微生物による除草剤Chlornitrofen (CNP)とそのアミノ誘導体の代謝

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Original Article

Microbial Metabolism of the Herbicide Chlornitrofen and Its Amino Derivative*

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Several strains of microorganisms which degrade chlornitrofen (2,4,6-trichlorophenyl 4-nitrophenyl ether, CNP) and its 4-amino derivative (CNP-amino) were isolated from soil, and some of them were identified as *Corynebacterium* sp., *Streptomyces* sp. and *Penicillium* sp. All of the isolates could not utilize these compounds as sole carbon sources in an inorganic salt medium but they rapidly degraded them in the presence of supplemental carbon sources in the medium. The reduction of the nitro group of chlornitrofen to CNP-amino and the successive *N*-acetylation or *N*-methylation, and the cleavage of the ether linkage were dominant metabolic pathways by these microorganisms. The metabolic pathways, however, differed depending on microorganisms and media. The replacement of the amino group of CNP-amino by a hydroxy group, the reduction and successive formylation of *p*-nitrophenol and the cleavage of the phenyl ring to produce CO_2 ultimately were also observed.

INTRODUCTION

The fate and behavior of diphenyl ether herbicides such as chlornitrofen and nitrofen in soil have been studied in our previous reports.[1-3] Diphenyl ether herbicides were rapidly degraded in flooded soil, and the phenyl ring was ultimately decomposed to CO_2. The first step of the main degradation pathway of chlornitrofen in soil under flooded conditions was reduction of the nitro group with Fe^{2+} produced by microorganisms in aerobic soil.[3] Reduction occurred even in sterile soil when soil was sterilized after preincubation under flooded conditions, although reduction did not occur when soil was sterilized before preincubation. Niki & Kuwatsuka,[4] Ichihashi *et al.*[5] and Ohyama & Kuwatsuka[6] have also suggested that soil microorganisms are responsible for the degradation of diphenyl ether herbicides in soil.

This paper reports the metabolism of ^{14}C-chlornitrofen and its amino derivative by microorganisms isolated from soil.

* Most of this study was presented at the Annual Meeting of Pestic. Sci. Soc., Japan, 1978.

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MATERIALS AND METHODS

1. *Radioactive and Nonradioactive Chemicals*

^{14}C-Chlornitrofen (2,4,6-trichlorophenyl 4-nitrophenyl ether, CNP), uniformly labeled at the nitrophenyl-ring, was synthesized by Daiichi Pure Chemicals Co. Ltd. and supplied by Mitsui Toatsu Chemicals Inc. A ^{14}C-labeled compound of the amino derivative of chlornitrofen (2,4,6-trichlorophenyl 4-aminophenyl ether, CNP-amino) was prepared by reduction of ^{14}C-chlornitrofen with $SnCl_2$ and HCl in ethanol and purified by thin-layer chromatography (TLC). The specific activity of ^{14}C-chlornitrofen and its amino derivative was 1.4 mCi/mmol, and the radioactive purity of both chemicals was more than 99%. Reference compounds and their thin-layer chromatographic behaviors have been described in our

previous paper.[1]

2. Soil Samples

Mineral soil of Chigasaki was collected from a paddy field. The sampling place and the property of the soil have been reported previously.[2]

3. Isolation of Chlornitrofen-Degrading Microorganisms from Soil

The Chigasaki soil was perfused with chlornitrofen to be enriched with microorganisms capable of degrading chlornitrofen by Collins' perfusion method.[7] The perfusion apparatus consisted of a soil column of 14.4×3.8 cm (i.d.) and a 600-ml flat-base round flask. Twenty grams of the undried soil sample of 2–5 mm particle size prepared by sieving was placed in the column of the apparatus. The soil was perfused with water for 24 hr preliminarily. Then, 300 ml of 0.3 ppm chlornitrofen aqueous solution was perfused at 25°C. The solution was renewed every day. After 30 days, 10 g of the perfused soil was suspended in 250 ml of water. Microorganisms capable of degrading chlornitrofen were isolated from the soil suspension by the dilution plate method by using ager plates of a medium containing 5 mg of chlornitrofen, 0.5 g of K_2HPO_4, 100 ml of soil extract, and 15 g of ager in 1000 ml of water (pH 6.8–7.0). The soil extract was prepared as follows: 1 kg of the Chigasaki soil, 1 g of $CaCl_2$ and 1000 ml of water in a 2000-ml Erlenmeyer flask were shaken for 5 min, autoclaved at 120°C for 20 min, and the content was filtered through a filter paper (Toyo No. 2) by suction. The medium inoculated with the soil suspension was incubated at 28°C for 2 weeks. Each colony developed on the ager plates was transferred to an albumin ager medium[8] containing 0.3 ppm chlornitrofen for bacteria and actinomycetes, and to a potato dextrose ager medium[8] containing 0.3 ppm chlornitrofen for fungi.

Microorganisms of each colony on the ager medium were inoculated into 100 ml of each medium containing 2 mg of chlornitrofen. The media were Medium-1 (peptone+$(NH_4)_2SO_4$ 2.6 g, KH_2PO_4 2.4 g, $K_2HPO_4\cdot 3H_2O$ 5.7 g, $MgSO_4\cdot 7H_2O$ 1 g, $CuSO_4\cdot 5H_2O$ 6 mg, $FeSO_4\cdot 7H_2O$ 1 mg, $MnCl_2\cdot 4H_2O$ 8 mg, $ZnSO_4\cdot 7H_2O$ 2 mg in 1000 ml of water at pH 7.0), Medium-2 ($NaNO_3$ 0.5 g, K_2HPO_4 0.5 g, $MgSO_4\cdot 7H_2O$ 0.2 g, $FeSO_4\cdot 7H_2O$ 1 mg in 1000 ml of water at pH 7.0) and Medium-3 ($K_2HPO_4\cdot 3H_2O$ 1.6 g, KH_2PO_4 0.4 g, NH_4NO_3 0.5 g, $MgSO_4\cdot 7H_2O$ 0.2 g, $CaCl_2\cdot 2H_2O$ 25 mg, $FeCl_3\cdot 6H_2O$ 1 mg in 1000 ml of water at pH 7.0) for bacteria; Medium-2, Medium-3 and Medium-4 (egg albumin 0.3 g, glucose 1 g, $K_2HPO_4\cdot 3H_2O$ 0.5 g, $MgSO_4\cdot 7H_2O$ 0.2 g, $Fe_2(SO_4)_3$ 1 mg in 1000 ml of water at pH 7.0) for actinomycetes; Medium-2, Medium-3 and Medium-5 (sucrose 30 g, $NaNO_3$ 3 g, $K_2HPO_4\cdot 3H_2O$ 1 g, $MgSO_4\cdot 7H_2O$ 0.5 g, KCl 0.5 g, $FeSO_4\cdot 7H_2O$ 1 mg in 1000 ml of water at pH 4.5) for fungi. The media were incubated while shaken at 28°C for 1 week and homogenized by Polytron (Kinematica, Switzerland) for 1 min. The mixture was shaken with 30 ml of hexane for 5 min. Chlornitrofen in the hexane extract was determined by gas chromatography as described in our previous paper.[2] Strains of microorganisms which resulted in more than 50% degradation in the medium were chosen as effective chlornitrofen decomposers. They were purified on the alubumin ager medium and then the potato dextrose ager medium containing chlornitrofen.

4. Isolation of CNP-Amino-Degrading Microorganisms from Soil

Two hundred grams(dry basis) of the Chigasaki soil passed through a 1-mm sieve put in a 500-ml Erlenmeyer flask, flooded with water up to 2 cm deep, and preincubated at 28°C for 2 weeks. CNP-amino was then amended with the soil at 100 ppm, and the mouth of the flask was closed with a rubber stopper. Then, CNP-amino was added to the soil at 100 ppm three times every 2 weeks during further incubation at 28°C. After the 4th incubation, 10 g of the enriched soil was suspended in 100 ml of water. Then, microorganisms capable of degrading CNP-amino were isolated from the soil suspension by the dilution method, described for the isolation of chlornitrofen-degrading microorganisms.

5. Metabolic Study of Chlornitrofen and CNP-Amino by Isolates

Each strain of the isolated microorganisms

was inoculated into each 20 ml of the described media in a test tube (20×2.7 cm). Then, 0.4 mg of ^{14}C-chlornitrofen dissolved in 0.2 ml of a mixture of acetone and ethanol (1 : 1 v/v) was added into the tube. The tube was maintained on a reciprocal shaker at 30°C for 5, 10 and 20 days. For determining $^{14}CO_2$ liberated from the incubating medium, CO_2-free air was discharged into the tube. The air from the outlet was passed through toluene to trap volatile organic materials and then through 4 N KOH solution to trap $^{14}CO_2$. The KOH solution and the toluene were renewed every 2 days. The tubes were maintained under sterile conditions. Radioactivity in the traps was measured as described previously.[1] Growth of the microorganisms was determined periodically by measuring the optical density at 600 nm, and calibrated based on the dry weight.

At the end of incubation, the medium was homogenized with 100 ml of acetone by Polytron. The homogenate was concentrated *in vacuo* and extracted successively with ether after adjusting to neutral, acidic and alkaline conditions. The extracts were dried with anhydrous Na_2SO_4, concentrated and subjected TLC as described previously.[1]

6. *Radioassay and Thin-Layer Chromatography*

The methods have been described in our previous paper.[1]

RESULTS

1. *Isolation of Microorganisms Degrading Chlornitrofen and CNP-Amino*

Microorganisms active for the degradation of chlornitrofen and CNP-amino were isolated from the soil enriched by amending with these chemicals. From 15 isolates, 3 strains of bacteria (B-3, -7 and -10), a strain of actinomycetes (A-6) and a strain of fungi (F-14) degraded chlornitrofen in the media (Medium-1 and -4). One (B-125) of three bacterial isolates degraded CNP-amino in the same media. These isolates, however, were unable to utilize chlornitrofen and CNP-amino as sole carbon sources in the inorganic salt media (Medium 2 and -3). The characteristics of bacterium B-10 were as follows: irregular rod(long) shape, gram-stain positive, none of dull mobility, positive catalase activity, negative gelatine liquation, negative starch liquation and negative arginine dehydrolase activity. From these results, the genus of isolate B-10 was identified to be *Corynebacterium* sp. Two isolates A-6 and F-14 were identified to be *Streptomyces* sp. and *Penicillium* sp., respectively, based on the morphological and physiological characteristics. Three strains of bacteria (B-3, -7 and -125) were not identified.

2. *Metabolic Fate of Chlornitrofen and CNP-Amino by Isolated Microorganisms*

Changes in the amount of chlornitrofen metabolites by the isolates (B-10, A-6 and F-14), and growth of the isolates are shown in Table 1. In all media, total radioactivity recovery from neutral, acidic and alkaline fractions of each medium exceeded 93% of the applied amount during 20 days of incubation. The number of the microbes reached a maximum after 5 days of incubation, and the degradation rate of chlornitrofen increased as microbes in the medium increased. In Medium-1 (peptone-inorganic medium), B-10 (*Corynebacterium* sp.) neither degraded chlornitrofen, nor grew until the 20th day of incubation. About 15 radioactive spots were detected on thin-layer chromatograms of the ether extracts, and main metabolites were identified by co-chromatography with some kinds of solvent systems. The amount of the main metabolites greatly differed depending on the isolates and the media examined. The main metabolic product by B-10 was CNP-acetylamino in Medium-4, and others were CNP-amino, CNP-methylamino and *p*-nitrophenol. In the metabolic products by A-6 (*Streptomyces* sp.), *p*-nitrophenol was the main product in Medium-1, while CNP-amino and CNP-methylamino were the main products in Medium-4. CNP-acetylamino and CNP-hydroxy were detected in small amounts in Medium-1, but neither CNP-amino nor CNP-methylamino was detected. CNP-acetylamino and *p*-nitrophenol were also detected in small amounts in Medium-4. The metabolites by F-14 (*Penicillium* sp.) in Medium-1 were CNP-amino, CNP-acetylamino and *p*-nitrophenol but none of them were accumulated during the incubation.

Table 1 Degradation products of chlornitrofen by microbial isolates (%).

Isolates[a]	Medium No.	Days of inc.	Distribution of radioactivity[b]										Growth of microbes (mg)
			CNP	CNP-NH_2[c]	CNP-$NHCOCH_3$[c]	CNP-$NHCH_3$[c]	CNP-OH[c]	Nitro-phenol	Other hydro-phobics	$^{14}CO_2$	Water soluble	Total radio-activity	
B-10	1	5	95.9	0	0	0	0	0	0	0	0	95.9	0
		10	95.3	0	0	0	0	0	0	0	0	95.3	0
		20	94.0	0	0	0	0	0	0	0	0	94.0	0
	4	5	78.4	0.6	2.0	0	0	0	1.7	0	14.0	96.7	18
		10	58.4	0.8	4.8	0.6	0	0.3	2.5	0.8	27.3	95.5	24
		20	51.0	0.5	8.1	0.9	0	0.4	4.1	3.4	27.5	95.9	24
A-6	1	5	25.1	0	3.3	0	0	40.9	3.5	0.5	23.7	97.0	57
		10	23.6	0	0	0	0.3	25.8	4.2	1.0	40.3	95.2	49
		20	15.9	0	0	0	0.5	19.2	3.6	3.4	53.5	96.1	57
	4	5	86.2	1.7	0	6.0	0	0	1.8	0	3.3	99.0	25
		10	51.6	4.0	0.4	10.2	0	0	3.5	0	24.7	94.4	30
		20	34.1	3.3	0.3	4.7	0	0.2	3.7	0.8	50.2	97.3	29
F-14	1	5	93.1	0	0.7	0	0	0	1.1	0	2.2	97.1	6
		10	87.3	0.9	1.2	0	0	0.3	0.9	0	7.6	98.2	6
		20	64.6	1.8	1.6	0	0	0.9	2.5	0.3	24.3	96.0	6
	5	5	70.9	1.0	10.9	0	0	0	2.3	0	14.0	99.1	33
		10	12.1	0.8	66.9	0	0	0	4.7	0	12.5	97.0	30
		20	10.6	0.7	52.0	0.5	0	0.4	4.3	0.9	29.4	98.8	30

[a] Microbial isolates; B-10: *Corynebacterium* sp., A-6: *Streptomyces* sp., F-14: *Penicillium* sp.

[b] Radioactivity applied is referred as 100%.

[c] CNP-NH_2: 2,4,6-trichlorophenyl 4-aminophenyl ether, CNP-$NHCOCH_3$: 2,4,6-trichlorophenyl 4-acethylaminophenyl ether, CNP-$NHCH_3$: 2,4,6-trichlorophenyl 4-methylaminophenyl ether, CNP-OH: 2,4,6-trichlorophenyl 4-hydroxyphenyl ether.

Table 2 Degradation of CNP-amino by isolated microorganisms (B-125) (%).

Medium No.	Days of inc.	Distribution of radioactivity[a]									Growth of microbes (mg)
		CNP-NH_2	CNP-$NHCOCH_3$	CNP-$NHCH_3$	Amino phenol	Hydro-xyform-anilide	Other hydro-phobics	$^{14}CO_2$	Water soluble	Total radio-activity	
1	5	43.9	12.6	0	0.3	0	5.2	0	33.8	95.8	12
	10	22.2	12.2	0	0.6	0.5	4.8	0	49.5	89.6	12
	20	12.9	10.3	0	0.5	0.8	6.0	1.2	57.3	89.0	12
4	5	44.6	10.3	0	0	0	3.1	0.6	41.3	99.9	15
	10	34.1	6.0	1.3	0	1.3	2.6	1.7	51.1	98.1	12
	20	8.9	47.1	0	0	3.8	4.1	4.4	26.6	94.9	13

[a] Radioactivity applied is referred as 100%.

In Medium-5, however, the main metabolite was CNP-acetylamino. CNP-amino, CNP-methylamino and *p*-nitrophenol were also detected in small amounts.

Changes in the amount of CNP-amino metabolites by bacteria (B-125), and growth of the isolate are shown in Table 2. There were no large differences in the growth of bacteria and the degradation rate of CNP-amino between Medium-1 and -4. More than 89% of applied radioactivity was recovered from both media during 20 days of incubation. CNP-acetylamino was produced in large amounts as the main metabolite, especially in Medium-4. Small amounts of *p*-aminophenol and CNP-methylamino were also detected in Medium-1 and -4. *p*-Hydroxyformanilide was produced in larger amounts in Medium-4 than -1.

$^{14}CO_2$ was liberated by these degraders by 0.3 to 4.4% from chlornitrofen and CNP-amino during 20 days of incubation. Ether-soluble unidentified metabolites were also detected, but none of them were more than 1% of applied radioactivity. The amount of water-soluble radioactive materials increased rapidly with time in all cases. It was presumed that hydrophilic metabolites in the water-soluble fractions were produced by these isolates, but such metabolites were not studied this time.

DISCUSSION

Our previous reports[1,9] have showed that chlornitrofen is rapidly degraded in soil under flooded conditions, and the degradation products are CNP-amino and its *N*-acetyl derivatives, etc. which are finally degraded to CO_2. In sterilized soil under reductive conditions, however, the degradation route involves only the reduction to CNP-amino, which is promoted with Fe^{2+} in soil. This finding suggests that microorganisms are involved in the degradation process to a large extent. In this study, five strains of chlornitrofen-degrading microorganisms were isolated from the soil enriched with chlornitrofen. One bacterium capable of degradating CNP-amino was also isolated from the soil enriched with CNP-amino. Chlornitrofen-degrading microbes were identified *Corynebacterium* sp. (B-10), *Strepto-*

myces sp. (A-6) and *Penicillium* sp. (F-14), but two bacteria were not identified. A bacterial isolate (B-125) capable of degrading CNP-amino was also left unidentified. All of the isolates could not utilize chlornitrofen and CNP-amino as sole carbon sources in inorganic salt media. Chlornitrofen and CNP-amino were rapidly degraded in the presence of supplemental carbon sources. These facts suggest that these microbes degrade diphenyl ethers by co-metabolism. No growth of *Corynebacterium* sp. (B-10), however, was observed in Medium-1 (peptone-inorganic salts), and chlornitrofen was not degraded by the isolate in the medium. The result indicates that the isolate (B-10) is unable to utilize chlornitrofen or peptone as a carbon source.

The amount of degradation products of chlornitrofen was largely dependent on the kind of the isolates and the nutrient media examined. The proliferation rates of the isolates were also dependent on the kind of media.

In all cases, about 15 degradation products of chlornitrofen and CNP-amino were detected in the ether extracts of media by TLC. Four main degradation products were identified as CNP-amino and its *N*-acetyl and *N*-methyl derivatives, and *p*-nitrophenol by cochromatography. CNP-hydroxy, *p*-aminophenol and *p*-hydroxyformanilide were also identified as minor metabolites. Possible metabolic pathways of chlornitrofen by isolated microorganisms are summarized in Fig. 1. Chlornitrofen was attacked at three sites of the molecule. The first step was the reduction of the nitro group to produce CNP-amino, which was subsequently acetylated to CNP-acetylamino. This main pathway is also the main degradation pathway in soil.[1] A large amount of CNP-amino was accumulated in flooded soil, while there was no tendency of its accumulation in the media for microbial metabolism, because the stabilization of CNP-amino by soil adsorption[10] does not occur in such media. The acetylation and methylation of CNP-amino occurred rapidly in the media. The degradation of chlornitrofen was remarkably retarded in sterilized soil in general. When flooded soil was sterilized after preincubation before the application of chlornitrofen, however, its nitro group was reduced with Fe^{2+} in reductive soil.[3] The reduction with Fe^{2+} was a dominant route in flooded soil, where the reduction of Fe^{3+} to Fe^{2+} is also attributable to microbial action.[11] These results indicate that microorganisms are involved directly and indirectly in the degradation of chlornitrofen at the initial step. In soil, CNP-amino was produced under both oxidative and reductive conditions.[1] The reduction of nitro group to produce CNP-amino was presumed mainly due to microbial action in soil under oxidative conditions, while it was due to chemical reaction with Fe^{2+} in soil under reductive conditions. The finding that acetylaniline compounds are easily hydrolyzed to the corresponding aniline derivatives by microorganisms.[12] It may indicate that the acetylation of CNP-amino is a reversible reaction. This pathway was identified as the main route by B-10 in Medium-4 and F-14 in Medium-1 and -5. The other metabolic pathway involved was the methylation of CNP-amino to CNP-methylamino, which was not detected as a degradation product in flooded soil. Because *N*-methylanilide derivatives are oxidized to amino derivatives by *Pseudomonas aminovorans*,[13] the methylation of CNP-amino may also be reversible. This seemed to be a dominant metabolic pathway by A-6 in Medium-4. The pathway also involved was the hydrolytic cleavage of the ether linkage of chlornitrofen to produce *p*-nitrophenol and it was the main

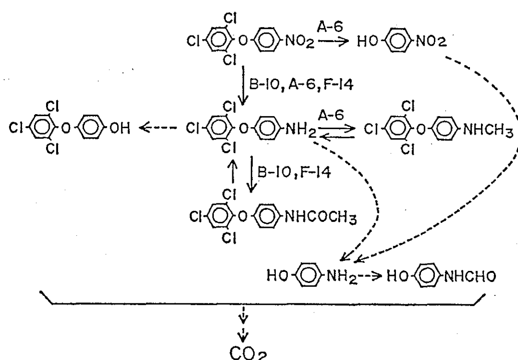

Fig. 1 Proposed metabolic pathways of chlornitrofen by microorganisms isolated from soil. ——: main pathways, ------: minor pathways. B-10: *Corynebacterium* sp., A-6: *Streptomyces* sp., F-14: *Penicillium* sp.

metabolic route by A-6 in Medium-1. The production of phenolic compounds was an important process in the degradation of chlornitrofen,[1] chlomethoxynil[4] and bifenox[6] in soil. A minor pathway was the substitution of the amino group by the hydroxy group to produce CNP-hydroxy by A-6 in Medium-4. This was also observed as a minor pathway in flooded soil.[1]

In the metabolic pathways of CNP-amino, *N*-acetylation and *N*-methylation were main processes. The ether linkage also cleaved to produce *p*-aminophenol, which was formylated to *p*-hydroxyformanilide. The formylation of CNP-amino in soil was also observed as one of the minor degradation pathways.[1]

The phenyl rings of chlornitrofen and CNP-amino were cleaved and oxidized to CO_2 by all the isolates examined. However, CO_2 was slightly liberated from chlornitrofen in soil[1] and chlornitrofen and CNP-amino degrading microorganisms. Water-soluble metabolites were produced in large amounts by the degrading microorganisms in all the cases.

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要　　約

微生物による除草剤 Chlornitrofen(CNP) とそのアミノ誘導体の代謝

小山田正美，鍬塚昭三

CNP およびそのアミノ誘導体を分解する数種の微生物を土壌から単離し，そのうち *Corynebacterium* sp., *Streptomyces* sp. および *Penicillium* sp. を同定した．無機培養液中において，それら分解菌は両化合物を唯一の炭素源として利用できなかったが，他の炭素栄養源の添加によって急速に分解した．分解菌による CNP の主要代謝経路は，ニトロ基の還元による CNP アミノ誘導体の生成とそれに続く *N*-アセチル化，*N*-メチル化およびアミノ基の水酸基への置換ならびにエーテル結合の開裂であった．これら代謝経路は供試した微生物および培養液の違いにより異なった．アミノフェノールおよびホルミル体も代謝物中に検出され，フェニル基は CO_2 に酸化された．