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Microbial Metabolism of N-Methylcarbamate Insecticide. III.[1] Time Course in Metabolism of *o-sec*-Butylphenyl N-Methylcarbamate by *Aspergillus niger* and Species Differences among Soil Fungi

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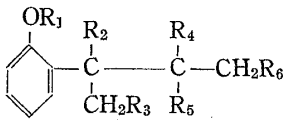
The biotransformation, time dependent changes of metabolites and differences of the mode of metabolism among five fungi, which were all isolated from the soil of rice field treated with *o-sec*-buthylpenyl N-methylcarbamate (BPMC) (I) were investigated using ^{3}H-BPMC. In every fungus used in the present study, a common metabolic pathway was that through hydroxylation at the alkyl side-chain. The metabolic breakdown of I by *A. niger* reached the maximum at 2 or 3 days cultivation and hydroxylation at alkyl side-chain of I was attained at the early stage of cultivation. Remarkable differences have been shown to exist among the various species as to formation of metabolites.

Agricultural chemicals undergo changes that are essentially dependent on the physical and chemical conditions of a given environment and, at the same time, are largely under the influence of biological factors, especially the effects of microorganisms in the soil. Characteristics of the transformation of agrochemicals by microorganisms consist in the production of a wide variety of metabolites, which provide information as valuable reference for studies on metabolic pathways in various species of organisms. Such data are also of great value to prediction of the metabolic potentiation.

Our previous study[3] demonstrated isolation of a series of various metabolites of *o-sec*-butylphenyl N-methylcarbamate (BPMC) (I) in cultures of *Aspergillus niger* (*A. niger*). Most of the metabolites are listed in Chart 1 and their chemical structures have been confirmed by comparison with the reference compounds obtained by chemical syntheses.[1]



M-1: $R_1=R_2=R_3=R_4=R_5=R_6=H$
M-2: $R_1=CONH_2$, $R_2=R_3=R_4=R_5=R_6=H$
M-3: $R_1=CONHCH_3$, R_4, $R_5=O$, $R_2=R_3=R_6=H$
M-4: $R_1=CONHCH_2OH$, $R_2=R_3=R_4=R_5=R_6=H$
M-5: $R_1=CONHCH_3$, $R_2=OH$, $R_3=R_4=R_5=R_6=H$
M-6 (*threo*): $R_1=CONHCH_3$, $R_4=OH$, $R_2=R_3=R_5=R_6=H$
M-7 (*erythro*): $R_1=CONHCH_3$, $R_4=OH$, $R_2=R_3=R_5=R_6=H$
M-8: $R_1=CONHCH_3$, $R_3=OH$, $R_2=R_4=R_5=R_6=H$
M-9: $R_1=CONHCH_3$, $R_6=OH$, $R_2=R_3=R_4=R_5=H$

Chart 1

The pattern of BPMC metabolism thus determined is considered to represent a typical example of microbial decomposition of the compound. The metabolism proceeded through hydroxylation of the *sec*-butyl side chain and some through cleavage or modification of the carbamoyl group. These metabolites were all shown to have less anticholinesterase activity than mother compound, BPMC, and hence no enhancement of toxicity by metabolism was taken place.[4]

Degradation of pesticides in a given environment owes much, as described above, to the action of soil bacteria or fungi. It would be of significance, therefore, to clarify in more

1) Part II: T. Suzuki and M. Takeda, *Chem. Pharm. Bull.* (Tokyo), **24**, 1976 (1976).
2) Location: *18–1, Kamiyoga 1–chome, Setagaya-ku, Tokyo, 158, Japan.*
3) T. Suzuki and M. Takeda, *Chem. Pharm. Bull.* (Tokyo), **24**, 1967(1976).
4) T. Suzuki, M. Takeda, and M. Uchiyama, *Eisei Kagaku*, **21**, 330 (1975).

detail the mode of decomposition of BPMC through metabolism by microorganisms isolated from the soil over which the drug has been applied. In view of this, the present study was performed to investigate the time course of ^{3}H–BPMC metabolism by *Aspergillus niger* in comparison with that by several other fungi isolated from the soil which had been sprayed with BPMC.

Materials and Methods

Incubation——A solution made up by dissolving 1.06 μCi of ^{3}H-BPMC[1] in 10 μl of acetone was added to the culture medium (30 ml), and broth cultures were prepared and incubated with the same media and in the same manner as described previously.[3]

Fungi——The organisms used included *A. niger* and several species of fungi. These were isolated from the soil which had been sprayed with a 4% granular preparation of BPMC four times at a dose of 4 kg/are. Their names were depicted in the legend of Table II.

Extraction of Metabolites and Determination of Radioactivity——In the course of incubation an aliquot was withdrawn from medium at various intervals and extracted with ether. Acidic metabolites (Fr. -1) were separated from neutral and phenolic metabolites (Fr. -2) by extracting the ether layer with $NaHCO_3$. Both fractions were further separated by thin-layer chromatography (TLC) of silica gel to obtain several metabolites, which were M-10, UK-2, and -3 from acidic Fr.-1 and M-1 through M-9 and UK-1 from neutral Fr. -2. The solvent system for each TLC was described in Fig. 2.

Radio-scannograms shown in Fig. 2 were recorded by Aloka TLC-chromatogram scanner. Each spot on plate was scraped off and extracted with MeOH and the radioactivity was determined by Aloka liquid scintillation spectrometer Type 650. Two kinds of scintillation mixture consisted of 2.0 g 2,5-diphenyloxazole oxide (PPO) and 0.2 g 1,4-bis[2(4-methyl-5-phenyloxazolyl)] benzene (DMPOPOP) in 500 ml of toluene and of 4.0 g PPO, 0.4 g POPOP, 100 g naphthalene in a mixture of 750 ml dioxane and 150 ml MeOH were used for organic solvent soluble and insoluble materials, respectively.

The remained broth which contains unextracted metabolites was applied onto a column of Amberlite XAD-2 to separate into two fractions, namely Fr.-3, eluate with H_2O and Fr.-4, eluate with MeOH.

Authentic Compounds——Ten kinds of authentic compounds listed in the legend of Fig. 2 were obtained by chemical[1] and microbiological[3] methods.

Results and Discussion

Time Course of BPMC Metabolism by *A. niger*

The time course of incorporation of radioactivity from ^{3}H–BPMC added to the broth culture of *A. niger* into various fractions was shown in Fig. 1. The average recovery of radioactivity was determined to be 63%. No significant change was observed in distribution of radioactivity in the various fractions after the seventh day of incubation. Thirty per cent of the original BPMC was noted to remain unaltered on the 3rd day of incubation, but to be 22% on 5th day, whereas Fr.-1 displayed a progressive increase in radioactivity with incubation, *viz.* 1.2%, 6.8% and 7.5% on 3rd, 5th and 7th days, respectively, but this radioactivity became practically constant thereafter. On the other hand, the radioactivity in Fr.-3 appeared to increase slowly but continuously throughout the incubation.

As shown in Fig. 2a, three different spots were obtained on the radiochromatogram of Fr.-1. One (M-10) with an *Rf* value of 0.48, which had been so far unidentified, was assumed to be *o*-(2-carboxy-1-methylethyl)phenyl N-methylcarbamate (X) based on the behaviors on TLC plates developed by some different solvent systems. The remaining two spots with *Rfs* 0.39 (UK-2) and 0.21 (UK-3) have been indicated to represent further oxidized forms derived from M-10 as viewed with respect to their physicochemical properties. No further attempt to identify these substances was done.

Fig. 2b shows the neutral and phenolic metabolites, M-1 through M-9, which were tentatively identified with the known metabolites[3] listed in Chart 1 by comparing the behavior on TLC plates.

Time dependent changes of various metabolites recovered from Fr.-1 and -2 were shown in Table I. It reveals that the side-chain hydroxylation takes place earlier than hydrolysis

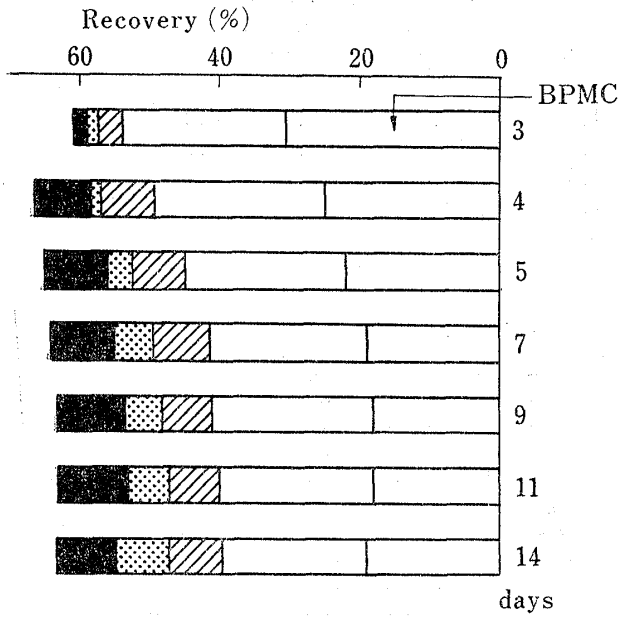


Fig. 1. Recovery of Radioactivity in Various Fractions in the Course of Cultivation of *A. niger* with ^{3}H-BPMC

■: Fr-1 □: Fr-2 ▦: Fr-3 ▨: Fr-4

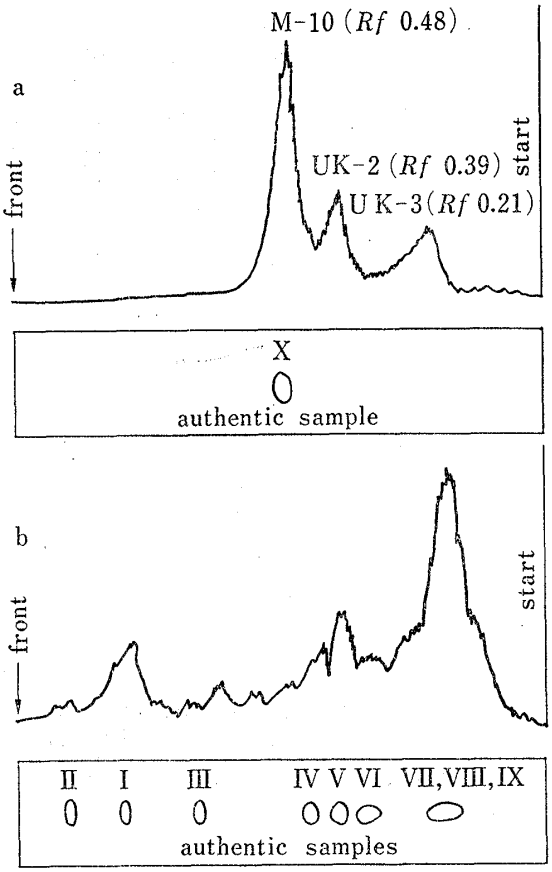


Fig. 2. Typical Radiochromatograms of Fr.-1(Fig. 2a) and Fr.-2(Fig. 2b) obtained by Incubation with *A. niger*

TLC
Fr.-1: $CHCl_3$–MeOH-AcOH (18: 1: 1)
Fr.-2: The plate was once developed with solvent system of MeCN-benzene (1: 9), and followed by solvent system of ether–hexane (1: 1, three times).

authentic samples
I: BPMC
II: *o-sec*-butylphenol (M-1)
III: *o-sec*-butylphenylcarbamate (M-2)
IV: *o*-(1-methylacetonyl)phenyl N-methylcarbamate (M-3)
V: *o-sec*-butylphenyl N-hydroxymethylcarbamate (M-4)
VI: *o*-(1-hydroxy-1-methylpropyl) phenyl N-methylcarbamate (M-5)
VII: a mixture of *threo- and erythro-o*-(2-hydroxy-1-methylpropyl) phenyl N-methylcarbamate (M-6 and M-7)
VIII: *o*-(3-hydroxy-1-methylpropyl) phenyl N-methylcarbamate (M-9)
IX: *o*-(1-hydroxymethylpropyl) phenyl N-methylcarbamate (M-8)
X: *o*-(2-carboxy-1-methylethyl) phenyl N-methylcarbamate (M-10)

of carbamoyl group and the acidic or carbonyl metabolites were brought by further oxidation of hydroxylated substances.

Fr.-3 and -4 are thought to contain, as described previously, more polar metabolites than those in Fr.-1 and -2, but the precise chemical characterization for the components in Fr.-3 and -4 was not performed. As one of the preliminary test for Fr.-4, neutral polar fraction, Fig. 3 shows two distinct spots on the radiochromatogram. One showing an *Rf* value of 0.91 was proved to contain metabolites M-6 through M-9 whereas the other with an *Rf* value of 0.59 was identical to none of the metabolites isolated.

Fig. 4 shows the radioactivity recovered in each fraction prepared from the culture media of various soil fungi. Predominant distribution of radioactivity was found in Fr.-2, neutral or phenolic metabolites including the original BPMC, except the case of *Cladosporium cladosporioides*. In the medium of *Cladosporium cladosporioides* total recovery of radioactivity was the lowest and, at the same time, a considerable portion of metabolites were occupied by acidic Fr.-1 and more polar substances. These facts indicates the effective metabolic conversion and decomposition of BPMC by *Cladosporium* sp.

Among the four other fungi, *Coniothyrium* sp. seems to produce fairly large amount of acidic Fr.-1 and polar Fr.-3, and the distribution of ^{3}H in Fr.-4 (polar metabolites) was highest

Table I. Recovery of Radioactivity from ^{3}H-BPMC by *A. niger*

Days	BPMC	M-1	M-2	M-3	M-4	M-5	M-6–M-9	M-10	UK-2	UK-3
3	30.8	0.05	0.46	0.24	0.95	1.0	10.9	0.31	0.21	0.25
4	23.8	0.08	0.32	0.23	1.2	1.3	13.0	2.5	0.90	0.52
5	21.9	0.14	0.33	0.40	1.6	1.6	14.3	3.1	0.75	1.1
7	19.1	0.14	0.34	0.38	1.8	1.8	12.6	3.6	0.91	2.0

Each value was shown as percentage toward total radioactivity added to the medium.

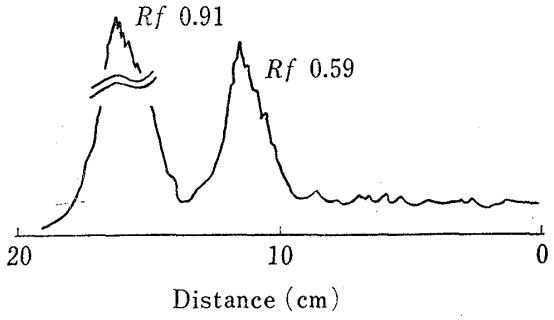


Fig. 3. Scanning Radiochromatogram of Fr-4 obtained by Incubation with *A. niger*

Solvent system: iso-PrOH–$CHCl_3$–MeOH–H_2O (37: 37: 19: 7)

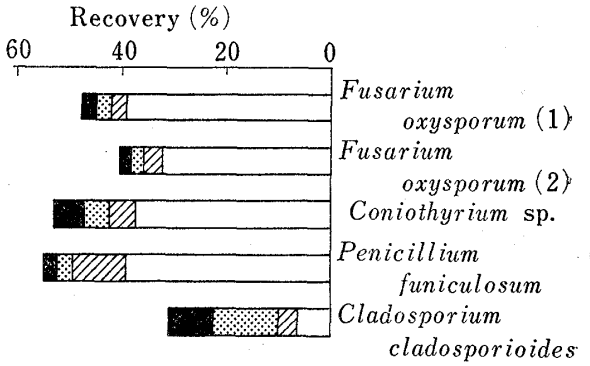


Fig. 4. Recovery of Radioactivity in Each Fraction in the Course of Cultivation of the isolated Soil Fungi with ^{3}H-BPMC

■: Fr-1 □: Fr-2 ▦: Fr-3 ▨: Fr-4

Table II. Radioactivity of the Metabolites by the Soil Fungi

Fungi	BPMC	M-1	M-2	M-3	M-4	M-5	M-6–M-9	M-10	UK-1	UK-2	UK-3
I	7.9	0.02	0.15	0.92	2.3	7.7	6.6	—	1.6	—	—
II	7.9	0.02	0.08	0.55	2.9	5.7	6.3	—	2.1	—	—
III	0.35	0.01	0.15	0.74	1.4	1.1	24.5	2.2	—	1.4	0.91
IV	0.04	0.01	0.03	0.43	0.62	5.6	26.7	0.55	—	0.14	0.04
V	0.51	0.49	0.49	0.83	1.3	0.69	0.72	3.3	—	1.5	0.88

Each value was shown as percentage toward total radioactivity added to the medium. I; *Fusarium oxysporum* Shlecht. ex Fr. (1), II; *Fusarium oxysporum* Shlecht. ex Fr. (2), III; *Penicillium funiculosum* Thom, IV; *Coniothyrium* sp., V; *Cladosporium cladosporioides* (Fres.) de Uries.

in the case of *Penicillium funiculosum*. These findings introduced from Fig. 4 were quantitatively explained by the results shown in Table II.

Table II includes per cent recovery of radioactivity in each metabolite isolated from Fr.-1 and -2 toward total radioactivity of substrates ^{3}H–BPMC as described in Table I for *A. niger*. It is obvious that *Cladosporium* sp. produced mainly acidic metabolites like M-10, UK-2 and UK-3, compared with other fungi, but total recovery was quite low indicating an efficient metabolic degradation was taking place. *Coniothyrium* sp. and *Penicillium* sp., both mentioned above, showed high distribution of ^{3}H in hydroxylated metabolites and only little residue of BPMC.

In the case of *Fusarium* sp. about one third of Fr.-2 was original BPMC, which reveals the less efficient metabolic conversion than other species.

On the chromatogram of Fr.-2 prepared from the medium of *Fusarium* sp. one new metabolite so far unidentified was detected, *Rf* value of which corresponded to that of *o*-(3-hydroxy-1-methylpropyl)phenol.[1] However, the dicarbamate ester of the new metabolite did not give the identical chemical constants and chromatographic behavior. Therefore, the metabolite was designated as UK-1 in Table II.

In conclusion BPMC was found to be metabolized to acidic or more polar substances *via* hydroxylated intermediates as demonstrated by the time course study of *A. niger*. Among some different kinds of soil fungi, *Cladosporium* sp. showed most efficient metabolic degradation of BPMC. *Coniothyrium* and *Penicillium* sp. showed similar degradation patterns and metabolized mainly BPMC to alkyl side-chain hydroxylated products. *Fusarium* sp. showed ineffective metabolic degradation but gave a wide variety of metabolites of BPMC.

Acknowledgement The authors are indebted to Dr. M. Ichinoe of this Institute for identification of fungi.