原　著

Synergistic Action of Sevin with *p*-Chlorophenyl *p*-chlorobenzenesulfonate. Masayuki KATO (Kyoto Herbal Garden, Research and Development Division, Takeda Chemical Industries Ltd. Ichijoji, Kyoto, Japan.) Received May. 8, 1965. *Botyu-Kagaku*, 30, 67, 1965.

14. Sevin に対する *p*-Chlorophenyl *p*-chlorobenzenesulfonate の共力作用

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数種の殺虫剤に対する *p*-chlorophenyl *p*-chlorobenzenesulfonate の共力作用をアズキゾウムシを用いた dry-film 法で検討した結果，特に carbamate 系殺虫剤に対して顕著な共力作用が見られた．Sevin に対する共力作用はイエバエ，チャバネゴキブリ，ハスモンヨトウなどにも見られ，その共力作用は殺虫剤の 4 倍量加用の時に最大であった．Sevin に対する *p*-chlorophenyl *p*-chlorobenzenesulfonate の共力作用の機作は，piperonyl butoxide の Sevin に対する機作と類似しており，昆虫表皮における殺虫剤の透過性に対する影響よりもむしろ昆虫体内における殺虫剤の酵素的解毒を阻害するためと推定された．

The synergisitic effect of methylenedioxyphenyl type of synergists, such as piperonyl butoxide, on carbamate insecticides was first demonstrated by Moorefield.[1] Moreover, it has been reported that such compounds, octachloro-dipropylether,[2] 2-(3, 5-dichloro-2-biphenylyloxy) triethylamine,[3] and organothiocyanates[4] can be demonstrated as having a synergistic effect in admixture with various carbamate insecticides.

The carbamate insecticides are easily detoxified by enzymatic hydrolysis in insect body,[5] so that they are of rather low effectiveness for many species of practical pest insects although they possess superior character of being low mammalian toxicity. The synergists, such as piperonyl butoxide, for carbamate insecticides effect their action through blockage of detoxication mechanisms and not through an effect on insecticide absorption or penetration.[5]

The compound, *p*-chlorophenyl *p*-chlorobenzenesulfonate which is one of excellent mitecides, has also potentiating activity for some chlorinated hydrocarbon insecticides, such as γ-BHC,[6] *p*, *p*'-DDT,[7] and aldrin[8] against mites or some insect species.

It is the object of this report to show the potentiation of the insecticidal activity of Sevin by the addition of *p*-chlorophenyl *p*-chlorobenzene sulfonate and the mechanism of the potentiating action.

Materials and Methods

The following 9 organic insecticides and 2 synergists were tested.

Malathion : *O*, *O*-dimethyl *S*-(1, 2-carboethoxyethyl) dithiophosphate

Mecarbam : ethyl [(diethoxyphosphino-thiolthio) acetyl] methylcarbamate

Dimethoate : *O*, *O*-dimethyl *S*-(*N*-methylcarbamoylmethyl) phosphorodithioate

γ-BHC : 1, 2, 3, 4, 5, 6-hexachlorocyclohexane

p, *p*'-DDT : 1, 1, 1-trichloro-2, 2-bis (*p*-chlorophenyl) ethane

Sevin : 1-naphtyl *N*-methylcarbamate

Zectran : 4-dimethylamino-3, 5-xylyl methylcarbamate

Carbamate 1: 4-chloro-3, 5-xylyl *N*-methylcarbamate

Carbamate 2: 2-chloro-3, 5-xylyl *N*-methylcarbamate

CPCBS : *p*-chlorophenyl *p*-chlorobenzenesulfonate

Piperonyl butoxide: 3, 4-methylenedioxy-6-propylbenzyl diethyleneglycolether

These samples were of technical grade except piperonyl butoxide which was of reagent grade.

The insecitcidal activity of the materials was primarily determined against 2-days-old adult males of azuki bean weevils (*Callosobruchus chinensis* L.) by the dry-film contact method in

the petri-dish, 9cm×2cm. Each test was repeated 3 times with 20 insects. Three-to 5-days-old adult females of house fly (*Musca domestica* L.) of a strain designated Lab-em-7-em, young adult males of German cockroach (*Blattella germanica* L.), and 3rd- to 4th-inster larvae of tobacco cutworm (*Prodenia litura* Fabricius) (50~80mg, 65mg in mean weight) were topically applied with 1.0 μl w/v solutions in acetone. The numbers of insects tested at each dosage were 30 flies, 20 roaches and 20 worms respectively. In the test with green rice leafhoppers (*Nephotettix apicalis cincticeps* Uhler), rice stems of 10cm length were dipped into emulsified aqueous solution for 10 seconds, and thereafter the stems were dried, one of the stems and 5 male adults of the leafhopper were put into a test tube (diameter 2.5cm, length 15cm), which was then covered the opening with a sheat of gauze. The LD-50 and LC-50 values were obtained from dosage mortality curves plotted on logarithmic-probability paper by the method of Litchfield and Wilcoxon.[9] The synergism was determined by the method previously reported.[9] All mortality counts were made after keeping the treated insects for 24 hours under the following conditions; supplying sugar and water for flies, a piece of potato for worms and water for roaches, at 25°C, 60~70 percent R.H. A continuous contact method was applied to azuki bean weevils and leafhoppers.

Anticholinesterase determinations were made by the Hesterin's colorimetric method.[10] Sevin and CPCBS (abbreviation for *p*-chlorophenyl *p*-chlorobenzenesulfonate) were purified by two times recrystallization of above-mentioned technical grade samples. The homogenate of house fly heads at the concentration of 6mg per ml of Ringer solution (NaCl 0.15M, $MgCl_2$ 0.04M) was used as the enzyme solution. The inhibitor was added into it and incubated for 40 minutes before adding the substrate (acetylcholine bromide, the final concentration was 0.003M). The reaction mixture was incubated at the pH7.2 and the temperature of 30°C with shaking for 30 minutes. The IN-50 values (the concentration of the inhibitors for 50 percent inhibition) were obtained from concentration-percent inhibition curves plotted on logarithmic-Probit paper by the method of Gardiner and Kilby.[11]

Detoxication study was performed as follows. Three grams of each species of insects, weevils, flies and worms (160mg in mean weight), were dipped into the emulsified 0.003 percent aqueous solution of Sevin with synergists (at the ratio of 1:4) or without synergists for 30 seconds. These solutions contained emulsifier (Tween-20) and solvent (benzene) at the concentration of 0.024 percent and 0.096 percent respectively. The insects dipped were transferred into a glassfilter to remove the excessive solution quickly by sacking, and then onto a filter paper placed in a petri-dish, 15cm×2.5cm. House flies treated were transferred into a cage after about 1 hour and then supplied with milk and sugar. After keeping at 25°C for 6 or 24 hours, the treated insects were washed with acetone for three times, and homogenized thoroughly by adding 20 ml of acetone using a small Waring blender. The homogenate was put into a centrifugal tube and centrifuged slightly. One ml of the supernatant acetone solution was pipetted into a Warburg flask, and evaporated *in vacuo*. The amounts of Sevin in insects were determined by means of the anticholinesterase method. The esterase determinations were made manometrically by the standard Warburg procedure[12] with $NaHCO_3$ buffer at 30°C in atomosphere of 5 percent carbon dioxide and 95 percent nitrogen. One ml of the above-mentioned enzyme preparation, 1ml of Ringer solution and 0.5ml of $NaHCO_3$ (0.138M) were pipetted into the treated Warburg flask, and 0.5ml of 0.06M acetylcholine bromide in the side arm. The substrate was added after pre-incubation for 40 minutes, and the evolution of carbon dioxide was determined after 30 minutes.

Results and Discussion

The carbamate insecticides are known to be inhibitors of acetylcholinesterase, and their anti-esterase activities are closely related to the mode of action of toxicity.[13] But these insecticides, such as Sevin, are easily detoxified in insect body by carbamate esterase.[5,14,15,16] The activity and mode of detoxication seem to be different among the insect species; the house fly metabolizes Sevin to three metabolites and the german

cockroach to six while the milkweed bug to one.[15] Since 1-naphthol in these insects formed the metabolites, the hydrolysis of Sevin to 1-naphthol was probably the critical step.[15]

The synergistic compounds for carbamate insecticides, such as piperonyl butoxide,[1] Sesamex,[17] MGK-264[18] and SKF-525A, inhibit the carbamate esterase *in vitro* at the concentration of 3×10^{-6}M.[19] The addition of synergists for carbamate insecticides decreases the rate of hydrolysis of insecticides to corresponding products in insect. The synergists potentiate the insecticidal activity of carbamates and appear an intrinsic toxicity which is responsible to anticholinesterase activity by the carbamate esterase inhibition and not by absorption or penetration of the carbamates.[6,16]

Comparisons between CPCBS and piperonyl butoxide as synergist for some organic insecticides at 4:1 ratio against azuki bean weevils are shown in Table 1. This table shows that the addition of CPCBS potentiates the toxicities of Sevin, carbamate 1, and γ-BHC as well as piperonyl butoxide. The most potentiated insecticides by the addition of CPCBS were carbamate insecticides; Sevin and carbamate 1. These carbamate insecticides were lower toxic than the other unpotentiated carbamate insecticides. Table 2 shows the synergisms of CPCBS and piperonyl butoxide for Sevin against various insect species. CPCBS potentiated the toxicities of Sevin against house flies, german cockroaches and tobacco

Table 1. Comparison between *p*-chlorophenyl *p*-chlorobenzenesulfonate and piperonyl butoxide as synergist for some organic insecticides at 4:1 ratio against azuki bean weevils.

Insecticide tested	Toxicity to azuki bean weevils, LD-50 (μg/cm²)		
	alone	with CPCBS[1]	with P. B.[2]
Sevin	1.5	1.1×10^{-1}*	1.9×10^{-1}*
Carbamate 1	4.2	3.2×10^{-1}*	2.2×10^{-1}*
Carbamate 2	4.1×10^{-2}	3.8×10^{-2}	4.1×10^{-2}
Zectran	5.4×10^{-2}	4.3×10^{-2}	4.4×10^{-2}
Mecarbam	1.4×10^{-1}	1.2×10^{-1}	7.2×10^{-2}*
Dimethoate	4.7×10^{-2}	4.6×10^{-2}	5.1×10^{-2}
Malathion	1.6×10^{-1}	1.5×10^{-1}	1.1×10^{-1}*
γ-BHC	1.1×10^{-1}	2.4×10^{-2}*	2.8×10^{-2}*
p, *p*'-DDT	2.2	2.2	2.3
CPCBS[1]	$>1.6\times10$		
P. B.[2]	$>1.6\times10$		

1) : *p*-chlorophenyl *p*-chlorobenzenesulfonate
2) : piperonyl butoxide
* : Significantly different between with and without synergist by the method of Litchfield and Wilcoxon.[9]

Table 2. Comparison between *p*-chlorophenyl *p*-chlorobenzenesulfonate and piperonyl butoxide as synergist for Sevin at 4:1 ratio against several insects.

Insect species tested		Sevin		
		alone	with CPCBS	with P. B.
House fly	LD-50(μg/g)	>750	100	26
German cockroach	〃 (〃)	>100	17	ca. 15
Tobacco cutworm	〃 (〃)	>100	55	71
Green rice leafhopper	LC-50(%)	1.8×10^{-3}	1.7×10^{-3}	1.8×10^{-3}

cutworms. CPCBS was less synergistic against house flies than piperoyl butoxide while CPCBS was more synergistic against azuki bean weevils and tobacco cutworms. To determine the most potentiating combination of CPCBS for Sevin, the experiments in which the ratio of CPCBS to Sevin was changed variously were carried out against the house fly and the azuki bean weevil. The data given in Tables 3 and 4 suggest that the ratio at 4 to 1 of CPCBS and Sevin was the most potentiating combination. Therefore, this ratio was used throughout the subsequent investigations.

In preliminary experiments given in Tables 1, 2, 3 and 4, the synergism was found when CPCBS was added to Sevin against house flies, azuki bean weevils, German cockroaches and tobacco cutworms as well as piperonyl butoxide was added. The experiment in Table 5 was conducted in order to know if the synergism of CPCBS depends upon the absorption or penetration of the insecticide. If the synergist is responsible for the absorption or penetration of the insecticide, the inseciticidal activity must be decreased when the insecticide and synergists are topically applied separately.[20] The data in Table 5 show that the separate application of the insecticide and the synergist results in decrease of its insecticidal activity undoubtedly, although the mortality is slightly higer than that on application of insecticide alone. From these data, there are no evidences to deny the effect of CPCBS on the

Table 3. Synergism of Sevin with *p*-chlorophenyl *p*-chlorobenzenesulfonate in various ratio against house flies.

Dosage (μg/g)		Percent Mortality
Sevin	CPCBS	
100	0	5.6± 5.62*
〃	50	10.5± 6.35
〃	100	24.4± 9.17
〃	400	52.8± 7.14
〃	1000	52.2± 15.7
0	1000	0.0

* : standard deviation (n=8~9)

Table 4. Synergism of Sevin with *p*-chlorophenyl *p*-chlorobenzenesulfonate in various ratio against azuki bean weevils.

Dosage (μg/cm^2)		Percent Mortality
Sevin	CPCBS	
1.1×10^{-1}	0	0.0
〃	5.5×10^{-2}	16.7± 7.07*
〃	1.1×10^{-1}	50.0±14.3
〃	4.4×10^{-1}	73.3±19.4
〃	1.1	71.2±16.4
0	1.1	0.0

* : standard deviation (n=7~9)

Table 5. Effect of *p*-chlorophenyl *p*-chlorobenzenesulfonate on the toxicity of Sevin against house flies topically applied on their thorax and/or abdomen.

Loci of application		Percent Mortality
Sevin(100μg/g)	CPCBS(400μg/g)	
Thorax		5.0± 5.5*
〃	Thorax	42.1±16.5
〃	Abdomen	16.0± 9.7
Abdomen		6.6± 7.8
〃	Thorax	7.1± 4.3
〃	Abdomen	13.3± 6.5

* : standard deviation (n=3~5)

Table 6. The joint synergistic action between *p*-chlorophenyl *p*-chlorobenzenesulfonate and piperonyl butoxide for Sevin against azuki bean weevils.

Sevin	(μg/cm^2)	0.11	0.11	0.11	0.11	0.11	0.11
CPCBS	(〃)	0.44	0.35	0.26	0.18	0.09	0
Piperonyl butoxide	(〃)	0	0.09	0.18	0.26	0.35	0.44
Observed Mortality	(%)	65	62	58	45	50	38
Expected Mortality	(%)	65	59.6	54.2	48.8	43.3	38
Potency*		100	104	107	92	115	100

* : (Observed Mortality/Expected Mortality)×100

absorption or penetration of the insecticide, however, it may be conceivable that the decrease of the insecticidal activity on the separate application is caused by separate distribution of the synergist and the insecticide in the insect body, and not caused by the direct effect of the synergist on absorption or penetration of the insecticide, so that separate application of the insecticide and the synergist is higher toxic than the insecticide alone.

To determine if CPCBS and piperonyl butoxide were acting at the same or separate sites, the combined effects of these synergists were evaluated. As shown in Table 6, the percentage mortality observed by the combined mixture coincided with calculated one by the methods of Sakai[21] and Horsfall.[22] From this fact, the action between CPCBS and piperonyl butoxide would be similar joint action, that is, the mechanism of the potentiating action of the toxicity by the addition of CPCBS to Sevin, and the site of action of CPCBS would be same to those of piperonyl butoxide.

Effects of CPCBS on the inhibition of acetylcholinesterase by Sevin *in vitro* was compared with that of piperonyl butoxide. From Table 7, the IN-50 values expressed as the concentration of Sevin were 1.9×10^{-7}M for Sevin alone, 2.0×10^{-7}M for Sevin with CPCBS (1:4 ratio in molar concentration), and 2.3×10^{-7}M for Sevin with piperonyl butoxide (*ditto*). Neither CPCBS nor piperonyl butoxide inhibited this enzyme at 10^{-4}M but both of them inhibited about 25 percent at 10^{-3}M. These data show that synergisms in insecticidal activity do not depend upon the potentiation of anticholinesterase activity of carbamate insecticide.

Table 7. Percentage inhibition of cholinesterase by Sevin with and without synergists in house fly.

Final concentration of Sevin (Mol.)	Sevin		
	alone	with CPCBS	with P. B.
4.5×10^{-6}	78.3	77.3	79.1
1.5×10^{-6}	67.0	68.0	68.8
5.0×10^{-7}	58.2	59.3	56.7
1.7×10^{-7}	50.4	49.5	49.0
5.5×10^{-8}	39.5	33.3	33.0
IN-50*($\times10^{-7}$)	1.9	2.0	2.3

* : concentration for 50 percent inhibition
Each synergist is added to Sevin at 4:1 ratio in molar concentration.

Many synergists for carbamate insecticides are considered to be inhibitor for detoxifing enzyme of carbamate insecticides as previously mentioned.[4,5,16,19] Then, the effect of CPCBS, as well as piperonyl butoxide, on detoxication of Sevin *in vivo* was examined. Insects were dipped into insecticidal solutions. The mortalities of the treated insects were negligibly small. Acetylcholinesterase catalizes the hydrolysis of acetylcholine to choline and acetic acid, so that the volume of carbon dioxide evolved indicates the activity of the esterase. Table 8 shows the evolution of carbon dioxide which is liberated from $NaHCO_3$ by reaction with acetic acid. From this table, it is shown that the addition of the synergists increases the content of anticholinesterase substances in the azuki bean weevil, while not in the house fly and the tobacco cutworm. As already mentioned, Sevin is strong inhibitor for this esterase. The previous experiments demonstrated that the synergist did not affect inhibition of the enzyme by Sevin, and that the mechanism of the potentiating action of toxicity by the addition of CPCBS for Sevin and the site of action of CPCBS would be the same to those of piperonyl butoxide which is strong inhibitor for carbamate esterase. Therefore, the increase of the content of the anticholinesterase substances would be due to the increase of residual Sevin in the azuki bean weevil body by inhibiting the Sevin detoxifing enzyme, while in the house fly and the tobacco cutworm, the concentration

Table 8. Anticholinesterase activity of acetone extract of insects treated with and without synergists. (CO_2 evolution, μl/30 min., 30°C)

Insect species tested	After treatment (hr.)	control	Sevin		
			alone	with CPCBS	with P. B.
Azuki bean weevil	6	151	102	75	76
Azuki bean weevil	24	169	142	81	96
House fly	24	201	140	153	146
Tobacco cutworm	24	193	199	178	197

of both synergists was not enough to inhibit the Sevin detoxifing enzyme.

Summary

1. The synergism of *p*-chlorophenyl *p*-chlorobenzenesulfonate for Sevin and its mode of action were studied.
2. *p*-Chlorophenyl *p*-chlorobenzenesulfonate potentiated insecticidal activities of some carbamate insecticides, such as Sevin, 4-chloro-3,5-xylyl N-methylcarbamate, and chlorinated hydrocarbon insecticides, for instance, γ-BHC against azuki bean weevils.
3. The synergism of CPCBS for Sevin was also observed against house flies, German cockroaches and tobacco cutworms. The most potentiating combination of Sevin and the synergist was 1 to 4 ratio in weight.
4. The action between CPCBS and piperonyl butoxide was considered to be similar joint action when both synergists used simultaneously with Sevin. It may be concluded that CPCBS as well as piperonyl butoxide does not effect on the absoption and penetration of Sevin.
5. CPCBS did not affect the anticholinesterase activity of Sevin *in vitro*.
6. CPCBS increased the anticholinesterase activities in azuki bean weevil body, and this increase seemed to depend upon the inhibition of the Sevin detoxifing enzyme.

Acknowledgment

The author is indebted to Dr. C. Harukawa, the Adviser of this company, and Mr. M. Sakai of the author's laboratory, for their useful suggestions and reading of the original manuscript. Thanks are also due to Mr. T. Okutani for his supply of the carbamate insecticides, 4-chloro-3, 5-xylyl *N*-methylcarbamate and 2-chloro-3, 5-xylyl *N*-methylcarbamate. The author is indebted to Mr. Y. Kojima and Miss M. Murakami for their technical assistance.

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