmed

by TLC, GLC, and MS.

Incubation of TOK in anaerobic soil conditions

One hundred grams of wet sample soils and 50 m*l* of distilled water were mixed up in 500 m*l* Erlenmeyer flasks, respectively, to provide flooded anaerobic conditions. Each 50 mg of TOK dissolved in a small amount of acetone was added to the above soils to give a concentration of 500 ppm. The mixtures were held at 30°C in an incubator for 2, 4, and 6 months, respectively. As the controls, the same soils without TOK were held in like manner.

Extraction of the soils treated with TOK

At the end of each incubation period, the soils were extracted with five 60 m*l* portions of methanol and four 60 m*l* portions of acetone. In each extraction, the soil was agitated with the solvents for 1 hr on a shaker, centrifuged at 14,000 g, and filtered.

Degradation of TOK in soil suspension

To a suspension made up of 95 m*l* of Glucose-inorganic salt medium (Medium A) and 5 g of Chung Ju soil (Soil B) in a 300 m*l*-Erlenmeyer flask was added the ethanol-dissolved TOK to a concentration of 25 ppm. This mixture was incubated aerobically by shaking at 30°C for 21 and 42 days. Three replicates were held for each incubation. As the control, 5 g of Chung Ju soil (Soil B) put in a 300 m*l*-Erlenmeyer flask were autoclaved at 121°C for 30 min. Autoclaving was repeated intermittently three times. To this autoclaved soil were added 95 m*l* of Medium A, and the suspension was autoclaved once more and added with TOK to a concentration of 25 ppm.

Extraction of the metabolites from soil suspensions

At the end of the incubation periods of 21 and 42 days, the soil and supernatant were separated from each other by centrifugation. The supernatant was extracted with three 60 m*l* aliquots of chloroform on a separatory funnel. The soil was extracted with six 50 m*l* aliquots of methanol by agitating for 20 min and centrifuging. All the extracts of three replicates were combined, dried on anhydrous sodium sulfate, and concentrated for the analyses by TLC and GLC.

TOK-degradability of soil extracts

Preparation of citrate buffers:

Buffer solutions of pH 4.83 and 6.43 which are the same as those of the sample soils were prepared with 0.1M sodium citrate and 0.1N hydrochloric acid.

Preparation of soil extracts:

One hundred grams of Chong Ju soil and 100 m*l* of the citrate buffer with pH 4.83 and 100 g of Chung Ju soil and 100 m*l* of the citrate buffer with pH6.43, respectively, were incubated in duplicate at 30°C for 11 days. At the completion of incubation, the mixtures were centrifuged at 3,000 rpm for 5 min and the supernatants were collected through filtration as soil extracts.

Incubation of TOK with soil extracts:

To 25 m*l* of each soil extract was added 1 mg of TOK dissolved in a small amount of acetone to give a concentration of 40ppm. This mixture was then incubated at 30°C for 21 days.

Extraction of degradation products:

The incubation mixtures were extracted with two 50 m*l* portions of redistilled chloroform and the extracts were concentrated on a flash evaporator for TLC and GLC analyses.

A test for TOK degradation by citrate buffer

In order to clarify that the degradation of TOK by soil extracts is due to soil microorganisms involved, a final concentration of 40 ppm of ethanol-dissolved TOK was added to 25 m*l* of citrate buffers which have the same pH's of 4.83 and 6.43, respectively, as those of the sample soils. Two replicates of the TOK-citrate buffer mixture were incubated for 7 months at 30°C and extracted with 10 m*l* of chloroform. The chloroform layer was separated, dried over anhydrous sodium sulfate, and concentrated to

a small volume for GLC.

Isolation of microorganisms from TOK-treated soils

Microorganisms were isolated from Chong Ju and Chung Ju soils which had been incubated with 500ppm of TOK, held in an incubator for two and four months, respectively, and extracted with organic solvents for collecting degradation products. The temporary designations are shown in Table 5. Since the isolates were selected and designated according to the morphological shapes of their colonies, some of them might be duplicated. The identification was not intended in the present investigation.

Incubation of the isolates with TOK

Each 150 m*l* of Medium C(Table 4) put in a 500-m*l* incubation flask and inoculated with each isolate was preincubated at 30°C for four days, respectively. Three milligrams of TOK dissolved in a small volume of acetone were added to each medium to provide a concentration of 20 ppm and incubated on a shaker for another 17 days. The incubated mixtures were extracted with two 60-m*l* portions of chloroform on a separatory funnel to collect the metabolites. The chloroform extracts were dried over anhydruos sodium sulfate and concentrated on a flash evaporator for TLC and GLC analyses.

Table 5. The designations of the isolates from the TOK-treated soils held for different incubation periods

Origin(soil)	Incubation period(month)	Designation
Chong Ju soil	2	T-1-1
		T-1-2
		T-1-3
	4	T-1-4
		T-1-5
		T-1-6
Chung Ju soil	2	T-2-1
		T-2-2
		T-2-3
	4	T-2-4
		T-2-5
		T-2-6

Comparison of TOK-degradability of some selected microorganisms in pure culture

Microorganisms tested for the TOK-degradability include *Aspergillus niger*, *Aspergillus versicolor*, *Chaetomium globosum*, *Fusarium oxysporum*, and *Penicillium luteum* as fungi. Bacteria which were examined are *Bacillus megaterium*, *Pseudomonas aeruginosa*, *Pseudomonas denitrificans*, *Pseudomonas putida*, Isolate A, and Isolate B.

Medium B and C were used for the cultures of fungi and bacteria, respectively. That is, 50 m*l* of each medium were put in large test tubes (28 cm×2.5 cm, O.D.), autoclaved at 121°C for 20 min, inoculated with the test microorganisms, and preincubated at 30°C for three days. To the incubation mixture was added the ethanol-dissolved TOK to the concentration of 30 ppm and the incubation continued for another 5 days. As the control, the media which were inoculated with the respective microorganisms without the addition of TOK were kept in like manner. At the completion of the incubation, each incubation mixture was extracted with two 18-m*l* portions of chloroform and the chloroform extracts were dried over anhydrous sodium sulfate, concentrated, and used for GLC.

Thin-layer chromatography

TLC was performed with 5196494 X, Art. 5554, DC-Alufolien, Kieselgel 60 F_{254}(25 Folien 20×20 cm), Schichtdicke 0.2 mm, E. Merck, Darmstadt. The developing solvents are:Solvent I, xylene-chloroform-acetone(40 : 25 : 35, v/v); Solvent II, chloroform-benzene(1 : 1, v/v); and Solvent III, benzene-ethyl acetate(19 : 1, v/v). The chromatograms were visualized under a UV lamp.

Gas-liquid chromatography

The analyses were performed with a Finnigan 9500 Gas Chromatograph and a Shimadzu Gas Chromatograph GC-6A, In both instruments, a flame ionization detector(FID) was eq-

uipped. The columns were a pyrex glass of 6 mm (OD)×6 ft packed with 5% SE-30 on Chromosorb W-HP and a stainless steel of 2m ×4 mm packed with 10% SE-30 on Chromosorb W-HP, respectively. Operating parameters for the Finnigan 9500 are as follows: Helium carrier flow, 30 ml/min; Oxygen, 400 ml/min; Hydrogen, 40 ml/min; Injector temp, 250°C; Detector temp, 250°C; Column temp, 90°C~250°C (6°C/min). Operating parameters for the Shimadzu GC-6A: Column temp, 240°C(Isotemp) and 180°C~250°C(10°C/min); Injector temp, 270°C; Detector temp, 270°C; Nitrogen flow, 80 ml/min; Air, 0.8kg/cm²; Hydrogen, 0.6 kg/cm². The area under each chromatogram peak was measured with a planimeter. For GLC-MS, all analyses were made by temperature-programming from 100°C to 260°C at a rate of 10°C/min. The other parameters were the same as the above.

Mass spectrometry

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

RESULTS AND DISCUSSION

Anaerobic degradation of TOK in flooded soil conditions

The organic solvent extracts of Chong Ju and Chung Ju soils incubated with 500 ppm of TOK were subject to TLC, GLC, and MS. On TLC, the Rf values of TOK and amino-TOK were 0.97 and 0.75 in Solvent Ⅰ, 0.90 and 0.58 in Solvent Ⅱ, and 0.90 and 0.63 in Solvent Ⅲ, respectively. The Rf values of the other metabolites could not be compared, because the authentic compounds were not available. Since the gas-liquid chromatograms of the solvent extracts from the soils exhibit almost the identical degradation products, only those of six-month incubation in both soils are shown in Fig. 1 and 2, respectively.

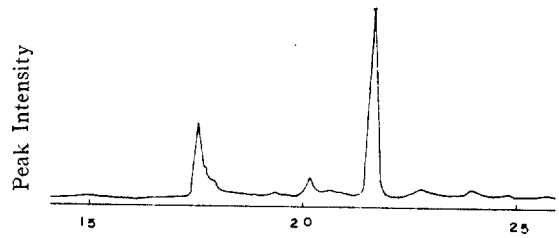


Fig. 1. Gas-liquid chromatogram of the solvent extract of Chong Ju soil treated with TOK and incubated for six months

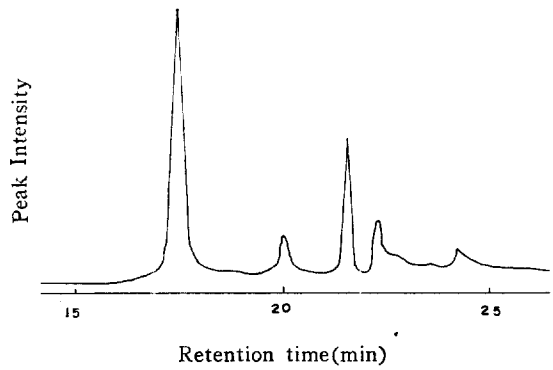


Fig. 2. Gas-liquid chromatogram of the solvent extract of Chung Ju soil treated with TOK and incubated for six months

Table 6. The metabolites obtained from the soils treated with TOK

Metabolite	Retention time(min)
4-chloro-4′-amino diphenyl ether	17.6
2,4-dichloro-4′-amino diphenyl ether (amino-TOK)	20.1
2,4-dichloro-4′-nitro diphenyl ether(TOK)	21.4
N-[4′-(4-chlorophenoxy)] phenyl formamide	23.6
N-[4′-(4-chlorophenoxy)] phenyl acetamide	24

On GLC, the retention times of the metabolites identified are presented in Table 6. The identification of the metabolites of TOK was mostly made by means of GLC-MS. Since the gas-liquid chromatograms of the samples showed almost

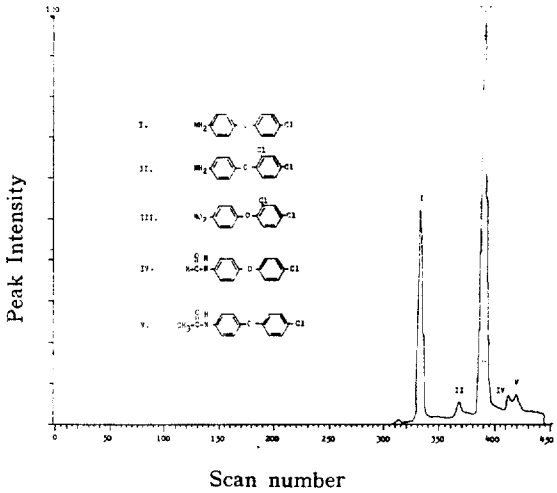


Fig. 3. Gas-liquid chromatogram for the mass spectra of the metabolites from Chung Ju soil treated with TOK and incubated for two months

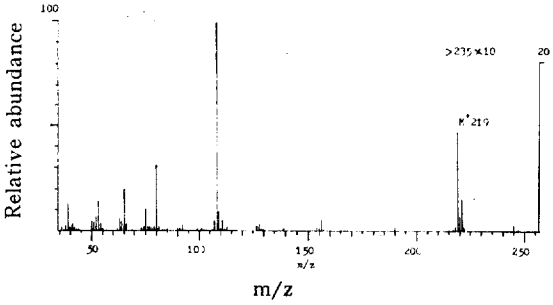


Fig. 4. Mass spectrum of metabolite Ⅰ, 4-chloro-4′-amino diphenyl ether

the same metabolites, the solvent extract of Chung Ju soil of two month incubation was subject to GLC-MS. Fig. 3 shows the gas-liquid chromatogram for MS, showing the relative amounts of the metabolites and the peak numbers scanned with the MS data system. The mass spectrum of each peak was taken. The mass spectrum of metabolite Ⅰ is shown in Fig. 4. As seen in Fig. 4, the molecular ion at m/z 219 and the M+2 peak indicating the isotopic abundance suggest the chemical structure, 4-chloro-4′-amino diphenyl ether. The small peak at m/z 245 is thought to be derived from a contaminant. In Fig. 5, the molecular ion at m/z 253 and the isotopic abundance ratio of the M+2 and M+4 peaks indicate structure, 2,4-dichloro-4′-amino diphenyl ether (amino-TOK) containing two chlorine atoms. Fig. 6 shows the mass spectrum of TOK which remains unchanged. The molecular ion appears at m/z 283. Fig. 7 is the mass spectrum of metabolite Ⅳ. In this spectrum, the molecular ion appears at m/z 247. The M+2 peak indicates the presence of one chlorine atom. The suggested structure is N-[4′-(4-chloro-phenoxy)] phenyl formamide. Fig. 8 shows the mass

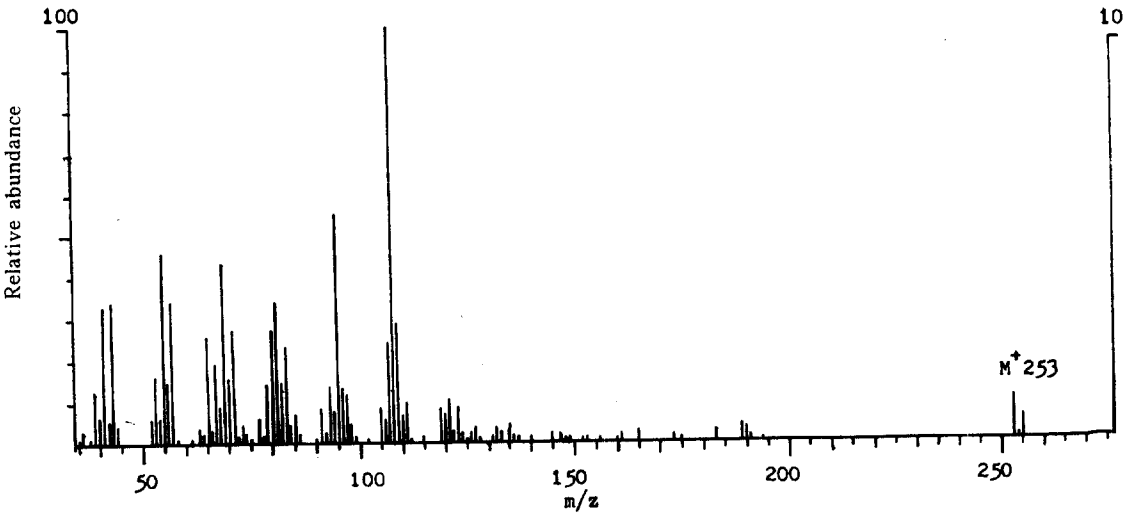


Fig. 5. Mass spectrum of metabolite Ⅱ, amino-TOK (2,4-dichloro-4′-amino diphenyl ether)

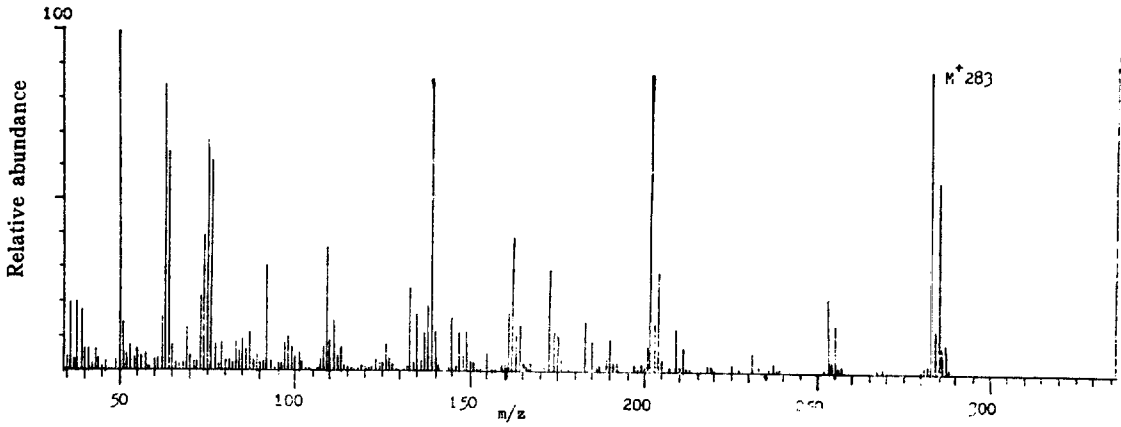


Fig. 6. Mass spectrum of metabolite Ⅲ, the residual TOK

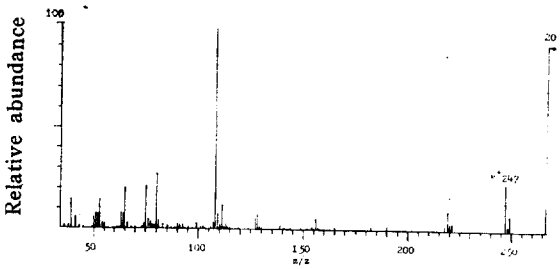


Fig. 7. Mass spectrum of metabolite Ⅳ, N-[4'-(4-chloro-phenoxy)] phenyl formamide

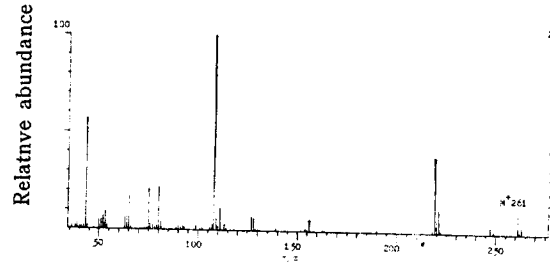


Fig. 8. Mass spectrum of metabolite Ⅴ. N-[4'-(4-chloro-phenoxy)] phenyl acetamide

Table 7. Comparison of the metabolites of TOK formed in soil with the residual TOK as an index as a function of the incubation periods, by means of GLC

Soil	Incubation period(month)	Metabolite*					
		Ⅰ	Ⅱ	Ⅲ	Ⅲ′	Ⅳ	Ⅴ
Chong Ju soil	2	3.35	2.52	100			trace
	4	28.37	4.96	100			trace
	6	50.17	8.77	100			trace
Chung Ju soil	2	42.91	2.05	100		2.37	3.03
	4	62.96	23.93	100	4.62	trace	3.20
	6	287.72	28.94	100	32.89		13.15

* Ⅰ : 4-Chloro-4'-amino diphenyl ether
Ⅱ : 2,4-Dichloro-4'-amino diphenyl ether(amino-TOK)
Ⅲ : 2,4-Dichloro-4'-nitro diphenyl ether(the residual TOK)
Ⅲ′: Unknown
Ⅳ : N-[4'-(4-chloro-phenoxy)] phenyl formamide
Ⅴ : N-4'-(4-chloro-phenoxy)] phenyl acetamide

spectrum of metabolite V. As seen in this spectrum, the molecular ion at m/z 261 and the isotopic abundance at the M+2 peak suggest the structure, N-[4'-(4-chloro-phenoxy)] phenyl acetamide. The degradation products of TOK in the two different soils as a function of the incubation periods are shown in Table 7. As seen in Table 7, the degradation of TOK turns out to depend on the physico-chemical properties of the soils onto which the herbicide was applied. In Chong Ju soil, the major metabolites were 4-chloro-4'-amino diphenyl ether and 2,4-dichloro-4'-amino diphenyl ether (amino-TOK). Only trace amounts of N-[4'-(4-chlorophenoxy)] phenyl acetamide were recognized. In other words, in Chong Ju soil, the replacement of one chlorine by hydrogen and the reduction of nitro group to amino group were the main features. Meanwhile, in Chung Ju soil, the metabolites identified include 4-chloro-4'-amino diphenyl ether, amino-TOK, N-[4'-(4-chloro-phenoxy)] phenyl formamide, N-[4'-(4-chloro-phenoxy)] phenyl acetamide, and some other unknowns, in addition to the residual TOK. The main features are also the replacement of one chlorine by hydrogen and the reduction of nitro group to amino group. In both cases, the degradation of TOK increases with incubation periods. Compared with the degradation in Chong Ju soil, TOK is more readily decomposed in Chung Ju soil as seen in Table 7. In Table 1, the major differences between the two soils are that the pH's of Chong Ju and Chung Ju soils are 4.83 and 6.43, that the textural classes silty loam and clayey loam, that the contents of Ca^{++} 2.33 and 10.5, and that the C.E.C. 7.7 and 14.14, respectively. Therefore, it is likely that the different rate of degradation of TOK in the two soils will be due to the different physico-chemical characteristics of the soils which, in consequence, will affect the activity of the microorganisms involved in the degradation.

According to Kuwatsuka(1971), the substituted diphenyl ethers, MO-338, TOK, X-52, and MO-500 produced their amino compounds only in flooded soils, the degradation was much slower under upland conditions than under flooded ones, and no amino derivatives were detected in soils under upland conditions. It was also shown that under flooded conditions, TOK and MO-500 were degraded much faster than MO-338 and X-52, and amino derivatives were the major metabolites in the earlier stages of incubation, whereas later, amino compounds were also metabolized to their compounds. Based on our results, at a relatively high concentration of 500 ppm, TOK undergoes the reduction of nitro group to amino group followed by the replacement of one chlorine by hydrogen to give 4-chloro-4'-amino diphenyl ether as the major metabolite in both soils under anaerobic flooded conditions. In the light of the fact that the amounts of amino-TOK are less than those of metabolite I, 4-chloro-4'-amino diphenyl ether in all cases, it is quite evident that the ring chlorines will be replaced by hydrogen with time.

The detection of metabolites IV and V indicates the formylation and acetylation of TOK in soils following the reduction of nitro group to amino group and the replacement of one chlorine by hydrogen. The proposed pathway of TOK degradation in anaerobic flooded soils is shown in Fig.9. In the metabolic studies of C^{14}-TOK in some weeds, Hawton, *et al.*(1971) concluded that at least two of metabolites were lipid-TOK conjugates or TOK polymers and others may be formed by cleavage of TOK at the ether linkage. Similar results were obtained from another diphenyl ether, Fluorodifen (*p*-nitrophenyl-α,α,α-trifluoro-2-nitro-*p*-tolyl ether). Bound residues of TOK in cereal grains and in rice and wheat straw were also reported (Wargo, *et al.*, 1975; Honeycutt, *et al.*, 1975). Gutenmann, *et al.*(1967) showed the amino-

TOK as a TOK metabolite in rumen fluid of dairy cows. TOK can be also metabolized in vivo to the acetamide, 2, 4-dichloro-1-(4-acetamidophenoxy) benzene (Adlr, *et al.*, 1971).

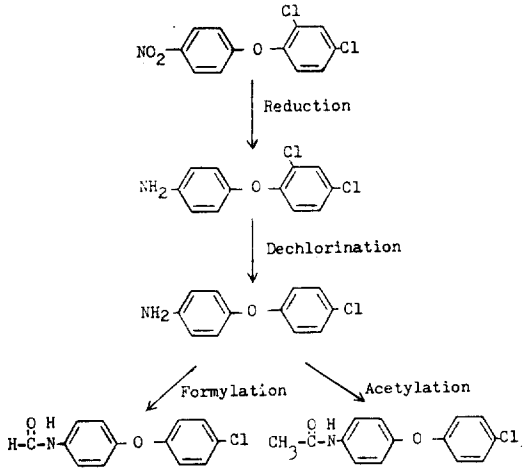
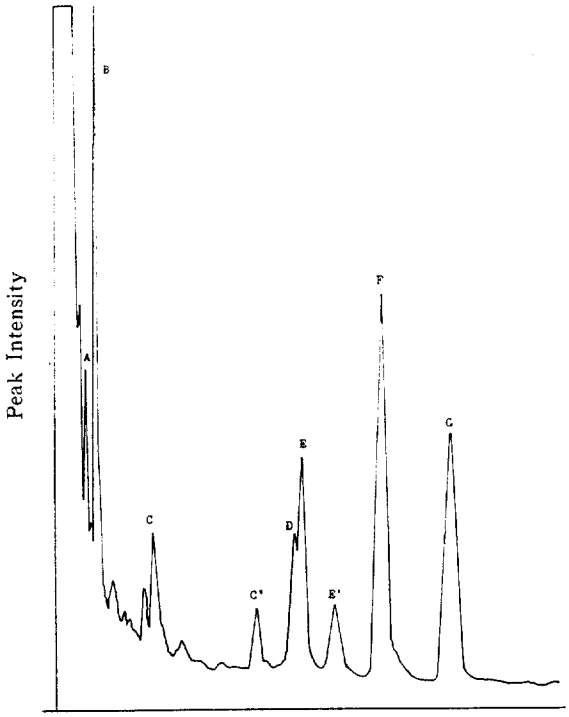


Fig. 9. Proposed pathway of TOK degradation in anaerobic flooded soils

The principal metabolite of diphenyl ether fed to rabbits was 4-hydroxydiphenyl ether which was excreted mainly as a glucuronide. The derivatives 4-methoxy- and 4, 4′-dimethoxy diphenyl ether were converted to the corresponding hydroxy derivatives by the rabbit(Williams, 1959). In the photodecomposition of TOK, Nakagawa, *et al.*(1974) showed that TOK undergoes rapid cleavage of the ether linkage to form 2, 4-dichloro-phenol and *p*-nitrophenol. As other prominent products, they identified hydroquinone, 4-nitrocatechol, amino-TOK, 4, 4′-bis(2, 4-dichloro-phenoxy) azobenzene, and an artifact N-[4-(2′, 4′-dichloro-phenoxy)] phenyl formamide. Other pathways involved the replacement of the ring chlorines by hydroxyl or hydrogen, ring hydroxylation, denitration by hydroxide prior to cleavage of the ether linkage, and possible intramolecular rearrangement of TOK to a biphenyl. Similar photolysis products of TOK were reported by Lee(1974) at nearly the same time. As reviewed above, even if the substituted diphenyl ether compounds were reported to be cleaved at the ether linkage in vivo or photochemically, in our experimental soil conditions where somewhat higher concentrations of TOK were used, no noticeable metabolites resulting from the ether linkage cleavage were recognized. In the photodecomposition of TOK in aqueous suspensions under sunlight, Nakagawa, *et al.*(1974) reported the formation of N-[4-(2′, 4′-dichloro-phenoxy)] phenyl formamide as an artifact resulting from the methanol used. In our investigation, however, acetylation and formylation during the degradation of TOK occurred in soils where no methanol was involved. According to Yih, *et al.*(1975), TOK is applied onto the surface of the soil in preemergence treatment. Incorporation of this compound into the soil drastically reduces its effectiveness as a herbicide. Therefore, it is believed that the reduction of nitro group to amino group, dechlorination, formylation, and acetylation will bring about a decrease in the toxicity and effectiveness of TOK as a possible enviromental contaminant.

Aerobic degradation of TOK in soil suspension

The gas-liquid chromatograms of the metabolites of TOK which was incubated aerobically in Chung Ju soil suspensions for 21 and 42 days are shown in Fig.10 and Fig.11, respectively. Based on the area of each peak, the relative amount of each metabolite was calculated with the residual TOK as an index and presented in Table 8. As seen in Table 8, in the case of 21 day's aerobic incubation, the viable soil suspension produced larger amounts of metabolites than the sterile soil suspension, even if small amounts of the metabolites were also detected in the latter. This result strongly indicates that the soil microorganisms are deeply involved in the degradation of TOK in soil suspension. In the meantime, the small amounts of metabolites in sterile soil suspension suggest the possibilities either of chemical decomposition or of the microbial contaminat-



ig. 10. Gas-liquid chromatogram of the degradation products of TOK incubated in viable Chung Ju soil suspensions for 21 days
A, B, C, C', D : Unknown
E : 4-Chloro-4'-amino diphenyl ether
E' : Unknown
F : Amino-TOK
G : TOK
*Column temp. : 180°C~250°C(10°C/min.) Programming

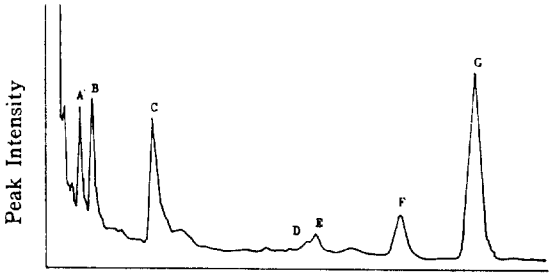


Fig. 11. Gas-liquid chromatogram of the degradation products of TOK incubated in viable Chung Ju soil suspensions for 42 days
A : Unknown
B, C, D : Unknown
E : 4-Chloro-4'-amino diphenyl ether
F : Amino-TOK
G : TOK
*Column temp. : 180°C~250°C(10°C/min.) Programming

Table 8. Comparison of the metabolites of TOK incubated aerobically in Chung Ju soil suspensions for 21 and 42 days with the residual TOK as an index

Metabolites	Incubation periods(days)			
	21		42	
	Sterile soil	Viable soil	Sterile soil	Viable soil
TOK	100	100	100	100
Amino-TOK	5.49	129.65	55.21	19.11
4-Chloro-4'-amino diphenyl ether	1.31	76.46	—	4.67

ion during incubation. In this connection, Niki, *et al.* (1976) have reported that diphenyl ether herbicides are more rapidly decomposed in flooded soils than in upland soils and that both microbial and chemical reactions are involved in this decomposition. In 42 day's aerobic incubation, the sterile soil produced more amino-TOK as a metabolite than the viable soil. This result is believed to arise either from the contamination during the rather long incubation period or from the chemical degradation largely due to the changed properties of the soil caused by autoclaving. It is noticeable that in viable soils, the 42 day's incubation produced less metabolites of amino-TOK and 4-chloro-4'-amino diphenyl ether than the 21 day's incubation, quite differently from the anaerobic flooded incubation. Based on this result, it might be possible that the reduction of nitro group to amino group might be reversible, depending on the environmental conditions. As another possible explanation, it might be due to the continuous degradation of the metabolites. However, no other metabolites leading to this conjecture were detected on GLC. In general, it is in good conformity with the report that the degradation of TOK is slower in aerobic conditions than in anaerobic flooded ones (Oh, *et al.*, 1981; Oyamada, *et al.*, 1979).

Degradation of TOK by soil extracts

mixtures, to which 40 ppm of TOK had been added, were subject to GLC analyses. Based on the GLC results, in the Chong Ju soil extract, the original TOK remained almost intact, whereas in the Chung Ju soil extract, 3.6 % of amino-TOK were formed by the reduction of the nitro group in the structure of TOK. In the light of the fact that TOK did not decompose in the pure citrate buffers of pH 4.83 and 6.43, respectively, in 7 months of incubation, it is evident that the soil extracts are capable of degrading TOK, due to the action of the enzymes excreted by the microorganisms involved in the incubation.

Degradation of TOK in pure culture of the microorganisms isolated

The GLC results of the chloroform extracts of Medium C which was incubated with each isolate and 20 ppm of TOK are presented in Table 9. Since no metabolites other than amino-TOK were formed from TOK, the percentage was calculated from the total of both TOK and amino-TOK. In the case of the Isolate T-1-1, almost all TOK remained unchanged, whereas in the Isolate T-2-3, amino-TOK was formed as much as 100%, no residual TOK remaining. Based on this result, it seems that the basic diphenyl ether moiety in the TOK structure is quite stable to the attack by the microorganisms. At the present time, even if the isolates are not identified, they are thought to belong to bacteria in appearance.

Table 9. Comparison of the residual TOK and the metabolite, amino-TOK in pure culture of microorganisms isolated from the two soils

Origin (soil)	Designation of the isolates	Residual TOK (%)	Amino-TOK, metabolite (%)
Chong Ju soil	T-1-1	100	trace
	T-1-2	97.8	2.2
	T-1-3	98.92	1.08
	T-1-4	80	20
	T-1-5	41	59
	T-1-6	82.7	17.3
Chung Ju soil	T-2-1	97	3
	T-2-2	31.8	68.2
	T-2-3	0	100
	T-2-4	97.6	2.4
	T-2-5	30.4	69.6
	T-2-6	92	8

Degradation of TOK in pure cultures of some selected microorganisms

Table 10 shows the degradation of TOK in pure cultures of the 11 selected strains of microorganisms. As seen in Table 10, TOK

Table 10. Comparison of degradation products of TOK in pure cultures of some selected microorganisms

Microorganisms	Strains	Metabolites(%)		
		4-Chloro-4'-amino diphenyl ether	Amino-TOK	TOK
Fungi	*Aspergillus niger*	0	0	100
	Aspergillus versicolor	0	0.60	99.40
	Chaetomium globosum	0	0	100
	Fusarium oxysporum	0	0	100
	Penicillium luteum	0	0	100
Bacteria	*Bacillus megaterium*	0	0	100
	Pseudomonas aeruginosa	0	6.44	93.56
	Pseudomonas denitrificans	0	2.92	97.08
	Pseudomonas putida	0	2.39	97.61
	Isolate A(T-2-3)	8.09	8.08	83.83
	Isolate B	11.83	19.64	68.53

remained intact in the cultures of all the fungi except for *Aspergillus versicolor*. Only *Aspergillus versicolor* produced a small amount of amino-TOK as the metabolite. In the meantime, all the bacteria except *Bacillus megaterium* degraded TOK to amino-TOK to a considerable degree. Isolate A and Isolate B produced 4-chloro-4'-amino diphenyl ether in addition to amino-TOK as the metabolites. Isolate B is the strain which was isolated from the soil B suspension of 42 day incubation. From this result, it is quite obvious that the microorganisms exposed to the xenobiotics for some time can develop the capability to decompose them for their subsistence in one way or another.

摘 要

1. 物理 化學的 特性이 다른 청주토와 충주토에 500 ppm의 TOK를 처리하고 2,4,6개월간 혐기적 담수상태로 배양시 生成된 分解産物은 4-Chloro-4'-amino diphenyl ether, 2,4-Dichloro-4'-amino-diphenyl ether(Amino-TOK), N-[4'-(4-Chlorophenoxy)] phenyl acetamide, 및 N-[4'-(4-chlorophenoxy)] phenyl formamide이었다. 即 담수된 혐기적 토양조건하에서 TOK는 $NO_2 \rightarrow NH_2$로의 환원, 脫鹽素化, acetylation 및 formylation 되었고 ether 結合은 붕괴되지 않았다. 그리고 pH가 6.43이고 Ca^{++} 함량과 C.E.C.가 높은 충주토에서는 pH 4.83이고 Ca^{++}함량과 C.E.C.가 낮은 청주토에서 보다 TOK의 分解가 더 용이하게 일어났다.

2. 충주토의 현탁액중에 25 ppm의 TOK를 加하고 好氣的으로 21일간 진탕배양시 생성된 분해산물로서 TOK: amino-TOK: 4-Chloro-4'-amino-diphenyl ether는 100 : 130 : 76의 비율이었고 42일간 배양시에는 100 : 19 : 5의 비율이어서 好氣的 狀態에서의 TOK의 分解는 배양기간에 반드시 비례하지 않음을 알 수 있었다.

3. 충주토의 citrate buffer 추출액은 TOK를 分解시키는 능력이 있었으며 이것은 토양 미생물의 작용임이 확인되었다.

4. TOK를 배양했던 토양으로 부터 12균주의 세균을 분리하여 순수배지中에서 TOK를 다시 배양한 結果 T-1-1 균주는 거의 分解力이 없는 반면 T-2-3 균주는 가장 分解力이 우수하였고 이때의 分解産物은 Amino-TOK이었다.

5. 순수 토양미생물에 의한 TOK의 분해에서는 곰팡이 보다는 *Pseudomonas*屬의 세균들이 分解能이 더 양호하였으나 충주로 현탁액으로 부터 分離한 菌株 B가 가장 분해능이 양호하였다.

REFERENCES

1. Adler, I.L., Roser, R.L., and Wargo, J.P., 1971. Rohm and Haas Company, unpublished data.
2. Crosby, D.G., and Nakagawa, M., 1971. Abstr. 162nd Meet. Amer. Chem. Soc. Washington, D. C., September.
3. Gutenmann, W.H., and Lisk, D.J., 1967. Metabolism of TOK Herbicide in the Dairy Cow. J. Dairy Sci. **50**, 1516~1518.
4. Hawton, D., and Stobbe, E.H., 1971. The Fate of Nitrofen in Rape, Redroot Pigweed, and Green Foxtail. Weed Sci. **19**, 555~558.
5. Honeycutt, R.C., and Adler, I.L., 1975. Characterization of Bound Residues of Nitrofen in Rice and Wheat Straw. J. Agric. Food Chem., **23**, 1097~1101.
6. Ichihashi, M., Takahashi, R., Kimura, F., and Yokomichi, Y., 1971. Abstr. 10th Meet. Weed Sci., Japan, **35**.
7. Kuwatsuka, S., 1971. Environmental Toxicology of Pesticides, U.S.-Japan Seminar, Oiso, Japan, October.
8. Lee, J.K., 1974. Solution Phase Photolyses of Substituted Diphenyl Ether Herbicides under Simulated Environmental Conditions. J. Kor. Agr. Chem. Soc., **17**, 149~176.
9. Moreland, D.E., Blackmon, W.J., Todd, H.G., and Farmer, F.S., 1970. Weed Sci. **18**, 636.
10. Nakagawa, M. and Crosby, D.G., 1974. Photodecomposition of Nitrofen. J. Agr. Food Chem., **22**, 849~853.
11. Niki, Y. and Kuwatsuka, S.. 1976. Soil Sci.

Plant Nutr. **22**, 233.

12. Oh, B.Y., Jeong, Y.H., and Lee, B.M., 1981. Studies on Degradation of Butachlor and Nitrofen in Different Soil Conditions. J. Kor. Agr. Chem. Soc. **24**—**2**, 112~119.
13. Oyamada, M. and Kuwatsuka, S., 1979. J. Pesticide Sci. **4**, 157.
14. Wargo, J.P., Honeycutt, R.C., and Adler, I.L., 1975. Characterization of Bound Residues of Nitrofen in Cereal Grains. J. Agric. Food Chem., **23**, 1095.
15. Williams, R.T., 1959. "Detoxification Mechanisms." pp. 324~330, Chapman and Hall, London.
16. Yih, R.Y., Swithenbank, C., 1975. New Potent Diphenyl Ether Herbicides. J. Agric. Food Chem., **23**, 592~593.