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A Study on the Degradation of 3,4-Dichloroaniline by a Soil Fungus, *Chaetomium globosum* (Part I)

With Special Emphasis on Acetylation

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土壤絲狀菌 *Chaetomium globosum*에 依한 3,4-Dichloroaniline의 變化에 關한 硏究 (第一報)

特히 Acetylation을 中心으로

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SUMMARY

In order to investigate mechanisms related to the microbial degradation of 3,4-dichloroaniline, it was incubated with a soil fungus, *Chaetomium globosum* and the following results were obtained.

(1) 3,4-Dichloroacetanilide turned out to be the major metabolite, indicating that acetylation is the major scheme.

(2) The presence of trace amounts of 3,4-dichloronitrobenzene, 3,3′, 4,4′-tetrachloroazobenzene, 3,4-dichlorophenylhydroxylamine, and 3,4-dichloroaniline is suggestive of the aromatic amine oxidation as the minor pathway.

(3) Other metabolites with m/e 112, 114, and 279 were also isolated, but their identities are under investigation.

(4) Dechlorination occurring during incubation indicates the possibility of forming hydroxylated and other metabolites.

INTRODUCTION

A variety of pesticides contain the aniline moiety which is liberated in the environment. Thereafter, this compound undergoes subsequent microbial degradation and synthetic reactions.

Condensation reactions resulting in the formation of both symmetrical and asymmetrical azobenzenes have been reported.[1,2,15,22]

The condensation of three molecules of 3,4-dichloroaniline to form 4-(3,4-dichloroanilino)-3,3′, 4,4′-tetrachloroazobenzene has also been reported.[23] Kaufman et al.[11,13] reported the formation of 3,3′, 4,4′-tetrachloroazoxybenzene during the metabolism of 3,4-dichloroaniline by the soil fungus *Fusarium oxysporum* Schlecht. The involvement of several labile intermediates in the formation of azobenzenes and anilinoazobenzenes has been indicated.[4,6,20,21,27] In microbial culture solution, it was found that the soil fungus *Geotrichum candidum* formed 3,3′, 4,4′-tetrachloroazobenzene[5] and *Fusarium oxysporum* produced 3,3′, 4,4′-tetrachloroazoxybenzene[13] from 3,4-dichloroaniline, respectively.

Meanwhile, Tweedy et al.[28] have reported acetylation as a major factor in the microbial degradation of p-bromoaniline. Later, Kaufman et

al.[11,12] revealed the presence of both acetylated and formylated chloroanilines. Further, they[14] reported the involvement of oxidation and acylation of the aromatic amine group in the degradation of 4-chloroaniline by *Fusarium oxysporum* Schlecht. There are some other reports on the acetylation of anilines by microorganisms.[3,24,26,30]

Recently, Russel et al.[25] reported formylation and acetylation of 4-chloroaniline by a *Streptomyces* species.

In the present investigation, 3,4-dichloroaniline (3,4-DCA) was incubated in PDB medium with the soil fungus, *Chaetomium globosum* with a view to establishing the nature of its metabolites and further elucidating the involved degradation mechanisms.

MATERIALS AND METHODS

Syntheses of 3,4-DCA metabolites.

3,4-Dichlorophenylhydroxylamine was prepared by a modification of the method for phenylhydroxylamine.[14,29] Ethanol-water (50 : 50) mixture was used as solvent for the reduction of 3,4-dichloronitrobenzene with zinc powder.

3,4-Dichloronitrosobenzene was synthesized by acidified sodium dichromate oxidation of 3,4-dichlorophenylhydroxylamine.[14,29]

Synthesis of 3,3′, 4,4′-tetrachloroazobenzene (TCAB) was accomplished from 3,4-DCA by the method of Lee et al.[17]

3,3′, 4,4′-Tetrachloroazoxybenzene was synthesized by the method described for azoxybenzene.[29] 3,4-Dichloronitrobenzene was used as starting material.

3,4-Dichloroacetanilide and 3,4-dichloroformylanilide were prepared by the methods for acetanilide and formylanilide,[29] respectively. For purification, the crude 3,4-dichloroacetanilide was dissolved in toluene and active carbon was added to remove the impurities. After refluxed on a water-bath, it was filtered while hot, and concentrated to a small amount and the resulting white crystal was washed with n-hexane. The identity was confirmed by ms and its purity was checked on tlc and glc.

Incubation of *C. globosum* with 3,4-DCA.

15mg of 3,4-DCA dissolved in 1ml of acetone was added to 300ml of potato dextrose broth[18] preincubated with *C. globosum* for 6 days. The mixture was reincubated at 28°C for 4, 7, 13 days, respectively. After each required incubation, the culture mixture was filtered with glass-wool and the filtrate was extracted with five 150ml aliquots of chloroform on a separatory funnel.

The chloroform layer was collected, dried over anhydrous magnesium sulfate, and evaporated. The resulting residue was redissolved in acetone for the subsequent analyses. Duplicate flasks containing the inoculum without 3,4-DCA and 3,4-DCA without inoculum were maintained as controls and tested in the same way.

Gas-liquid chromatography.

The analyses were performed with a TRACOR (TM) 550 Gas Chromatograph equipped with a flame ionization detector. The column was a stainless steel of 3′×1/4″ packed with 10% SE-30 on 60-80 mesh Chromosorb W. Operating parameters were as follows: nitrogen carrier flow, 47ml/min; injection temp, 200°C; detector temp, 190°C. All analyses were made by temp-programming from 80°C to 230°C at a rate of 5°C/min. The chartspeed was 0.25 in/min.

Thin-layer chromatography.

It was accomplished on precoated analytical plates of silica gel HF-254 with fluorescent indicator (Art. 5554, DC-Alufolien Kieselgel 60 F_{254}, 25 Folien 20×20cm Schichtdicke 0.2mm, E. Merck, Darmstadt) using solvent systems, benzene-methanol (85 : 15v/v) and methanol-water-ammonia (30 : 12 : 1v/v). The separated substances were detected under a uv lamp.

Infrared spectrometry.

Infrared spectra were obtained as liquid films (0.025mm thickness) on an IR-430 Infrared-Spectrophotometer, Shimadzu,

Detection of 3,4-dichlorophenylhydroxylamine.[14]

3,4-Dichlorophenylhydroxylamine production

was detected by the trisodium pentacyanoammine ferroate complex.[7,14]

The development of a magenta color with the addition of 4 drops of a 0.2% (w/v) aqueous trisodium pentacyanoammine ferroate solution to 1ml of the centrifugate was indicative of the presence of the 3,4-dichlorophenylhydroxylamine-trisodium pentacyanoammine ferroate complex.

Detection of 3,4-dichloronitrosobenzene.[14]

3,4-Dichloronitrosobenzene was detected by reaction of 1ml of the centrifugate with 0.5ml of 5% (w/v) aqueous hydroxylamine hydrochloride followed by 0.5ml of 5% (w/v) aqueous N-1-naphthylethylenediamine dihydrochloride and 4ml of distilled water. A reddish-brown color develops immediately in the presence of 3,4-dichloronitrosobenzene.

Detection of 3,4-dichloroacetanilide.[14]

It was visualized by first exposing the tlc plates to nitrous oxide fumes in a closed container for 15-20 min, and then spraying with a 0.5% (w/v) N-1-naphthylethylenediamine dihydrochloride in ethanol. Nitrous oxide, N_2O[9] was obtained by thermal decomposition of ammonium nitrate in the melt at 250-260°.

Determination of chlorine ion.

Chlorine ion released from 3,4-DCA during incubation was checked by the method of Iwasaki et al.[10,18] for the elucidation of metabolites. The absorbance of each sample was measured at 580nm after 0, 4, and 8-day incubations, respectively.

RESULTS

Gas-liquid chromatograms of the metabolites of 3,4-DCA.

The gas-liquid chromatograms of 3,4-DCA metabolites obtained from the incubation with *C. globosum* for 4, 7, and 13 days are presented in Fig. 1, 2, and 3, respectively.

As seen in Fig. 1, 2, and 3, 3,4-dichloroacetanilide turned out to be one of the major metabolites by this microorganism, whereas trace amounts of 3,4-dichloronitrobenzene, TCAB and 3,4-DCA which is thought to remain unchanged

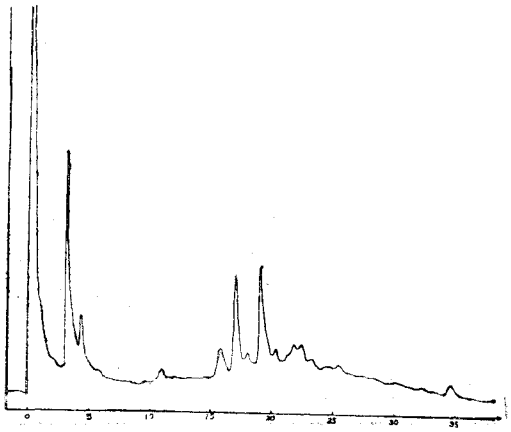

Fig. 1. Gas-liquid chromatogram of 3,4-DCA metabolites obtained from the 4-day incubation mixture

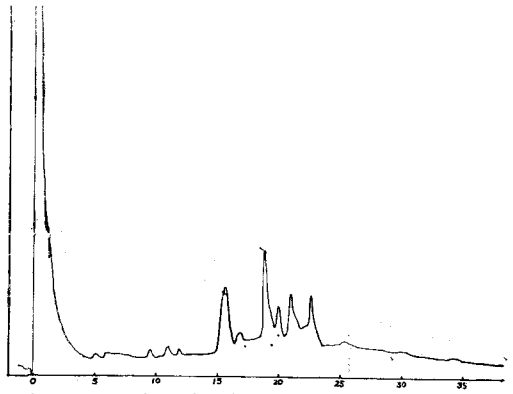

Fig. 2. Gas-liquid chromatogram of 3,4-DCA metabolites obtained from the 7-day incubation mixture

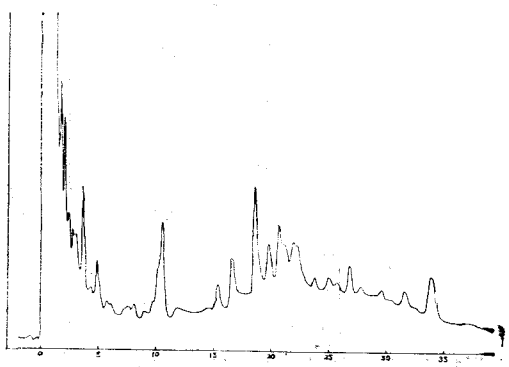

Fig. 3. Gas-liquid chromatogram of 3,4-DCA metabolites obtained from the 13-day incubation mixture

or was transformed reversibly from 3,4-dichloroacetanilide were also detected in all the different incubation periods. When incubated for 40 days, almost the same metabolites seemed to be formed in separate experiments.

Some unidentified peaks which were thought to result from the control incubation were subtracted from the peaks present in Fig. 1,2, and 3. Table 1 shows the expected metabolites of 3,4-DCA which could be identified on glc or not.

Table 1. Possible metabolites of 3,4-DCA by *C. globosom* on glc

Metabolites	Retention time (min)	Remarks
3,4-dichloronitrobenzene	9.4	detected
3,4-DCA	11	detected
3,4-dichloroformylanilide	18.5	—
3,4-dichloroacetanilide	18.8	detected
TCAB	31.6	detected
3,3′,4,4′-tetrachloroazoxybenzene	35	—

Thin-layer chromatography.

The thin-layer chromatogram of metabolites of 3,4-DCA is shown in Fig. 4.

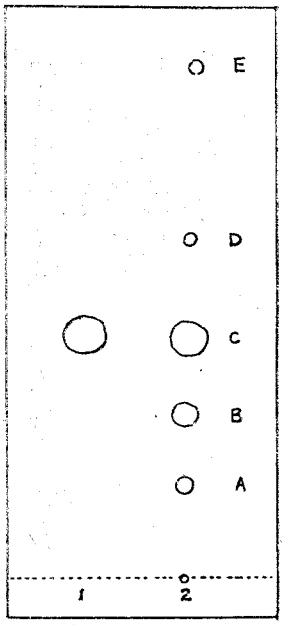

Fig. 4. Thin-layer chromatogram of metabolites of 3,4-DCA by *C.globosum*
(1) Authentic 3,4-dichloroacetanilide
(1) Metabolites of 3,4-DCA

Table 2 shows the metabolites and their Rf's.

Table 2. Metabolites of 3,4-DCA and their Rf's on tlc

Metabolites	Rf's	Remarks
A	0.16	Unidentified
B	0.30	〃
C	0.43	3,4-dichloroacetanilide
D	0.59	Unidentified
E	0.90	m/e 279

As shown in Fig. 4 and Table 2, metabolite C corresponds to 3,4-dichloroacetanilide. Metabolites A,B,D, and E were not identified or characterized as yet. Of these, metabolite E has the moldcular weight of m/e 279.

Infrared spectrometry of metabolite C (3,4-dichloroacetanilide)

The infrared spectra of authentic 3,4-dichloroacetanilide and metabolite C are presented in Fig. 5.

In these two spectra, they are almost identical, showing the characteristic amide C=O absorpti on at 1,650 cm^{-1}, the CH_3 absorption of acetyl at 1,450cm^{-1}, and the ph-NH absorption at 1,280 cm^{-1}. On account of the scanty sample, the spectrum of metabolite C is not satisfactory.

Detection of 3,4-dichloroacetanilide.

The presence of 3,4-dichloroacetanilide was confirmed by exposing the tlc plates to nitrous oxide and then spraying with N-1-naphthylethylenediamine dihydrochloride in ethanol. It was visible as a yellow spot.

Detection of acetic acid.

In order to support the presence of 3,4-dichloroacetanilide, formation of acetic acid during incubation by the microorganism was checked and identified by glc. Accordingly, it is considered that acetic acid which was formed will be involved in the formation of 3,4-dichloroacetanilide by action of some unknown enzymes produced by *C. globosum*,

Identification of 3,4-dichlorophenylhydroxylamine.

Since 3,4-dichlorophenylhydroxylamine is an

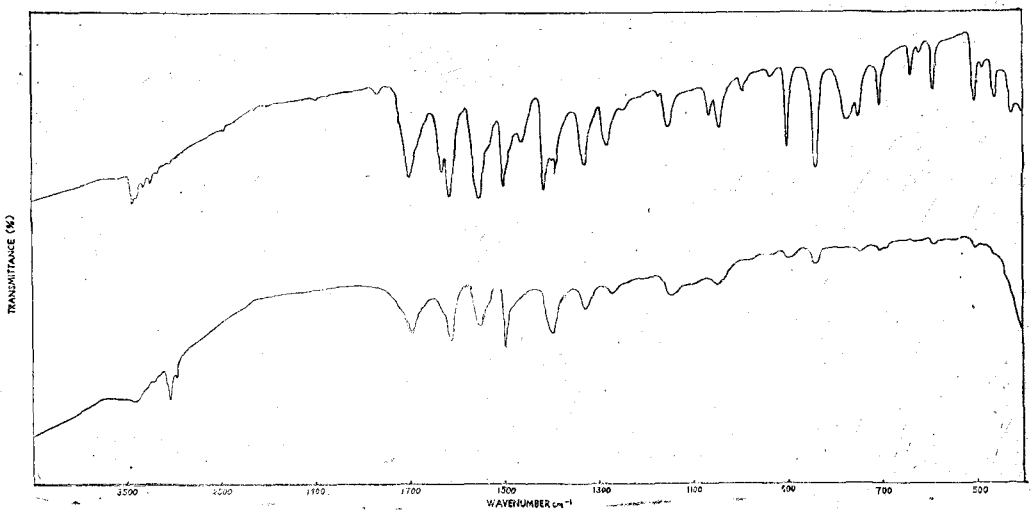

Fig. 5. Ir spectra of authentic 3,4-dichloroacetanilide (upper) and metabolite C (lower)

unstable compound, it was isolated as a complex of trisodium pentacyanoammine ferroate. On tlc analysis, the Rf value of the authentic and metabolite 3,4-dichlorophenylhydroxylamine was the identical 0.83 in methanol-water-ammonia(30 : 12 : 1). Only a trace amount was noticeable, indicating that oxidation of the aromatic amine is a minor pathway in this microorganism.

Identification of 3,4-dichloronitrosobenze ne.

It was difficult to detect the presence of this metabolite due to the color of the incubation mixture which is brown in itself.

Determination of chlorine ion.

For the sake of confirming the release of chlorine ion from 3,4-DCA, the absorbance of each sample taken after 0-, 4-, and 8-day incubations was measured. The result is shown in Table 3.

Table 3. Determination of chlorine ion

Incubation period(days)	Absorbance (at 580nm)
0	0.024
4	0.17
8	0.275

As seen in Table 3, it is believed that chlorine ion is considerably released from the compound. The absorbance after 8-day incubation was not checked due to the experimental difficulties.

DISCUSSION

Tweedy et al.[28] have reported that *Talaromyces wortmanii*, *Fusarium oxysorum*, *Chlorella vulgaris*, and a species of *Bacillus* produced p-bromoacetanilide from p-bromo-aniline in culture solution, suggesting that acetylation of substituted anilines in soils could be a major factor in preventing accumulation of the aniline derivatives and also could be competitive with oxidative coupling to the azobenzenes, at least in the case of the bromine-containing aniline.

Later, Kaufman et al.[11,12] revealed the presence of both acetylated and formylated chloroanilines, indicating that the process of acylation provides an alternative pathway for the degradation of chloroanilines.

Further, they[14] reported that the degradation of 4-chloroaniline by the soil fungus *Fusarium oxysporum* Schlecht, indicating that at least two metabolic pathways were involved; oxidation of the aromatic amine group was the major mechanism of 4-chloroaniline metabolism, but acylation of the aromatic amine group also occurred.

Weber[30] showed that acetylation was a major metabolic pathway of foreign anilines especially in mammalian tissues.

Smith et al.[26] found the conversion of aniline by four microorganisms acetanilide, 2-hydroxyacetanilide, 4-hydroxyaniline, and two unknown phenolic products.

Acetylation also took place in a *Paracoccus* sp. using aniline, 2-, 3-, or 4-chloro-aniline.[3,24] Russel et al.[25] found 4-chloroformylanilide and 4-chloracetanilide as the metabolites of 4-chloroaniline in a liquid growth medium by a *Streptomyces* sp. which was isolated from soil.

Kearney et al.[16] reported the formation of 3,4-dichloroformylanilide from 3,4-DCA in soil.

In the present investigation, it was disclosed that when 3,4-DCA was incubated with the soil fungus, *C. globosum* in PDB culture solution, 3,4-dichloroacetanilide was formed as one of the major metabolites. The minor metabolites detected include 3,4-dichloronitrobenzene, 3,4-dichlorophenylhydroxylamine, 3,4-DCA, and TCAB. Their metabolic scheme could be presented as in Fig. 6.

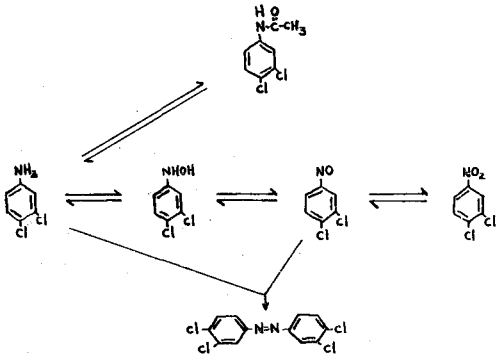

Fig. 6. Possible scheme for the metabolism of 3,4-DCA by *C. globosum*

In this scheme, the reversibility beween the metabolites was verified by Kaufman et al.[14] and the reduction of nitro aryl compounds to the corresponding arylamine by *F. oxysporum*[19] and other microorganisms[8] has also been observed by some others.

While Kaufman et al.[14] reported that in the degradation of 4-chloroaniline by *F. oxysporum*, oxidation of the aromatic amine was the major mechanism, the degradation of 3,4-DCA by *C. globosum* in this investigation yielded 3,4-dichloroacetanilide as the main metabolite. The trace amounts of 3,4-dichloronitrobenzene, 3,4-dichlorophenylhydroxylamine, and TCAB were indicative of the possibility of the oxidative process. The result obtained confirms the fact that acylation and oxidation of the aromatic amine in pesticides depend on the microorganisms involed in the degradation of the chemicals used.

On the glc-ms analysis, the metabolites with m/e 112, 114, and 279 were also detected, but their identities could not be determined. These and other metabolites are under investigation.

要　　約

3,4-DCA의 微生物에 依한 分解機構를 究明하기 위하여 土壤絲狀菌 *C. globosum*과 培養하여 다음의 結果를 얻었다.

1. 3,4-Dichloroacetanilide가 主된 代謝產物로 밝혀졌으며 이는 Acetylation이 주된 代謝經路임을 示唆해 준다.

2. 少量 生成된 3,4-dichloronitrobenzene, TCAB, 3,4-dichlorophenylhydroxylamine, 그리고 3.4-DCA의 存在는 副次的인 經路로 芳香族 Amine의 酸化를 暗示해 준다.

3. m/e 112, 114 및 279인 代謝產物이 分離되었으나 이들의 構造는 계속 究硏中이다.

4. 培養中 發生하는 脫鹽素 反應은 水酸化 및 其他 代謝產物의 形成 可能性을 示唆해 준다.

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