Original Article

Bound Residues of 4-Aminophenyl 2,4,6-Trichlorophenyl Ether and 4-Aminophenyl 2,4-Dichlorophenyl Ether in Paddy Soil Treated with CNP (Chlornitrofen)*

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An efficient method for the liberation of CNP-amino (4-aminophenyl 2,4,6-trichlorophenyl ether) and nitrofen-amino (4-aminophenyl 2,4-dichlorophenyl ether) from soil bound residues was studied. A soil sample was obtained from a paddy field treated with CNP-granules (4-nitrophenyl 2,4,6-trichlorophenyl ether) more than nine months earlier. In the acetone extract of the soil, 3.64 ppm of CNP, 0.50 ppm of CNP-amino, and 0.01 ppm of nitrofen-amino were detected. However, large quantities of these amines (11.40 ppm and 0.88 ppm, respectively, at maximum) were observed in extracts from soils treated under strongly alkaline or acidic conditions. Fractionation of soil organic matter pre-extracted with acetone revealed that the amino compounds were largely associated with the humin fraction of the soil. A maximum amount of CNP-amino from soil bound residue was obtained by heating the acetone-extracted soil with 5 N potassium hydroxide and sodium sulfide for 20 hr at 100°C.

INTRODUCTION

Diphenyl ether herbicides, chlornitrofen (CNP, MO®), nitrofen (NIP, TOK®), and chlomethoxynil (X-52) have been widely used in Japan for the control of weeds in rice paddy fields. These herbicides are applied to flooded soil during the months of May and June. The herbicides disappear rather rapidly from treated soil during the summer, and may increase again slightly in autumn as the soil becomes dry.¹⁰⁾

Many degradation products from the diphenyl ether herbicides have been detected in soils under flooded conditions in the laboratory.^{4,5)} Their amino analogs were easily derived by direct microbial action,²⁾ and by chemical

action with ferrous ions produced metabolically by soil microorganisms.⁴⁾ The amino analogs were very reactive in soils and it was difficult to determine their exact contents in a free state.¹⁴⁾ Acetamide and formamide analogs were also detected and, in some cases, nitrofen derivatives were produced by dechlorination of CNP.^{4,5)}

A considerable amount of CNP-amino and a small amount of CNP were detected in paddy field soils sampled in autumn and extracted with acetone and 1 N potassium hydroxide.⁸⁾ The author¹¹⁾ and Tamagawa *et al.*⁷⁾ observed a significant increase in the amounts of CNP-amino and nitrofen-amino released from soil samples when alkaline treatments were strengthened over the conditions reported by Tatsukawa *et al.*⁸⁾ A tentative method was established to obtain maximum amounts of amino analogs from soil.^{12,13)} By this method, soil samples (40 g, 50% of moisture) were

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added with 30 ml of 10 N potassium hydroxide and 1–10 g of sodium sulfide and kept for 4 hr at 80°C before extraction with acetone. Under the conditions of analysis, CNP and nitrofen in the soil samples were quantitatively reduced to CNP-amino and nitrofen-amino.¹²⁾

It was suggested that the amounts of amino analogs obtained by the above analytical method were the combined amounts of those from free amino analogs, nitro compounds, acylamide analogs, and "soil bound residues."^{1,3,6)} It was reasonable to determine the combined amounts of amino analogs to survey the total residual substances which produce amino analogs. The amount of "CNP-amino" in soil was calculated from the combined amount by subtracting the amount of CNP-amino changeable from CNP in the course of the alkaline treatment. The amount of "nitrofen-amino" in soil was calculated by the same means. The contents of CNP and nitrofen in the soil sample were determined by other methods.^{11–14)}

Many soil samples collected from different areas of Japan were surveyed for residues of CNP, nitrofen, and their amino analogs using this method of analysis.^{11–14)} The gas-liquid chromatography (*glc*) peaks of these compounds were identified with the authentic compounds by GC-MS.¹²⁾ Field surveys revealed that the amino analogs of CNP and nitrofen persist for many years as stable soil bound residues, and accumulate more significantly in the northern region of Japan.

This paper is concerned with further studies on the chemical character of bound residues in Kamikunii soil which shows the highest residue level of CNP-amino among the surveyed soil samples,^{10–14)} and on the establishment of optimum conditions for the liberation of CNP-amino from soil bound residues.

MATERIALS AND METHODS

1. Soil Sample

A sample of Kamikunii soil was selected for this study from among many types of paddy field soil,^{10–14)} as it was exceedingly contaminated with CNP and its derivatives. The soil was sampled to a depth of 5 cm from a rice paddy field at Kamikunii in Mito city in Japan on February 14, 1975. The characteristics of this

alluvial clay loam were as follows: sand 57.9%, silt 22.5%, clay 19.4%, field capacity 102.2%, pH (H₂O) 5.55, total carbon 6.3%, total nitrogen 0.57%, C.E.C. 28.9 me/100 g and free iron oxide 8.3 mg/100 g. MO-granules (CNP 3.6 kg/ha, on May 5) and Haikat-granules (CNP 2.1 kg and ethyl ester of MCPA 0.21 kg/ha, on May 30) were applied to the field in 1973 and in 1974. Nitrofen had never been applied to this field. The field was then sampled 9 months after the final application of CNP. It was unusually wet even after drainage, and the moisture content of the soil sample was 56%. The sample was homogenized in mortar without additional water.

2. Chemicals

CNP, nitrofen, CNP-amino and nitrofen-amino of *glc* standards were cordially provided by Mitsui-Toatsu Inc. Purity of the standards was confirmed by examining their melting points and GC-MS. The melting point were CNP 107.5°C, nitrofen 71°C, CNP-amino 106°C and nitrofen-amino 59–60°C.

3. Alkaline or Acidic Treatment of Soil

The homogenized soil sample (40 g as wet soil) was weighed in a stoppered conical flask. As shown in Table 1, addition of 30 ml of 10 N KOH to the sample resulted in a final normality of 5.8 N, 30 g of solid KOH and 10 ml of water in 11.4 N, 30 ml of 3.5 N HCl and 10 N H₂SO₄ in 2.0 N and 5.8 N, respectively, and conc. H₂SO₄ (17 ml) and water (10 ml) in 12.5 N. For samples Nos. 2 and 3 in Table 1 crystalline sodium sulfide (Na₂S·9H₂O) was added to accelerate the rate of amino analog formation from nitro compounds. The treated soil mixtures were heated at varying temperatures and time periods in a water bath with frequent shaking. After treatment, the mixtures were shaken for 1 hr with 100 ml of acetone and centrifuged. The supernatant was purified for estimation of CNP, nitrofen, and the amino analogs by *glc*.

4. Fractionation of CNP-Amino in Soil

CNP-amino in the soil was estimated after fractionation as shown in Fig. 1. Each extraction procedure from the soil was carried out by shaking with solvent for 30 min at room tem-

Fig. 1 Flow diagram for fractionation of CNP-amino from CNP-treated paddy field soil.

perature, centrifuging at 3,500 rpm for 8 min at 5°C, and filtering through a glass filter. The residual soil was treated with a hot alkaline solution and extracted with acetone. The aqueous extracts IV, V and VI were each shaken with 30 ml of hexane for 5 min, and the hexane layers IVF, VF and VIF were separated. To the remaining aqueous layers were added 10 g of crystalline Na₂S and enough solid KOH to adjust the basicity to a 10 N concentration. These solutions were heated for 20 hr in a water bath at 85°C and shaken with 30 ml of hexane for 5 min at room temperature to obtain hexane extracts IVB, VB and VIB. CNP-amino in each organic extract was cleaned up and estimated by *glc* analysis (Table 2).

5. Optimum Conditions for Release of Bound CNP-Amino

For the study, 2 g of the dry soil powder prepared by extracting soil with acetone (Fig. 1) was treated with 25 ml of KOH varying in concentration from 1 to 15 N as shown in Figs. 3 and 4. Crystalline Na₂S was added to the soil mixture in amounts ranging from 0 to 10 g. The samples were heated at various temperatures for various periods as shown in Fig. 3. The released CNP-amino was extracted

with 100 ml of hexane by shaking for 1 hr.

6. Cleanup of Crude Extracts and Trifluoroacetylation

A 30 ml of aliquot of the crude acetone extract was added to 300 ml of aqueous 0.1 N KOH and 2% Na₂SO₄ held in a separatory funnel. This mixture was shaken with 80 ml of hexane and the lower aqueous layer discarded. The hexane layer was shaken twice with 60 ml of 0.5 N HCl and checked for CNP and nitrofen. The aqueous layers of the two extractions were combined, adjusted to pH 10 with 10 N KOH, and shaken with 30 ml of hexane for 5 min. For trifluoroacetylation of amino analogs in the hexane extracts, 1 ml of 1% anhydrous trifluoroacetic acid in benzene and 1 ml of 0.2% triethylamine in benzene were added to 8 ml of the hexane solution and shaken vigorously for 1 min. The reaction mixture was washed with 2 ml of water and dried with 1 g of anhydrous Na₂SO₄.

7. Gas-liquid Chromatography

The *glc* analyses of CNP, nitrofen, and their trifluoroacetylated amino derivatives were performed with a Shimadzu GC-5A equipped with (⁶³Ni) EC detector. Separation was achieved on

Table 1 Relationship of alkaline or acidic treatment with the amounts of CNP, CNP-amino, and nitrofen-amino extracted from Kamikunii soil.

No.	Soil treatment ^{a)}						Extracted amounts ^{b)}		
	Alkali or acid	Final soln. normality (N)	Final vol. (ml)	Na ₂ S 9H ₂ O (g)	Heating		CNP (ppm)	CNP-amino (ppm)	Nitrofen-amino (ppm)
					Temp. (°C)	Time (hr)			
1	Control	0	22	0	20	0	3.64	0.50	0.01
2	KOH	5.8	52	1	80	4	1.30	7.24	0.14
3	KOH	11.4	47	10	90	10	t ^{c)}	11.40	0.88
4	HCl	2.0	52	0	20	0	3.74	0.66	0.01
5	H ₂ SO ₄	5.8	52	0	80	4	2.08	2.49	0.04
6	H ₂ SO ₄	12.5	49	0	90	10	0.12	4.87	0.04

^{a)} Forty grams of soil containing 22 g of moisture was treated.

^{b)} Amounts based on dry matter.

^{c)} Trace amount.

a 2 m × 3 mm i.d. glass column containing 5% OV-1 coated on Chromosorb W AW DMCS 60–80 mesh. The operating conditions for *glc* were: N₂ carrier gas 100 ml/min, injection port 230°C, column 220°C, and detector 260°C.

RESULTS

The amounts of CNP, CNP-amino and nitrofen-amino extracted from Kamikunii soil after alkaline or acidic treatment are given in Table 1. Without the treatment, 3.6 ppm of CNP, 0.5 ppm of CNP-amino and 0.01 ppm of nitrofen-amino were obtained by extraction with acetone (control). After heating the soil with KOH and Na₂S, the recovery of CNP was clearly decreased, while the recoveries of both amino analogs increased significantly. The maximum recoveries of amino analogs were obtained with 11.4 N KOH and 10 g of Na₂S (No. 3). A gas chromatogram of the extract of No. 3 is shown in Fig. 2. Acid treatments resulted in increased amounts of amino analogs, but the values were not as significant as those resulting from alkaline treatment (Table 1).

Table 2 shows the amount of CNP-amino determined for each soil fraction obtained from the paddy field soil by the method shown in Fig. 1. The total amount of CNP-amino extracted from 40 g of Kamikunii soil was 170.4 μg (9.7 ppm based on dry soil). The sum of CNP-amino in the acetone extracts I, II and III, which was present in a free state in the soil, was only 6% of the total extracted amount. In the aqueous extracts, small amounts of

Fig. 2 An example of a gas chromatogram of trifluoroacetamide derivatives of CNP and nitrofen extracted from soil.

The chromatogram was obtained by injection of 5 μl aliquot of an extract obtained under the conditions of No. 3 in Table 1 or a solution derived from a mixed standard solution of amino analogs at each 0.1 ppm. C: CNP-trifluoroacetamide, N: nitrofen-trifluoroacetamide.

CNP-amino were detected. IVF, VF and VIF represent CNP-amino which was released from soil bound residues in the course of fractionation. IVB, VB and VIB represent CNP-amino which was fractionated into the aqueous extracts in a bound state with the soluble humic substances (humic acid + fulvic acid) and liberated by the strong alkaline treatment with heat. The total amount of CNP-amino in

Table 2 Fractionation of CNP-amino in Kamikunii soil.

Fractions		CNP-amino extracted		
		μg	μg	%
Acetone extract (I)		6.8	10.4	6.1
Acetone extract (II)		2.6		
Acetone extract (III)		1.0		
0.1 M sodium pyrophosphate extract (IV)	Free amino (IVF)	0.6	4.3	2.5
	Bound amino (IVB)	3.7		
1 N KOH extract (V)	Free amino (VF)	1.6	4.8	2.8
	Bound amino (VB)	3.2		
10 N KOH extract (VI)	Free amino (VIF)	0.3	0.9	0.5
	Bound amino (VIB)	0.6		
Alkali treated soil acetone extract (VII)			150.0	88.0
Total			170.4	100.0

Soil (40 g) with a dry matter content of 17.6 g was analyzed. IVF, VF and VIF were estimated from the hexane extract of the aqueous solution. IVB, VB and VIB were estimated after hot alkaline treatment of the residual aqueous phase.

these three aqueous extracts was 5.8% of the extracted CNP-amino. The major part of CNP-amino (88%) in Kamikunii soil was detected in the acetone extract of the residual soil (including humin) after the hot alkaline treatment (VII). In addition to CNP-amino, CNP and nitrofen-amino were detected in the extracts I-III and VII, respectively.

The conditions for release of CNP-amino from soil bound residues are shown in Fig. 3. The quantity of CNP-amino released from Kamikunii soil pre-extracted with acetone increased with an increase in time and temperature of alkaline treatment. Maximum values of CNP-amino were obtained with 5 N KOH. Addition of 0, 2.5, 5 and 10 g of crystalline Na_2S to 5 N KOH followed by heating for 20 hr at 100°C obtained CNP-amino at concentrations of 7.2, 8.4, 8.1 and 7.9 ppm, respectively. Figure 4 shows that the amount of CNP-amino was reduced with heating periods in excess of 40 hr. Substitution of NaOH for KOH gave essentially the same result. These studies revealed that the maximum amount of CNP-amino from soil bound residues was obtained by heating samples at 100°C for 20 hr in the presence of 5 N KOH and 2.5 g of Na_2S .

Fig. 3 Release of bound CNP-amino from Kamikunii soil by potassium hydroxide treatment.

Two grams of soil pre-extracted with acetone was treated with 25 ml of KOH aqueous solution. In case of treatments of at 100°C , 5 g of crystalline Na_2S was added. Heating periods in hour are given in the figure.

Temperature of treatments; -----: 20°C (room temp.), ----: 84°C , —: 100°C .

Fig. 4 Release of bound CNP-amino from Kamikunii soil by alkaline treatment.

Two grams of soil pre-extracted with acetone was treated at 100°C with 25 ml of 5 N KOH and 5 g of crystalline Na₂S.

DISCUSSION

In this study, large quantities of CNP-amino and nitrofen-amino were released by strong alkaline treatments of Kamikunii soil. As the sources of the amino analogs, free amino analogs, nitro compounds (CNP and nitrofen),¹²⁾ acylamide analogs,^{4,5)} and "soil bound residues" of the amino analogs^{1,6)} were suspected. An interim definition of soil bound residue is "that unextractable and chemically unidentifiable pesticide residue remaining in fulvic acid, humic acid, and humin fractions after exhaustive sequential extraction with non-polar organic and polar solvents."³⁾ By the assay of acetone extracts of the soil, moderate amounts of CNP and CNP-amino, and a trace amount of nitrofen-amino were detected. Acylamide analogs were not detected in Kamikunii soil. Fractionation of the residual soil revealed that the major part of the released amino analogs was derived from the insoluble humin fraction. The detected nitrofen-amino was supposed to be derived from the treated CNP in the field, and put into the same bound status as CNP-amino.^{4,12)}

Optimum conditions for release of CNP-amino from the soil bound residues were as follows: heating 2 g of the acetone-extracted

soil with 25 ml of 5 N KOH and 2.5 g of crystalline Na₂S for 20 hr at 100°C. Although a wide variety of alkaline conditions has been applied for the determination of the amino analogs,⁸⁻¹⁴⁾ the above-mentioned conditions are recommended.

Hsu *et al.*¹⁾ suggested that ¹⁴C-labeled 3,4-dichloroaniline in soil is covalently incorporated into the humic matrix structure, becoming partially unhydrolyzable even under a strong alkaline condition. The authors are not sure that the bound CNP-amino and nitrofen-amino are completely released under the above-mentioned optimum conditions, but certainly the maximum amounts are available.

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要 約

CNP 処理水田土壤中の CNP-アミノ体と NIP-アミノ体の結合型残留

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土壤に結合して残留する CNP-アミノ体と NIP-アミノ体の有効な解離法を検討した。分析用土壤は、水田に CNP 粒剤を散布し、9 カ月以上経過してから採取した。この土壤のアセトン抽出により、3.64 ppm の CNP、0.50 ppm の CNP-アミノ体と 0.01 ppm の NIP-アミノ体が検出された。しかし、土壤を強くアルカリまたは酸で処理してから抽出すると、大量の CNP-アミノ体と NIP-アミノ体（最高で、11.4 ppm と 0.88 ppm）が検出された。アセトン抽出後、土壤有機物を分画すると、アミノ体は主に土壤中の humin 区分と結合していることがわかった。土壤結合型残留物質から抽出される CNP-アミノ体の最高値は、アセトン抽出後の土壤に 5 規定の水酸化カリウム水溶液と硫化ナトリウムを加え、100°C で 20 時間加熱することによって得られた。