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The Absorption and Metabolism of Fenobucarb and Carbofuran by Susceptible and Carbamate Insecticide-selected Strains of the Brown Planthopper (*Nilaparvata lugens* Stål)

Fenobucarb 및 Carbofuran의 저항성 벼멸구 체벽 투과량과 체내 대사에 관한 연구

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ABSTRACT Cuticular penetration and detoxication as mechanisms of resistance to the carbamate insecticides in fenobucarb-selected(R_f) and carbofuran-selected(R_c) strains of the brown planthopper (*N. lugens* Stål) were investigated. Rates of penetration were not significantly different in the susceptible and resistant strains. However, total amount of excretion of the R_f and R_c strains were much larger than that of the susceptible strain. Fenobucarb and carbofuran were in vivo metabolized much faster in the R_f strain than in the susceptible strain. OSBP (o-sec-butyl phenol) and 3-ketocarbofuran phenol were in vitro the major metabolites of fenobucarb and carbofuran in the brown planthopper, respectively. Total amount of the two major metabolites were produced about 2 times larger in the R_f and R_c strains compared to the susceptible strain. OSBP and 3-ketocarbofuran phenol were not so toxic to the brown planthopper (LD_{50})100μg/g hopper). Based on our data, detoxication plays a large role in resistance to fenobucarb and carbofuran in the resistant strain of BPH, although several resistance factors may be involved.

KEY WORDS Brown planthopper, insecticide resistance, fenobucarb (BPMC), carbofuran, penetration, metabolism, metabolites

초 록 벼멸구의 살충제 저항성 기구를 구명하고자 fenobucarb 와 carbofuran으로 벼멸구를 18세대 이상 누대 선발하여 얻어진 저항성 벼멸구를 대상으로 저항성 기구를 조사하였으며, 얻어진 결과 중 살충제의 체벽 투과량, 대사 및 대사산물에 대하여 보고하고자 한다. Fenobucarb 및 carbofuran은 처리 1시간 이내에 처리된 양의 50%이상이 벼멸구 체벽을 투과하였고, 계통별 체벽 투과량 차이는 크지 않았으나, 저항성 계통에서 적었으며, 배설된 양도 처리 3시간 이후 저항성 계통에서 증가하여 5시간 후에는 감수성 계통에 비해 각각 1.9배, 1.6배가 많았다. 벼멸구 체내에서는 두 살충제 모두 감수성 계통에 비하여 저항성 계통에서 2시간 정도 빠르게 분해되었다. 벼멸구 homogenate를 이용하여 대사산물을 조사한 결과 fenobucarb의 주 대사산물은 OSBP(o-sec-butyl phenol)이고, crbofuran의 주 대사산물은 3-ketocarbofuran phenol이었으며, 대사산물량은 저항성 계통에서 2배 정도 증가하였다. 주 대사산물인 OSBP 및 3-ketocarbofuran phenol의 감수성 벼멸구에 대한 반수치사약량은 100μg/g 이상으로 독성이 없었다. 살충제의 빠른 분해대사는 벼멸구의 주요한 저항성 기구중 하나인 것으로 나타났다.

검 색 어 벼멸구, 살충제 저항성, fenobucarb(BPMC), carbofuran, 체벽 투과량, 대사, 대사산물

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Insecticide resistnace mechanisms of the insects are classified into 3 categories such as decreased absorption, alterd site of action and increased detoxication (Plapp 1976, Oppenoorth 1984). Decreased absorption was revealed in the house fly (Forgash et al. 1962, Farnham et al. 1965), mosquito (Matsumura & Brown 1963), tobacco budworm (Vinson & Law 1971) etc. Decreased absorption was reported to be controlled by a gene on chromosome Ⅲ in the House fly (Hoyer & Plapp 1968) and mosquito (Pillai & Brown 1965). But, penetrated amount of DDT and dieldrin in the resistant house fly was only a half of that of thec susceptible house fly(Plapp & Hoyer 1968).

Thereupon, it is believed that decreased absorption is an assistant factor for detoxifying enzymes to degrade larger amount of insecticide by extention of penetration time (Sawicki & Lord 1970).

Metcalf et al. (1967) reported that metabolites of nine carbamate insecticides were not different, but speed of degradation was faster in the resistant house fly than the susceptible one. Similar results were also reported in the cattle tick and two-spotted spider mite (Schnitzerling et al. 1974, Smissaert et al. 1970). Kao et al. (1984), however, told difference in amount of α-monoacid of malathion between the resistant and susceptible house fly.

Information on resistance mechanism is very important to understand resistance itself and to develop tools for suppression of resistnace problems. In this respect, we carried out some experiments on the resistnace mechaninsm of the BPH. Continued to the previous paper (Park & Choi 1991), penetration into integument and metabolism of ^{14}C-labeled fenobucarb and carbofuran in the fenobucarb-selected(R_f) and carbofuran-selected (R_c) brown planthopper strain is presented in this paper.

MATERIALS AND METHODS

Brown planthopper strains

The brown planthopper (BPH) strains were selected with fenobucarb(BPMC) and carbofuran more than 18 generations. Each selected strain was designated R_f and R_c strain, respectively. Two strains were 50.3 and 49.2 times less sensitive to corresponding insecticides than the susceptible strain. Rearing method of the BPH were identical to the previous report (Park & Choi 1991).

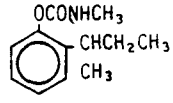
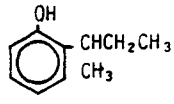
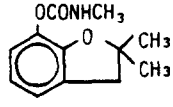
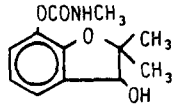
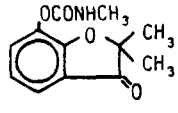
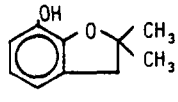
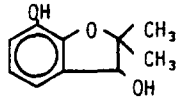
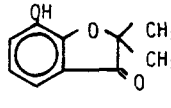
Chemicals

^{14}C-fenobucarb and ^{14}C-carbofuran were used. Specific activity (mCi/mmole) were 14.29 and 18.58, respectively. Radiochemical purity of both chemicals was more than 99%, ^{14}C-fenobucarb and ^{14}C-carbofuran were labeled uniformly in benzene ring and benzofuran ring-3, respectively. The labeled chemicals were donated from International Atomic Energy Agency. Some authentic metabolites were also used. Structural formula of metabolites, presented by Chemistry Division of Agricultual Chemicals Research Institute, were illustrated in Table 1.

Penetration studies

Four day old female planthoppers were treated topically on the dorsal thorax with 2.1 ng fenobucarb and 0.38 ng carbofuran per hopper in 0.25 μl of acetone. These dosages corresponded to the LD_{20} for the susceptible strain. Hundred female (50 hoppers/vial) were held in the scintillation vials (20 ml volume) for 0, 0.5, 1, 3 and 5 hours. Then, the surface of the BPH's from each treatment were washed 3 times with 25 ml of acetone. Acetone fraction with 3-4 drops of nujol®(Merck Co.) as the keeper solution was evaporated and followed by direct addition of 15 ml scintillation mixture (READY-SOLV EP®, Beckman Co.). It accounted for surface wash fraction. The washed BPH's were homogenized in 20 ml methanol, and centrifuged twice for 20 minutes at 1,000 g. The supernatant with 3-4

Table. 1. Structural formula of fenobucarb and carbofuran, and its principal metabolites

	Structural formula	Chemical name
Fenobucarb (BPMC)		2-sec-butylphenyl methylcarbamate
OSBP		o-sec-butylphenol
Carbofuran		2, 3-dihydro-2,2-dimethylbenzofuran-= 7-yl methylcarbamate
3-Hydroxycarbofuran		2,3-dihydro-2,2-dimethyl-3-hydroxy-= benozofuran-7-yl methylcarbamate
3-Ketocarbofuran		2,3-dihydro-2,2-dimethyl-3-ketoben-= zofuran-7-yl methylcarbamate
Carbofuran phenol		2,3-dihydro-2,2-dimethyl-7-benzofu-= ranol
3-Hydroxycarbofuran phenol		2,3-dihydro-2,2-dimethyl-3-hydroxy-= 7-benzofuranol
3-Ketocarbofuran phenol		2,3-dihydro-2,2-dimethyl-3-keto-7-= benzofuranol

drops of nujol was evaporated, and followed by direct addition of 15 ml mixture EP (extracted fraction). One ml of BTS-450,® (Beckman Co.) was added to residue to solubilize the tissue. After standing overnight at room temperature, 4-5 drops of 30 % H_2O_2 was added to the solubilized sample to reduce the quenching. After 2 hour standing, 15 ml of mixture (READY-SOLV NA®), little amount of Triton X-100 and glacial acetic acid were added(unextracted fraction). Inner wall of the scintillation vial which had contained the treated BPH was washed with 15ml of mixture EP (excreted fraction). The redioactivity in all the samples were counted by the liquid scintillation counter (LSC: Beckman model LS 100C)

In vivo metabolism studies

^{14}C-ring labeled fenobucarb and carbofuran were treated to the BPH(R_f and susceptible strain) by the same method mentioned above. Investigation was carried out at 0.5, 1, 3 and 5 hours after treatment, and 100 females were subjected per each treatment. After the appropriate lapse of time, the BPH's were washed 3 times with each 25 ml acetone. The BPH's

were homogenized in 20 ml of methanol and filtered with glass filter. The filtrate was evaporated and partitioned between 25 ml of dichloromethane (twice), 25 ml of ether(1 time) and 10 ml of distilled water. Organic-soluble fraction was added 3-4 drops of nujol and evaporated. Finally, 15 ml of mixture EP was added to the organic-soluble fraction. Water-soluble fraction was made up of 2 ml of partitioned water and 15 ml of mixture EP. Radioactivity in all the samples were counted by the LSC.

In vivo metabolism studies

Two gram of each R_f, R_c and susceptible strain were homogenized in 10 ml of 0.067M phosphate buffer(pH 7.2) containing 0.3M sucrose by means of Potter's glass homogenizer at 4℃. After homognization, centrifugation was carried out at 1,000 g(2℃) without filtration. The supernant was filtered through the 6-layer cheese cloth and the filtrate was used as the enzyme source. Three ml of phosphate buffer, 2 ml of enzyme source and 10 μl of each labeled insecticide (fenobucarb: 2.07 μg, 31, 500dpm; carbofuran: 2.21 μg, 42,600 dpm) were mixed together. The mixture was incubated at 30℃ for 2 hours in shaking incubator, and partitioned between 20 ml of dichloromethane(twice), 20 ml of ether (1 time) and 25 ml of distilled water. Organic-soluble fraction with 3-4 drops of nujol was evaporated, and made it to 10 ml with methanol. Three ml of methanol was evaporated with N_2 gas and diluted into 0.5 ml of acetone. Then, 100 μl was applied to the thin layer chromatographic plate to fractionate metabolites. The developing solvent systems were diethyl ether and benzene(3 : 1, v/v) for fenobucarb and vice versa for carbofuran. Some authentic metabolites were cochromatographed to certify the fractionated metabolites. After 15 cm upward developing, 0.1 % ρ-nitrobenzenediazonium fluoroborate, dissolved in mixture of 1N methanolic NaOH and acetone/methanol(1 : 1, v/v), was sprayed to visualize the cochromatographed standards. Each colored region was scraped and radioactivity was determined by the LSC after addition of 15 ml of mixture EP. Above authentic metabolites were tested to check insecticidal activity to the BPH by topical micro-application method.

RESULTS AND DISCUSSION

Penetration studies

The LD_{20} dose caused slight hyperactivity and a little mortality in the susceptible strain, but not in the resistant strains during the test. Results of the fate of ^{14}C-fenobucarb and carbofuran topically applied to susceptible and resistant strains of the BPH are shown in Figure 1. Total recovered radiocarbon were more than 80% throughout the test. In the Fig. 1, extracted and unextracted fraction are united to internal fraction, and external fraction means surface wash fraction. The external fraction decreased sharply regardless of strains and insecticides. Internal fraction of both insecticides in the susceptible strain was gradually increased during the test intervals. But in the R_f strain, internal amount of fenobucarb was lower at the beginning, and started to decrease about 3 hours after treatment. Internal fraction of ^{14}C-crabofuran treated to the R_c strain showed similar trend. It has been reportd that reduced penetration plays a role in resistance to the insecticides. A reduced penetration mechanism has been identified as a 'Pen' factor on chrosome Ⅲ of the house fly which alone gives no resistance or enhanced resistance slightly (two to five folds) and acted as an intensifier of other resistance factors (Sawicki & Farnham, 1968, Plapp & Hoyer, 1968). Vinson and Law (1971) reported the increase of cuticular lipid and protein amount in the resistant tobacco budworm, and suggested that ascorbic acid was concerned to decreased absorption. In the green rice leafhopper (Moriya & Maeda

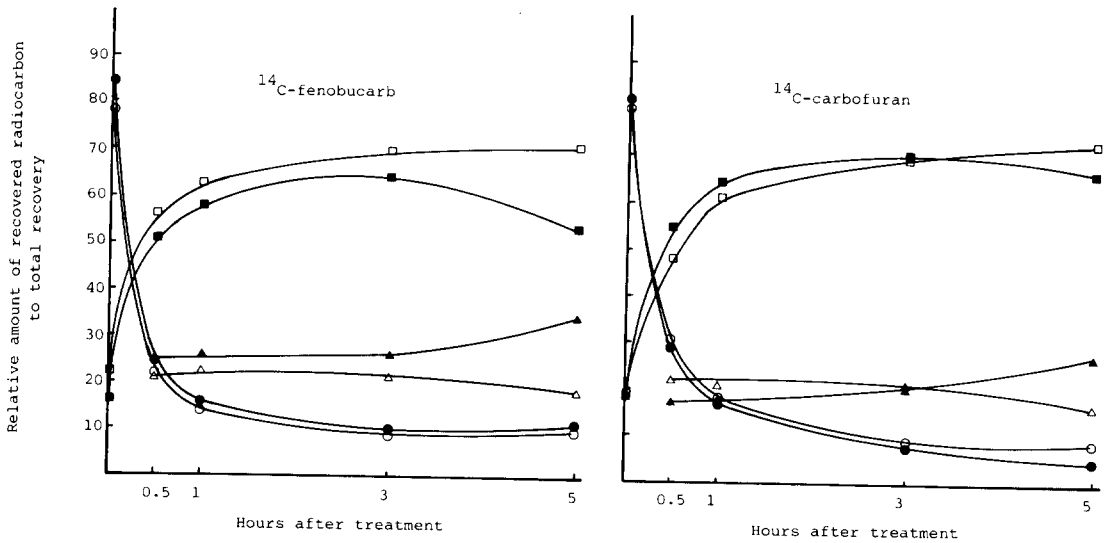


Fig. 1. Distribution of radiocarbon in external, internal and excreted fraction of the resistant and susceptible brown planthopper strains.

□—□ : Internal fraction of susceptible strain
△—△ : Excreted fraction of susceptible strain
○—○ : External fraction of susceptible strain
■—■ : Internal fraction of resistant strain
▲—▲ : Excreted fraction of resistant strain
●—● : External fraction of resistant strain

1976, Kazano et al. 1978, Hama et al. 1979) and smaller brown planthopper (Kazano et al. 1978), however, there was no difference in penetration amount between the susceptible and resistant strains.

Contrary to internal fraction, excreted fraction in the resistant strains was gradually increased and vice versa in the susceptible strain. It means that the speed of degradation and excretion are different between the resistant and susceptible strains. Metcalf et al. (1967) also reported that there was no difference in the sort of metabolites between the resistant and susceptible house fly strains, but the rates of degradation was higher in the resistant strain. Similar results were also reported in the cattle tick (Schnitzerling et al. 1974) and two-spotted spider mite (Smissaert et al. 1970). Based upon our data and earlier findings, it is concluded that increase in the speed of metabolism is one of the important resistance mechanisms.

In vivo metabolism studies

There were considerable difference in degradation rate of fenobucarb and carbofuran between the

Table 2. Distribution of radiocarbon between organic-soluble (O.S.) and water-soluble (W.S.) fractions in extracts of the resistant and susceptible female brown planthopper strains (*In vivo*)

Strains	Fractions	Recovered radiocarbon(%)							
		^{14}C-Fenobucarb				^{14}C-Carbofuran			
		0.5[a]	1	3	5	0.5	1	3	5
R_f	O.S.	60.0	50.8	27.1	—	25.2	23.0	16.1	9.7
	W.S.	40.0	49.2	72.9	—	74.8	77.0	83.9	90.3
S	O.S.	84.2	72.5	61.1	27.6	44.2	40.0	23.0	17.8
	W.S.	15.8	27.5	38.9	72.4	55.8	60.0	77.0	82.2

[a] Hour(s) after treatment.

Table 3. Isolation of some metabolites of ^{14}C-fenobucarb by thin layer chromatography after incubation with homogenates of the resistant(R_f) and susceptible female brown planthopper strains

Isolates	Isolated radiocarbon (%)	
	R_f	S
Fenobucarb	76.9	87.3
OSBP	17.2	9.0
Others	5.6	3.7

* Cochromatography was carried out with OSBP.

Table 4. Isolation of some metabolites of ^{14}C-carbofuran by thin layer chromatography after incubation with homogenates of the resistant (R_c) and susceptible brown planthopper strains

Isolates	Isolated radiocarbon (%)	
	R_c	S
3-Hydroxycarbofuran	0	0
3-Hydroxycarbofuran phenol	0	0.4
3-Ketocarbofuran	0.2	0.3
Carbofuran	72.4	81.1
3-Ketocarbofuran phenol	26.3	16.7
Carbofuran phenol	0.1	1.5
Others	1.0	0

* Cochromatography was carried out with 5 authentic metabolites.

resistant and susceptible strains (Table 2). Higher degradation rate and amount of the test insecticides were obtained in the R_f strain than in the susceptible one. Less than 50 % of organic-soluble fractions of fenobucarb and carbofuran in the R_f strain were diminished after 1 hour and within half an hour, respectively. More than 50 % of water-soluble fraction of fenobucarb, however, was remained after 3 or more hours in the susceptible strain. There were great differences in speed of degradation between fenobucarb and carbofuran, though R_f and R_c strains showed similar penetration patterns (Fig. 1). It might be attributable to the difference between strains tested, because only R_f strain was used in this experiment.

This result suggests that enhanced detoxication of the test insecticides in the carbamate insecticide-resistant brown planthoppers is one of the resistance mechanisms as pointed out in penetration studies. Moriya and Maeda (1976) reported that water-soluble portion of BPMC and MIPC was only 5-10 % compared to organic-soluble fraction 6 hours after treatment in the green rice leafhopper. It means that there are inter-specific difference in speed of degradation of the same insecticide.

In vivo metabolism studies

Metabolites of fenobucarb and carbofuran in the resistant and susceptible strains were presented in Table 3 and 4, respectively. OSBP(o-sec-butyl phenol) was the main metabolite of fenobucarb and other unknown metabolites were also detected. The amount of OSBP in the R_f strain was 2 times higher

Table 5. The LD_{50} value($\mu g / g$) of the metabolites of fenobucarb and carbofuran against the susceptible female brown planthopper strains, and their R_f values

Insecticides	Metabolites	R_f[a]	LD_{50}
Fenobucarb	Fenobucarb	0.60	1.21
	OSBP	0.70	)100
Carbofuran	3-Hydroxycarbofuran	0.08	4.36
	3-Hydroxycarbofuran phenol	0.21	)100
	3-Ketocarbofuran	0.27	3.35
	Carbofuran	0.33	0.33
	3-Ketocarbofuran phenol	0.43	)100
	Carbofuran phenol	0.52	)100

[a] Solvent system for fenobucarb and its metabolites; diethyl ether : benzene = 3 : 1(v/v), and for carbofuran and its metabolites; diethyl ether : benzene = 1 : 3 (v/v).

than that of the susceptible strain, and that of other unknown metabolites were also larger in the R_f strain. Ogawa et al. (1976, 1977) reported that amount of OSBP and OIPP(o-sec-isopropyl phenol) was relatively higher than other metabolites when treated in rice and paddy soil. Although their reports agreed with our report, further comprehensive investigations are needed in the insect body because of lack of fenobucarb metabolism study in insect.

The major metabolites of carbofuran was 3-ketocarbofuran phenol. The amount of 3-ketocarbofuran phenol was about 90% of all the metabolites detected in both strains. Its amount was also larger (1.6 times) in R_c strain than that of the susceptible strain. Metcalf et al. (1968) and Dorough (1968) demonstrasted that 3 - ketocarbofuran phenol and 3-hydroxycarbofuran were the main metabolites in the house fly.

OSBP is the product by hydrolysis of fenobucarb, but 3-ketocarbofuran phenol is acquired by oxidation and hydrolysis of carbofuran. It is supposed that degradation pathway of fenobucarb and carbofuran is dissimilar depend upon their different molecular structures as suggested by Kuhr (1970).

The amount of parent compounds were much more higher than in vivo metabolism studies. The reason is thought that data of in vivo metabolism studies (Table 2) was the comparison of the organic-soluble and water-soluble fractions, but in vitro metabolism studies, organic-soluble fraction only was used to seperate metabolites.

Toxicities of the metabolites of fenobucarb and carbofuran were checked (Table 5). OSBP and 3-ketocarbofuran phenol as major metabolite of fenobucarb and carbofuran were non-toxic to the BPH. The LD_{50} values of each compound were more than 100 μg/g, indicating that OSBP and 3-ketocarbofuran phenol were at least 80-300 times less toxic to the BPH than the parent compounds. However, other metabolites of carbofuran with carboxyl ester bond in their molecular structure were only 10 times less toxic than carbofuran. Acetylcholinesterae inhibition of OSBP was found to be 600 times less active than that of the parent compound (Ogawa et al. 1976). Oonnithan and Casida (1968) reported that AChE inhibition of metabolites with carboxyl ester bond in their molecular structure (metabolites by oxidation) had almost the same activity as carbaryl and propoxur.

Based upon our data and other reports, it is presumed that 3-hydroxycarbofuran and 3-ketocarbofuran, which are toxic to the BPH, are the first step metabolites as suggested by Lee (1988), and the final metabolites are OSBP and 3-ketocarbofuran phenol which are hydrolytic and non-toxic compounds of fenobucarb and carbofuran, respectively.

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