Genetical and Biochemical Studies on Joint Action of Insecticides. Tsutomu KASAI (Department of Genetics, Medical School, Osaka University*, Osaka, and Agricultural Experimental Station of Japan, Agricultural Chemicals and Insecticide Co., Kawachi-Nagano, Osaka, Japan**.) Received June. 28, 1965. *Botyu-Kagaku*, 30, 73, 1965.

15. 殺虫剤の連合作用に関する遺伝生化学的研究

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2種以上の殺虫剤または共力剤の混合によってもたらされる殺虫効力は joint action, synergistic action あるいは antagonistic action などと称せられ多くの研究者によって研究されてきたが，それらは昆虫の生理学的な面からの研究が多かった．Joint action は元来薬理学的な面から発展してきた概念であるために，殺虫剤の joint action に対する考え方も1個体における薬理作用と同一の概念で考えられてきたのもやむをえなかったと思われる．したがってこれまでの研究者らの分類によれば2種の殺虫剤が同一個体内で同一の作用点に同時に働くか，あるいは異なった作用点に働いた結果 joint action がもたらされたとする考えのもとにその分類がなされてきたのである．しかし実際に殺虫剤の joint action を解析する場合に対象となるのは1個体の昆虫ではなく集団であるために，その集団を構成する昆虫の殺虫剤感受性の不均一性を無視することはできない．しかしながら従来の joint action の分類には，これら殺虫剤の作用を受ける昆虫集団を考慮した研究はほとんどない状態である．

そこで著者は作用を受ける昆虫集団を問題とした研究を進めるために遺伝学的あるいは生化学的な面から joint action の解析をおこなった．すなわち，共力剤の具備すべき基本的な要因についての解析を進め，さらに2種の殺虫剤が昆虫の生理作用にもたらす相互的な影響を遺伝生化学的に解析し，さらに集団遺伝学的な立場より joint action を論じた．本論文の第1の部分では殺虫剤の膜透過性を高めることが共力作用の1要因であることを示した．第2の部分では環境条件が殺虫効力におよぼす影響を論じ，水分によって殺虫剤のガス化が高められるためにもたらされる共力作用を示した．第3の部分では異なったエステラーゼの阻害作用をもつ2種の殺虫剤の相互作用について薄層電気泳動法で研究し，一方の殺虫剤が他方の透過性をさまたげ，あるいは2種の殺虫剤がエステラーゼ阻害に関して competitive にはたらく例を示した．そして第4の部分では，お互いに殺虫機構を異にする殺虫剤の混合によって，遺伝的に不均一な昆虫集団にもたらされる joint action を論じた．以上の研究の結果から著者は殺虫剤を受ける昆虫集団の遺伝的構成を考慮した joint action の新しい分類方法を提案した．

Introduction

For a variety of reasons two or more chemicals having physiological effect on insects are contained in each of many formulations used for insect control. Firstly, the use of a mixture offers possibilities of more effective insect control, and of the development of supplemental agents which may extend the usefulness of various toxicants. Secondly, each material may contribute to a mixture its desirable specific properties. Thus the use of a mixture consisting of insecticides exhibiting independent, uncorrelated actions may be useful in controlling insect populations of various species.

Mixing of chemicals, which leads to a so-called joint action, synergistic action, activation or antagonistic action, has been studied by many investigators from various angles. The formalized treatment of the joint action of insecticides was presented by Bliss[1] (1939), Finny[2] (1942), Hawlett and Plackett[3] (1950), and Sakai[4] (1960). Reviews on this subject have been made in detail by Metcalf[5] (1955), Sakai[4] (1960), and Hawlett[6] (1960). These authors have discussed mainly the synergism between pyrethrin and pyrethrin synergists such as piperonyl butoxide, sulfoxide and N-propyl isome, and synergism between DDT and its derivatives. However, only a few studies on the mechanisms of joint action of two insecticides

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and of synergistic action seem to have been reported. It was found by Summerford *et al.*[7] (1951), March *et al.*[8] (1952) and Speroni[9] (1952) that certain compounds structurally related to DDT, but essentially nontoxic, may potentiate the action of DDT when applied in combination with DDT to DDT resistant house flies. Sun and Johnson[10] (1960) studied synergistic and antagonistic action of pyrethrin synergists in combination with many organophosphorus and chlorinated insecticides, and indicated that a synergistic or antagonistic action caused by pyrethrin synergist appeared to be mainly due to the inhibition of certain biological oxidations which either activate or detoxify the compounds. Ware and Roan[11] (1957) suggested that an antagonistic action of malathion with piperonyl butoxide might be due to the decreased permeability of malathion through the insect body wall.

These authors, however, failed to review the constitution of insect population on an insecticide resistance basis. Recently, many species of insects have developed resistance to various insecticides. In controlling resistant insects, a combined use of two or more insecticides proved to be useful. The concept of joint action should further cover the constitution of insect population to be applied with insecticides. During the present study an attempt was made to study the joint action of insecticides from a genetical and biochemical viewpoint. It was intended to analyze various factors responsible for synergistic or antagonistic action, and the mechanisms of joint action. Thus, it was required to establish combination of insecticides available for controlling resistant insects, with the selection of the best systematic screening method for synergists.

The present paper deals with various factors responsible for synergistic or antagonistic action and interactions between two insecticides having different physiological action. A new scheme for the classification of joint action considering the factors of synergists or antagonists, interactions between two insecticides, and joint action observed by heterogeneous constitution of insect population are discussed.

Materials and Methods

1. *Insects.*

Insecticide-resistant and susceptible strains of the house fly, *Musca domestica*, preserved at Osaka University were used. They included; (1) *NAIDM*, a susceptible strain obtained from Dr. T. Yamasaki of Tokyo University, (2) *Takatsuki*, a susceptible strain obtained from Dr. S. Nagasawa of Kyoto University, (3) *ro* ; *ct* ; *cm*, a susceptible and multichromosomal mutant strain (2 ; 4 ; 5=*rough* eyed ; *cut* wings ; *carmine* eyed) obtained from Dr. T. Hiroyoshi of Osaka University, (4) *RP*, a diazinon-resistant strain obtained from Dr. K. Yasutomi of National Institute of Health in Tokyo, and (5) *203d*, a multiple resistant (not only to diazinon but also to DDT, γ-BHC and sevin) strain obtained from Dr. M. Tsukamoto of Osaka University. All strains were reared on a larval medium consisting of equal portions of wheat bran, powdered Oriental Compressed Diet (Oriental Yeast Co., Ltd.) and fish meal. Adults were reared with powdered milk and water.

Resistant and susceptible strains of *Drosophila melanogaster* were obtained from the laboratory stocks at Osaka University. The strains used were as follows (Tsukamoto *et al*, 1954,[12] 1956[13]);

- *Hikone-R* : resistant not only to DDT, but also to various insecticides such as BHC, parathion, nicotine sulfate, etc.
- *KSL* : resistant to DDT, BHC, parathion, etc., but susceptible to nicotine sulfate.
- *bwa-*S_{16} : susceptible to DDT, BHC, parathion, etc., but resistant to nicotine sulfate.
- *bw* ; *stss* : susceptible not only to DDT, but also BHC, parathion, nicotine sulfate, etc.

2. *Insecticides and synergists.*

With the exception of common insecticides and synergists, names and structures of chemicals to be tested are given in Tables 1, 8 and 9. Technical grades of DDT, γ-BHC, sevin, UC-10854 (*m*-isopropylphenyl N-methylcarbamate), C-3 (*m*-methylphenyl N-methylcarbamate), dibrom, malathion, diazinon, DDVP, parathion, paraoxon, piperonyl-butoxide and γ-BHC emulsion (15g of γ-BHC and 10g of surface active agent diluted to 100ml with xylene) were used for tests. All of these chemicals were obtained from Japan Agricultural Chemicals and Insecticides Co., Ltd.

3. *Evaluation of insecticidal action.*

Evaluation of insecticidal action of insecticides or mixtures consisting of two or more chemicals was carried out with four methods. (a) *Topical application*: house flies about 2 days old were treated with acetone solution of insecticides or mixtures, and their mortality rates were counted after 24 hours. (b) *Contact method*: a filter paper (Toyoroshi Co., Ltd., No. 2) was put into petri dish, 9cm in diameter and 2cm high, and acetone solution of insecticides or mixtures was pipetted on the filter paper. After 60 minutes exposure ten house flies were put into the petri dishes. Knock down counts were performed at appropriate intervals. (c) *Immersion method*: a given concentration of emulsion or solution of insecticides with or without chemicals was prepared and maintained at 25°C. Larvae of the house fly were immersed into the emulsion or solution for 30 minutes. Then the insects were taken out and put into petri dishes to preserve at 25°C. Number of flies was counted when emergence of flies occurred within several days, after unaffected insects pupated. (d) *Larval test*: in the case of *D. melanogaster*, 50 first-instar larvae were transferred into a small glass vial containing 15ml of dry yeast medium (agar 2g, dry yeast powder 3g, sugar 4g, water or solution of chemicals 100ml), and the percentage emergence was determined. If necessary, some modifications were made to fit the experimental purpose.

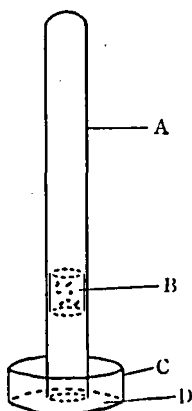

Fig. 1. Diagram of glass tube used for examination of insecticidal action of vapor.
A : test tube, 3cm in diameter, 25cm in length.
B : small glass tube, 2.8cm in diameter, 3cm in length, in which the insects were confined.
C : petri dish.
D : filter paper impregnated with chemicals.

For studying insecticidal action with vapor, a test tube 3cm in diameter, 25cm high was employed (Fig. 1). A little glass tube, 2.8cm in diameter and 3cm high, in which test insects were confined and both edges were closed with a cheese cloth, was put into the test tube in the distance of 5cm or 10cm from the opening, and the test tube was stood on the filter paper impregnated with insecticides, as the opening was downward. The knock down counts were made at appropriate intervals.

4. *Measurement of succinic dehydrogenase activity.*

Two methods were employed for measurement of effect of chemicals on succinic dehydrogenase activity, i.e. the method with decolorization of methylene blue and that with reduction of T. T. C.

Fifty house flies were homogenized in 5 ml of M/15 phosphate buffer (pH 7.2) with a glass homogenizer for 1 minute. One ml of homogenate was put into Thumberg tubes, and the side-arms were filled with 1ml of 1×10^{-3}M methylene blue, 1ml of 1×10^{-1}M sodium succinate and 1ml of 4×10^{-1}M chemicals such as glycerol-derivatives (dissolved in M/15 phosphate buffer, pH 7.2). The tubes were connected to a water suction for 15 minutes to produce a vacuum. The time needed for decolorization was measured, after the contents were mixed at 37°C, and the rate of inhibition was calculated as follows;

percent inhibition

$$=\left(1-\frac{\text{time needed for decolorization without chemicals}}{\text{time needed for decolorization with chemicals}}\right)\times100$$

Another procedure was as follows; Two hundred adult *D. melanogaster* were homogenized in 4ml of M/15 phosphate buffer, pH 7.2, with a glass homogenizer for 1 minute. One ml of homogenate, 1ml of 1×10^{-2}M sodium succinate solution, 1ml of 2×10^{-3}M T.T.C. (triphenyl tetrazolium chloride) and 1ml of 4×10^{-2}M chemicals were mixed in test tubes. After incubation at 37°C for 30 minutes, the reaction was stopped with 1ml of 20% trichloroacetic acid solution, and the red product was extracted with 8ml of ethylacetate. The optical density of ethylacetate fraction was measured

by a Beckmann spectrophotometer at 500mμ, and the rate of inhibition was calculated.

5. *Quantitative analysis of γ-BHC incorporated into living bodies.*

Chemical estimation of γ-BHC was performed by the method of Schechter and Hornstein[14] (1952). Insects treated with insecticides were put into reaction flask with 15ml of acetic acid, then 2g of malonic acid and 1g of zinc dust were added. Five ml of nitrating acid mixture ($HNO_3 : H_2SO_4 = 1:1$ in volume) was added to a nitrating column. The flask was heated for 60 minutes. Then the nitrating acid was treated following the procedures described by Gehrke and Beviet[15] (1956). After the nitrating acid was diluted, the *m*-dinitrobenzene contained in this solution was extracted with ether. The ether fraction was washed three times with 2% aqueous NaOH and twice with saturated sodium chloride solution. After the evapolation of ether, the color reaction was performed by adding methylethylketone and 40% aqueous KOH.

Measurement of γ-BHC permeated the epidermal membrane of an onion bulb was as follows; The epidermal membrane of an onion bulb was placed between the wall of two adjoining chambers each pierced by a hole 5mm in diameter (Fig. 2). Four ml of 4mM γ-BHC with or without added chemicals, emulsified or dissolved in M/15 phosphate buffer (pH 7.2), was put in the A-chamber; and 4ml of buffer solution was poured into the B-chamber to serve as the control. After being held for 24 hours at 25°C, the solution in B-chamber was removed and the γ-BHC which had penetrated from the A-chamber to the B-chamber through the membrane was extracted with meth ylenechloride; after evaporation of this solvent, the γ-BHC was assessed chemically by Schechter Hornstein's colorimetric method.

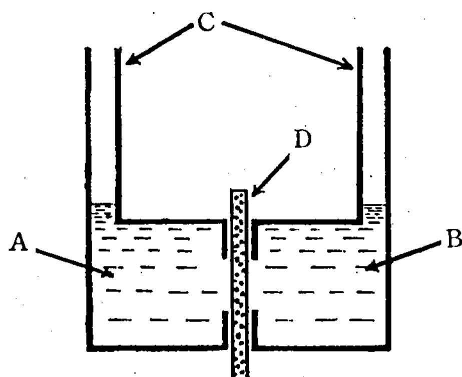

Fig. 2. Diagram of double chamber.
A : 4ml of phosphate buffer containing insecticide and chemicals.
B : 4ml of phosphate buffer alone.
C : double chamber with a holl of 5mm in diameter.
D : membrane employed for permeability test.

6. *Thin layer electrophoresis and zymograms.*

With a glass homogenizer house flies were ground in the presence of half their weight of deionized water. The homogenates were centrifuged at 12,000g for 60 minutes to remove debris. The strips of filter paper or oil free cotton threads which had been dipped in the homogenates were transferred to an agar-gel layer or embedded in the agar. Thin layer electrophoresis was carried out following the procedures described by Ogita [16]~[19] (1962, 1963a, b, 1964); that is, a medium containing 0.7g of agar and 2g of P.V.P. (polyvinylpyrrolidone in 100ml of potassium phosphate buffer (pH 6.8) of ionic strength 0.025 was used for the esterase separation. The medium was gelatinized on a glass plate (16.5×15cm) in such a way as to produce a layer with a very smooth surface and 0.7mm or 0.9mm thickness. A constant current of 1.5 to 2.0mA per cm width of the gel layer was applied for 90 to 120 minutes. After electrophoresis, the agar-gel plates were wetted with deionized water, and were sprayed with 1% β-naphthylacetate acetone solution. The plates were allowed to stand for 30 to 60 minutes at 37°C. Hydrolyzed products were detected by the spray of 0.5% aqueous naphthanil diazoblue B, and in order to preserve the plates, they were washed in running water and dried in a current of hot air.

Acetylcholine esterases were detected by a pH-indicator method (Ogita and Kasai 1964,[20] 1965[21]). The agar-gel plate after electrophoresis was immersed into the following substrate pH-indicator solution for 30 minutes at room temperature.

Components of reaction mixture

acetylcholine chloride	0.2g
sodium bicarbonate	21mg
BTB 1% ethylalcohol solution	2ml
H_2O	100ml

Then the agar-gel plates were preserved at 37°C

Table 2. The rate of emergence from media containing γ-BHC or the mixture of γ-BHC and chemicals. (Expressed as percentage compared with that emerged from untreated media. Based on 500 larvae by larval test method.)

Concentration of chemicals γ-BHC	Chloralacetone-chloroform	*bw;st ss*	*bw;HR₃*	*KSL*	*Hikone-R*
0.5×10^{-2}mM		98.6	85.0	—	—
0.7		81.7	103.8	—	—
1.0		67.6	73.8	—	—
1.5		26.7	93.8	—	—
2.0		31.0	77.5	95.4	94.9
3.0		0	1.3	78.1	92.4
4.0		0	0	94.3	101.3
6.0		0	0	56.3	68.4
8.0		0	0	2.3	63.3
10.0		0	0	0	7.6
12.0		0	0	0	2.5
0.5×10^{-2}mM	+0.5mM	27.0	26.3	90.5	103.3
0.5	1.0	3.2	1.3	78.1	103.3
0.5	1.5	0	0	74.0	77.2
0.5	2.0	0	0	45.2	83.6
0.5	4.0	0	0	11.0	35.9
0.1×10^{-2}mM	+2.0mM	16.9	58.7	—	—
0.2	2.0	2.8	—	—	—
0.3	2.0	0	0	—	—
0.5	2.0	0	0	36.8	—
0.7	2.0	0	0	26.4	74.7
1.0	2.0	0	0	19.6	51.9
1.5	2.0	0	0	6.9	79.8
2.0	2.0	—	—	14.9	11.4
3.0	2.0	—	—	4.0	1.3
0	2.0mM	90.5	96.1	116.4	102.2
0	4.0	55.6	82.9	104.1	104.3
0	0	100.0	100.0	100.0	100.0

in a moist chamber for 30~60 minutes, till the esterase activities were revealed as yellow bands in greenish-blue background by the acid produced.

For the demonstration of *in vivo* inhibition of esterases by organophosphates or carbamates, tissue extracts were prepared 3 hours after the topical application of the insecticides to house flies. For the special purpose such as the experiment for inhibition of esterases after the treatment of a carbamate and an organophosphate at different time intervals, the tissue extract was prepared at the time presented. The *in vitro* inhibition of the esterases was studied by incorporating the inhibitors into the agar-gel medium. Experimentally, 1ml of acetone solution of organophosphate or carbamate was pipetted into 100ml of the hot agar-gel medium, and after vigorous shaking an agar-gel layer was immediately prepared. Otherwise, the agar-gel plate following electrophoresis was allowed to react with a buffered solution containing the organophosphates or carbamates.

Results and Discussion

1. *Synergistic action of glycerol-derivatives and related chemicals with γ-BHC against larvae of the house fly and those of D. melanogaster.* *

The synergistic action of glycerol-derivatives was studied with the immersion method or the larval test method. Halogenohydrins such as

* A preliminary report on this subject has been published in *ITIS (Insect Toxicologists' Information Service)*, 5, 125, 1962.

ethylenechlorohydrin, glycerol α,γ-dichlorohydrin, and glycerol α,γ-dibromohydrin were found to act as synergists (Table 1). These chemicals have a hydroxy radical and halogens in their chemical constitution. On the other hand the parent chemicals, ethyleneglycol and glycerol, which have hydroxy radicals alone, or dichloroethylene in which all the hydroxy radicals are substituted with halogens proved to have no or less synergistic activity. Therefore chlorals were also examined if they were synergists for γ-BHC, demonstrating that chloralurea, chloralurethane and chloralacetonechloroform were synergistic (Table 1).

The synergistic action of chloralacetonechloroform with γ-BHC at various concentrations was

Table 1. Chemical constitutions and index of synergism of the chemicals mixed with γ-BHC against larvae of the house fly (*Takatsuki*-strain).

Chemical	Chemical constitution	Percentage* emergence from treatment of 5000 p. p. m. chemical alone	Index of synergism**	
			γ-BHC 1000 p. p. m. *** added to 5000 p. p. m. chemical	added to 2000 p. p. m. chemical
Ethyleneglycol	$HOCH_2CH_2OH$	97.0	1.09	0.80
Ethylenechlorohydrin	$ClCH_2 \cdot CH_2OH$	102.2	0.16	0.46
Dichloroethylene	$ClCH_2 \cdot CH_2Cl$	87.8	0.47	0.75
Glycerol	$HOCH_2 \cdot CHOH \cdot CH_2OH$	98.7	1.00	1.05
Glycerolα-monochlorohydrin	$ClCH_2 \cdot CHOH \cdot CH_2OH$	102.4	1.09	1.05
Glycerolα, γ-dichlorohydrin	$ClCH_2 \cdot CHOH \cdot CH_2Cl$	100.6	0.02	0.21
Glycerolα, γ-dibromohydrin	$BrCH_2 \cdot CHOH \cdot CH_2Br$	100.7	0	0
Diacetin	$CH_3COOCH_2 \cdot CHOH \cdot CH_2OCOCH_3$	98.3	0.54	0.60
Triacetin	$CH_3COOCH_2 \cdot CHOCOCH_3 \cdot CH_2OCOCH_3$	99.7	1.07	1.21
Chloral-urea	$CCl_3 \cdot CH(OH) \cdot NHCONH_2$	86.8	0	—
Chloral-thiourea	$CCl_3 \cdot CH(OH) \cdot NHCSNH_2$	100.0	1.09	—
Chloral-urethane	$CCl_3 \cdot CH(OH) \cdot NHCOOC_2H_5$	102.2	0.11	—
Chloral-phenylthiourea	$CCl_3 \cdot CH(OH) \cdot NHCSNHC_6H_5$	114.2	0.77	—
Chloral-acetonechloroform	$CCl_3 \cdot CH(OH)OC(CH_3)_2(CCl_3)$	104.9	0.03	0.53

* Corrected by comparing with untreated control (based on 50 larvae in each treatment).

** Index of synergism

$$= \frac{\text{Percentage adult emergence from larvae which were treated by } \gamma\text{-BHC with chemicals.}}{\text{Percentage adult emergence from larvae which were treated by } \gamma\text{-BHC alone.}}$$

*** Treatment of 1,000 p. p. m. γ-BHC alone brought about percentage emergence ranging 56.7 ~82.2%.

Table 3. Relations between immersion period and percentage emergence of house flies (*Takatsuki* strain).

Chemical	Concentrations (p. p. m.)	Immersion period (min.) 3	10	30
		percentage emergence (%)		
γ-BHC	4000	85.7	91.2	46.4
	2000	92.5	95.7	78.4
Glycerol α,γ-dichlorohydrin	5000			93.9
γ-BHC + Glycerolα,γ-dichlorohydrin	4000+5000	81.2	31.6	2.6
	2000+5000	96.1	31.9	0
Chloral-acetonechloroform	5000			92.0
γ-BHC + Chloral-acetonechloroform	4000+5000	69.2	52.0	2.6
	2000+5000	79.2	53.2	13.8

tested against several strains of *D. melanogaster* (Table 2). The rate of emergence was markedly decreased by adding the above chemicals to γ-BHC. This synergistic action was evident in resistant strains as well as susceptible ones. Table 3 shows relations between the period of exposure and the degree of insecticidal effect; the rate of emergence was decreased as the time of immersion was prolonged. For immersion period of 10 or 30 minutes, addition of the chemicals to γ-BHC markedly decreased the rate of emergence. Following a 3 minute immersion, however, the synergistic action of the chemicals upon γ-BHC was scarcely detectable.

It was assumed that the mechanism of synergistic action was closely associated with the narcotic activity of glycerol derivatives or chlorals. This assumption led to a further study on relations between synergistic activity and narcotic activity. Many experiments have been reported on the effect of narcotics on respiratory systems. Chlorals and urethanes inhibited dehydrogenase activity; the former inhibits respiration in mammals (Thumberg[22] 1936). The narcotic action of barbituric acid derivatives is due to uncoupling (Brody *et al.*[23] 1951), and Quastel[24] (1952) demonstrated that narcotics inhibited the synthesis of ATP. When the effect of glycerol derivatives and chlorals on succinic dehydrogenase activity was assessed, the chlorals showed an inhibitory action on the enzyme. Of glycerol derivatives, glycerol α, γ-dibromohydrin, glycerol α, γ-dichlorohydrin and glycerol α-monochlorohydrin inhibited the succinic dehydrogenase activity in the house

Table 4. Effect of chemicals on succinic dehydrogenase activity of house flies (*NAIDM* strain) measured by decolorization of methylene blue.

Chemicals	Time needed for decolorization of Mb. (min.)	Percentage inhibition (%)
Glycerol	11.5	4
Glycerol α-monochlorohydrin	14.0	21
Glycerol α, γ-dichlorohydrin	18.0	39
Glycerol α, γ-dibromohydrin	∞	100
Diacetin	14.0	0
Chloralurethane	22.0	34
Piperonyl butoxide	17.0	15
Control	11.0–14.5	—

fly (Tables 4 and 5). The extent of inhibition of the enzyme was in association with the degree of synergistic action in glycerol derivatives. Therefore, the synergistic action of glycerol derivatives for γ-BHC against house fly larvae may be due to their inhibitory action on dehydrogenase activity.

It is interesting that there is a hypothesis that narcotics effect the permeability of the cell membrane, though they do not always enhance penetration of various compounds through cell membrane (Danielli[25], 1955). Chemicals which have narcotic action either increase or decrease permeability of a compound, with a varying degree depending on the kind of the cell membrane, narcotic, and compound. In order to investigate the mechanism of synergistic action of these

Table 5. Effect of chemicals on succinic dehydrogenase activity of *D. melanogaster* (*bwa-S*$_{16}$ strain and *Hikone-R* strain) measured by reduction of T. T. C.

Chemicals	Final concentration ($\times 10^{-3}$M)	Percentage inhibition (%) *bwa-S*$_{16}$	*Hikone-R*
Chloralurea	10	73.1	62.0
Chloralthiourea	10	54.4	60.8
Chloralurethane	10	72.0	65.0
Chloral·acetonechloroform	10	81.9	77.2
Chloroform	10	76.0	84.3
p-Nitrophenol	10	93.5	93.0
2, 4-Dinitrophenol	10	93.0	91.8
Control	—	0	0

Table 6. Amounts of γ-BHC observed in living bodies, measured by Schechter-Horstein method immediately after 16 or 24 hrs. after treatment of insecticides against larvae of house fly.

Chemicals	Concentration (p. p. m.)	Time after treatment (hr.)	No. of insects	Amount of γ-BHC measured (μg)	Amount of γ-BHC per insect ($\times 10^{-3} \mu g$)
γ-BHC	1000	0	200	22.8	11.4
γ-BHC+Chloralacetonechloroform	1000+5000	0	200	48.0	24.0
γ-BHC	1000	24	200	10.7	5.4
γ-BHC+Chloralacetonechloroform	1000+5000	24	200	17.5	8.8
γ-BHC	200	16	250	8.3	3.3
γ-BHC+Chloralacetonechloroform	200+5000	16	250	29.2	11.7
γ-BHC	1000	0	170	20.4	11.9
γ-BHC+Glycerol α,γ-dibromohydrin	1000+5000	0	170	27.4	16.1
γ-BHC	200	16	170	2.8	1.6
γ-BHC+Glycerol α,γ-dibromohydrin	200+5000	16	170	8.0	4.7

chemicals, the γ-BHC absorbed by the bodies of larvae was measured chemically. The concentrations of γ-BHC used in the emulsion were 1,000 p. p. m. and 200p. p. m., because the insects treated with mixtures of 1,000p. p. m. γ-BHC and 5,000 p. p. m. chemicals were considerably affected in 16 or 24 hours. The results are shown in Table 6. The amount of γ-BHC absorbed into larvae was greater when γ-BHC solution in which the larvae had been immersed was supplemented with chemicals such as chloral-acetonechloroform or glycerol α,γ-dibromohydrin. This result may suggest that these chemicals increase an epidermal permeability of γ-BHC into the insect body. The epidermal membrane of an onion bulb was used as model for an experiment of permeability, though it was necessary to ascertain if permeability of γ-BHC through the insect epidermis was increased by addition of glycerol derivatives or chlorals. Permeability of γ-BHC through the epidermal membrane was increased by the addition of these chemicals as shown in Table 7. In a preliminary experiment where the same method was used, permeability of methylene blue through the epidermal membrane of an onion bulb or abdominal epidermis of a frog was increased by the addition of these chemicals. These results suggest an increase in the amount of γ-BHC detected in insects was due to an increased permeability of this insecticide when glycerol derivatives or chlorals were added as narcotics.

Table 7. Amount of γ-BHC permeated the epidermal membrane of an onion blub.

Chemicals	Concentrations of chemicals in A-chamber (mM)	Amount of γ-BHC measured in B-chamber after 24 hrs. (μg)
γ-BHC	4	10.0
γ-BHC+Glycerol	4+20	9.8
γ-BHC+Glycerol α,γ dichlorohydrin	4+20	17.6
γ-BHC+Diacetin	4+20	12.2
γ-BHC+Chloralacetone-chloroform	4+20	17.0
γ-BHC+ Chloralurethane	4+20	22.8

Thus, one of the possible mechanisms of synergistic action seems to increase permeability of the insecticide by adding synergists.

2. *Synergistic action produced by chemicals having a hygroscopic character*.*

Synergistic action of glycerol derivatives with γ-BHC or dibrom against adult house flies and *D. melanogaster* was studied with the contact method (Tables 8, 9, 10). Unexpectedly, glycerol α,γ-dichlorohydrin which had the most effective synergistic action against larvae of the house fly, exhibited no synergistic effect, while glycerol itself showed the most effective synergistic action against adult house flies. Ethyleneglycol showed

* A preliminary report on this subject has been published in *ITIS*, 5, 125, (1962).

Table 8. Synergistic action of chemicals with dibrom shown as KT-50 against *D. melanogaster* (*bw* ; *III Hikone-R* strain).

Chemicals	Chemical constitution	(KT-50 min.) Without* dibrom	With** dibrom
Glycerol	$CH_2OH \cdot CHOH \cdot CH_2OH$		4
Glycerol α-monochlorohydrin	$CH_2Cl \cdot CHOH \cdot CH_2OH$		8
Glycerol α,γ-dichlorohydrin	$CH_2Cl \cdot CHOH \cdot CH_2Cl$	42	20
Glycerol α,γ-dibromohydrin	$CH_2Br \cdot CHOH \cdot CH_2Br$	7	4
Ethyleneglycol	$CH_2OH \cdot CH_2OH$		4
Propyleneglycol	$CH_3CHOH \cdot CH_2OH$		4
Polyethyleneglycol 200	$HOCH_2(CH_2OCH_2)_3CH_2OH$		6
Polyethyleneglycol 300	$HOCH_2(CH_2OCH_2)_5CH_2OH$		6
Polyethyleneglycol 1500	$HOCH_2(CH_2OCH_2)_{33}CH_2OH$		12
Carbowax (35 Bridges)	$HOCH_2(CH_2OCH_2)_{34}CH_2OH$		14
Piperonyl butoxide			10
Dibrom alone			14

* Glycerol α,γ-dichlorohydrin and glycerol α,γ-dibromohydrin have their own knock down activity (narcotic action).
** 0.2mM dibrom was employed.

Table 9. Synergistic action of chemicals with γ-BHC shown as KT-50 against *D. melanogaster* (*bw* ; *III Hikone-R* strain).

Chemicals	Chemical constitution	KT-50 with γ-BHC* (min.)
Glycerol	$CH_2OH \cdot CH(OH) \cdot CH_2OH$	5
Ethyleneglycol	$CH_2OH \cdot CH_2OH$	5
Glycerol monoethylether	$CH_2OH \cdot CHOH \cdot CH_2OCH_3$	8
Glycerol diethylether	$C_2H_5OCH_2 \cdot CHOH \cdot CH_2OC_2H_5$	9
Monochloral glycerol	$CH_2OH \cdot CHOH \cdot CH_2OCH(OH)CCl_3$	6
Trichloral glycerol	$CH_2OCH(OH)CCl_3 \cdot CHOCH(OH)CCl_3 \cdot CH_2OCH(OH)CCl_3$	6
Ethyleneglycol monomethylether	$CH_2OH \cdot CH_2OCH_3$	12
Ethyleneglycol monoethylether	$CH_2OH \cdot CH_2OC_2H_5$	14
Diethyleneglycol	$CH_2OH \cdot CH_2OCH_2 \cdot CH_2OH$	10
γ-BHC alone		9

* Acetone solution of 1 mM γ-BHC with 5% chemical was used for the contact method.

Table 10. Synergistic action of chemicals with γ-BHC shown as KT-50 against house flies (*NAIDM* strain).

Chemicals	KT-50 with γ-BHC*(min.)
Glycerol	47
Monochloral glycerol	45
Trichloral glycerol	40
Ethyleneglycol	55
Propyleneglycol	55
γ-BHC alone	80

* 0.05% γ-BHC was employed.

synergistic action as well as glycerol. On the basis of an assumption that synergistic activity of chemicals was dependent on the hygroscopic character of chemicals, the absorption of water by filter paper containing chemicals was measured (Table 11). It was found that the hygroscopic activity of glycerol α,γ-dichlorohydrin was low, while that of glycerol was marked. It was revealed that piperonyl butoxide which is commercially used as a synergist for pyrethroids had also a hygroscopic character. A close correlation was found between the synergistic activity of glycerol

Table 11. Absorption of moisture in glycerol, glycerol derivatives and piperonyl butoxide.

Chemicals	Humidity condition	Weight of papers: Before preservation (mg)	Weight of papers: After 7 days preservation (mg)	Increase of weight (mg)	Rate of increase (%)
Glycerol	wet	3990	4970	+980	+24.56
	dry	3975	4076	+101	+ 2.54
Glycerol α-monochlorohydrin	wet	4038	4520	+482	+11.94
	dry	3974	3952	− 22	− 0.55
Glycerol α, γ-dichlorohydrin	wet	3937	4320	+383	+ 9.73
	dry	3901	3910	+ 9	+ 0.23
Piperonyl butoxide	wet	3953	4653	+698	+17.67
	dry	3950	4085	+135	+ 3.42
Control	wet	3913	4274	+334	+ 8.54
	dry	3954	3852	−102	− 2.58

One ml of 5% acetone solution of each chemical was pipetted on filter papers. After the solvent was evaporated, the filter papers were kept at dry (in a $CaCl_2$ desiccator) or wet (in a moistened chamber) conditions for 7 days, then the weight of the filter papers was measured.

Table 12. KT-50 of dibrom with or without glycerol derivatives against *D. melanogaster* (*bw* ; *III Hikone-R* strain), at various humidity conditions by contact method.

Chemicals*	KT-50 (min.)											
Days of preservation	1 day A	1 day B	1 day C	7 days** A	7 days** B	7 days** C	14 days*** A	14 days*** B	14 days*** C	21 days**** A	21 days**** B	21 days**** C
Humidity condition	dry	inter-mediate	wet	dry	interm-ediate	wet	wet	interm-ediate	dry	wet	wet	wet
Dibrom	39	3	2	50	12	3	6	15	75	80	71	67
Dibrom+glycerol	15	2	1	15	8	3	4	15	37	57	52	66
Dibrom+glycerol α, γ-dichlorohydrin	53	3	1	70	13	4	5	14	80	90	68	83

* One ml of 0.2 mM acetone solution of dibrom with or without 5% chemicals was dropped on a filter paper.

** The filter papers used in the experiment after 1 day preservation were preserved for the next 6 days in the same conditions.

*** The filter pepers used in the 2nd experiment were preserved for the next 7 days in the reverse condition.

**** All filter papers used in the 3rd experiment were preserved for the next 7 days in wet condition.

derivatives and the hygroscopic activity of the chemicals.

It was considered that the presence of water in filter paper was a very important factor if insecticidal action was tested by the contact method. Table 12 shows the changes of insecticidal action, when filter paper impregnated with an insecticide with or without glycerol was incubated under various degrees of humidity. Insecticidal action under wet conditions was marked but no synergistic action of glycerol was observed. Under dry conditions, it was slight and a synergistic action of glycerol was evident. It was indicated that insecticidal action of an insecticide during the contact method was affected by humidity. The vapor activity of insecticides with or without glycerol was measured by separating insects from filter paper impregnated with insecticides (Tables 13 and 14). It was stronger with glycerol than without it. Then several insecticides were tested for synergistic action with glycerol (Table 15), revealing that glycerol was favorably used with

Table 13. Vapor activity of dibrom evaluated by contact method.

Chemical*	KT-50 (min.)	KT-50 by vapor** (min.)
Dibrom	11	23
Dibrom+glycerol	3	12

* One ml of 0.2mM acetone solution of dibrom with or without 5% glycerol was used.

** Filter paper was covered with cheese cloths, on which insects were confined.

the contact method. However, the first assumption is nonacceptable, because the synergistic action of glycerol was evidenced even when the insects were kept away from the filter paper by cheese cloth, or when the vapor activity alone was measured. Nor is second assumption, because the synergistic action of glycerol was unspecific for various insecticides, and because the insecticidal action of insecticides exposed on filter paper was about as high when the filter paper was preserved in the same humidity after being preserved under different wet conditions. As a result, it may be considered that the synergistic action of glycerol with an insecticide was not due to a chemical change of the insecticide, but due to evaporation of insecticide itself which was accelerated by the presence of water. Weidhaas *et al.*[26] (1960) and Acree *et al.*[27] (1963) reported that in water suspension of DDT, higher co-distillation rate of DDT was observed than that expected from its vapor pressure. Kalkat *et al.*[28] (1961) also reported that relative humidity of experimental procedure affected on insecticidal action. It is suggested that insecticidal action by the contact method is affected by the vapor of an insecticide rather than the insecticide which penetrates the insect body through the epidermis.

Table 14. Vapor activity of γ-BHC and dibrom.

Chemical	Humidity condition	KT-50 (min.) Distance from insecticide 5cm	10cm
γ-BHC	dry	95	115
γ-BHC+glycerol	wet	68	85
Dibrom	dry	40	—
Dibrom+glycerol	wet	29	—

One ml of 5% acetone soluton of γ-BHC or dibrom with or without 5% glycerol was dropped on a filter paper. The filter paper impregnates was preserved at dry (in $CaCl_2$ desiccator) or wet (in a moistened chamber) conditions for 24 hours.

Table 15. Synergistic action of glycerol with several insecticides against *D. melanogaster* (*bw* ; *III Hikone-R* strain).

Chemical	KT-50 (min.) dry	wet
γ-BHC	16	9
γ-BHC+glycerol	10	9
DDVP	28	9
DDVP+glycerol	15	8
Diazinon	25	8
Diazinon+glycerol	18	5

Insecticides to be tested were; acetone solution of 1mM γ-BHC, 0.2mM DDVP and 1mM diazinon with or without 5% glycerol.

DDVP and diazinon as well as γ-BHC or dibrom.

The possible mechanisms for synergistic action were as follows:

(1) Insects' consumption of an insecticide together with the water on the filter paper resulted in more intake than the insecticide alone. (2) An insecticide, being affected by water, produced a new substance or substances which proved more powerful than the original. (3) Much insecticide was evaporated by water, and the vapor activity was vigorous under a closed condition as found in the contact method.

It follows that one of the mechanisms of synergistic action, or increase in insecticidal activity is enviromental factor such as relative humidity of the atmosphere where insecticides are used.

3. *Interaction between organophosphates and carbamates.*

a. *In vivo inhibition of esterases by organophosphates and carbamates*

To study inhibition of esterases by organophosphates or carbamates, house flies were treated with these chemicals with topical application and were homogenized 3 hours later, then the resultant brei was applied to thin layer electrophoresis, and the esterase activity was detected. The dose of insecticides adopted was about twice LD-50, probably bringing about 90~100% mortality in 24 hours.

Non-specific esterase zymograms of tissue extracts from untreated house flies of *ro* ; *ct* ; *cm* and *RP* strains are illustrated in Fig. 3 (Ogita 1962,[16] Ogita and Kasai 1965[29]). Inhibition of esterases by insecticides is shown in Fig. 4. Organophosphate insecticides such as diazinon, malathion and DDVP inhibited faster migrating esterase bands E_2 and E_4 markedly and E_9, E_{13} less markedly, while they failed to inhibit E_{18}, E_{19}, and E_{23} bands. On the contrary, carbamate insecticides such as sevin, UC-10854 or C-3 did not inhibit faster migrating E_2, E_4 bands, while slower moving bands like E_{18}, E_{19} and E_{23} bands were markedly inhibited. Acetylcholinesterase activity was markedly inhibited by organophosphates, while it was not inhibited by carbamate insecticides (Fig. 4 B).

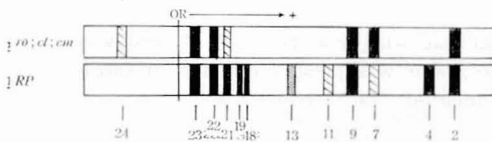

Fig. 3. Diagram showing zymogram patterns of esterases in two strains of the house fly. Relative intensities of the bands are indicated by shading. OR indicates the origin.

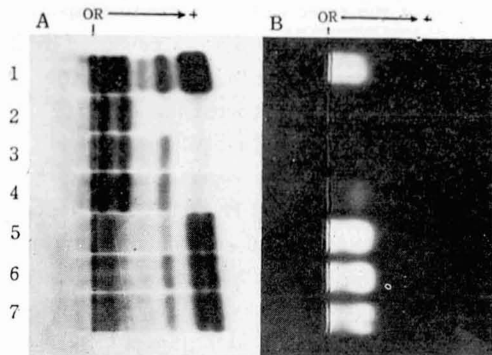

Fig. 4. Esterase zymograms of tissue extracts from house flies treated with organophosphates or carbamates (*RP* strain);

A : Non-specific esterase zymograms detected with β-naphthylacetate as a substrate and naphthanil diazoblue B as a coupler.

B : Cholinesterase zymograms detected with the pH-indicator method in which acetylcholine chloride is used as a substrate.

1. Control 2. Diazinon 0.4 μg/fly 3. Malathion 0.7 μg 4. DDVP 0.1 μg 5. Sevin 50 μg 6. UC-10854 2 μg 7. C-3 3 μg

Many organic phosphorus insecticides have proved to be active inhibitors of cholinesterases and other esterases in mammals and in insects in both *in vivo* and *in vitro* studies. In general there is good correlations between the toxicity of phosphate esters and their ability to inhibit ChE *in vitro*. Van Asperen[30] (1958) demonstrated that in house flies poisoned by exposure to vapors of DDVP, aliphatic esterase (Ali-E) activity of body homogenates was more inhibited than the acetylcholinesterase of head homogenates at the time of knock down. Plapp and Bigley[31] (1961a) reported that Ali-E in adult female house flies was rapidly inhibited *in vivo* following treatment with parathion or malathion, that cholinesterase activity was more slowly and less reversibly inhibited and that the inhibition was closely correlated with the observed symptoms of poisoning. Carbamate insecticides are known to be similar in many ways to organophosphorus insecticides. Plapp and Bigley[32] (1961b) have presented *in vivo* and *in vitro* inhibition of Ali-E and ChE by carbamate insecticides such as sevin and isolan.

The present results indicated that the esterases susceptible to organophosphates were different from those susceptible to carbamate insecticides. Insecticidal action of carbamates was considered to be due to their ability to inhibit ChE activity (Georghiou and Metcalf 1962[33]). However, the carbamate insecticides employed in this study did not inhibit acetylcholinesterase activity but inhibited the activities of E_{18}, E_{19} and E_{23} esterases (Fig. 4) which were present in the body fluid of house flies and were not inhibited by organophosphates or eserine (Ogita and Kasai 1965[29]). These results suggest that the mode of action of carbamate insecticides may be the inhibition of E_{18}, E_{19} and E_{23} esterase activity.

b. *Joint action of organophosphate and carbamate insecticides.*

The present finding that esterases are individually susceptible to organophosphate or carbamate insecticides reasonably leads to a possibility that a mixture of the two kinds of chemicals will prove more effective. Therefore a joint action was studied by mixing two insecticides at various concentrations (Fig. 5). However, no increase in insecticidal action was observed in any combination of organophosphates and carbamates in *RP* or *203d* strains. Another strain (*ro*;*ct*;*cm*) showed an increase in insecticidal action. However, biochemical studies were only limited to *RP* strain in this study. In order to observe the joint action of organophosphates and carbamates at a level of esterase inhibition, esterase zymograms of house

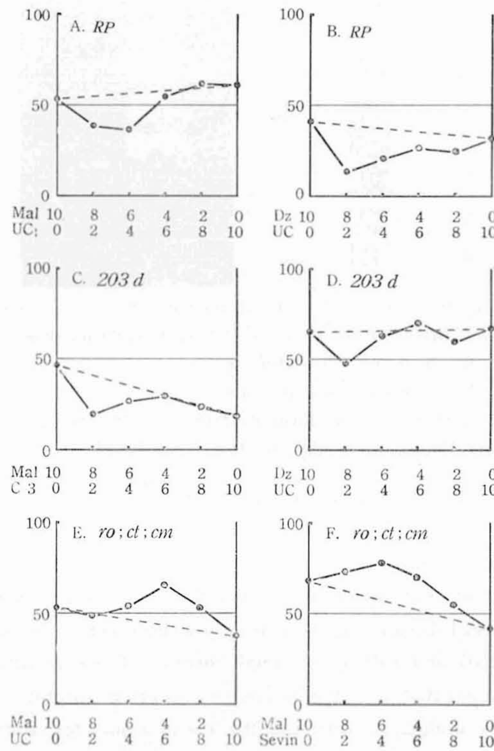

Fig. 5. Joint action between organophosphate and carbamate insecticides against several strains of the house fly. The abscissa represents the proportion of insecticides which were prepared for LD-50. The ordinate shows mortality in 24 hours. Fifty females and 50 males were used as a group for each dose.
Mal : Malathion, Dz : Diazinon, UC : UC-10854.

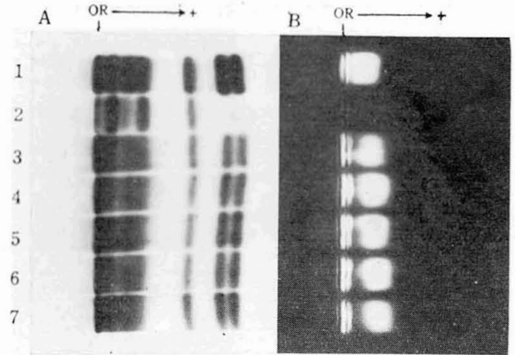

Fig. 6. Esterase zymograms of house flies (*RP* strain) treated with a mixture of malathion and UC-10854 at various doses.
A : Non-specific esterases.
B : Acetylcholinesterases.

	Doses (μg/fly)			Doses (μg/fly)	
	Malathion	UC-10854		Malathion	UC-10854
1.	0	0	2.	0.4	0
3.	0.32	0.2	4.	0.24	0.4
5.	0.16	0.6	6.	0.08	0.8
7.	0	1.0			

flies treated with either the insecticide-mixture or insecticide alone were comparatively studied. Unexpectedly, though inhibition of E_{18}, E_{19} and E_{23} esterase bands by carbamate insecticide such as UC-10854 was obvious, E_2 and E_4 esterase bands were not inhibited by malathion in mixtures at various concentrations (Fig. 6A). Moreover, addition of a small amount of UC-10854 into malathion resulted in a failure of inhibition of ChE activity (Fig. 6B). Effects of malathion and UC-10854 on esterases were further studied with house flies treated with a mixture or both chemicals applied individually at different time (Fig. 7). House flies pre-treated with UC-10854 and then treated with malathion showed higher activity of E_2 and E_4 esterases and ChE than those treated with malathion alone. On the other hand, house flies treated with malathion and then with UC-10854 showed a marked inhibition of E_2, E_4, E_{18}, E_{19} and E_{23} esterases. Flies treated with the mixture showed a slightly higher activity in E_2 and E_4 esterases and a higher activity in ChE than those treated with malathion alone. Fig. 8 shows the esterase zymograms of house flies treated with a sub-lethal dose of UC-10854 (0.5μg per fly) and malathion at different time intervals. Pretreatment with UC-10854 at 16 or 4 hours

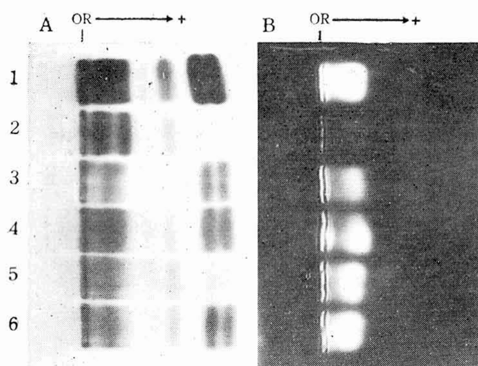

Fig. 7. Effect of malathion on esterase activity of house flies (*RP* strain) suffered pre- or post-treatment with UC-10854.

A : Non-specific esterases.
B : Acetylcholinesterases.
1 : Control.
2 : Malathion 0. 7μg/fly
3 : Malathion 0. 7μg was applied to flies which had been pre-treated with 0. 5μg of UC-10854.
4 : Mixture of malathion 0. 7μg and UC-10854 0. 5μg employed.
5 : Malathion 0. 7μg was firstly applied and then post-treated with 0. 5μg of UC-10854.
6 : UC-10854 0. 5μg/fly.

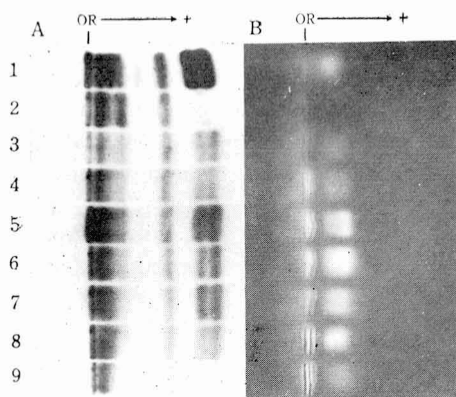

Fig. 8. Effect of malathion on esterases in house flies being pretreated or post-treated with UC-10854 (*RP* strain).

A : Non-specific esterases.
B : Acetylcholinesterases.

1. Control, 2. Malathion alone, 3. Pre-treatment* 16 hrs., 4. 4, 5. 2, 6. 1, 7. 0. 5, 8. 0**, 9. Post-treatment*** 1

* House flies pre-treated with 0. 5μg of UC-10854 per fly at the time presented, and subsequently treated with 0. 5μg of malathion per fly.
** Mixture (malathion and UC-10854) employed.
*** House flies treated with malathion and subsequently with UC-10854.

before malathion treatment resulted in a recovery from knock-down by carbamate poisoning, showing little prevention of esterase inhibition by malathion. However, pretreatment with UC-10854 at 2, 1 or 0. 5 hours before malathion treatment left E_2, E_4 esterase activity and ChE activity unchanged. A maximum effect of UC-10854 on decreased esterase inhibition by malathion might be brought about by pretreatment about 1 hour before.

To estimate the effect of concentrations of carbamates on organophosphate insecticides, several doses of UC-10854 were treated for house flies 1 hour before malathion treatment. The results in Fig. 9 indicate that as a dose of UC-10854 increased from 0. 01 to 3μg per fly esterase

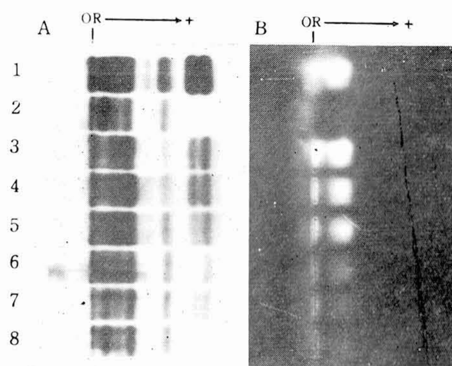

Fig. 9. Effect of malathion on esterases in house flies pre-treated with UC-10854 at various doses.

A : Non-specific esterases.
B : Acetylcholinesterases.

1. Control, 2. Malathion alone, 3. Pre-treatment 3μg/fly, 4. 1, 5. 0. 3, 6. 0. 1, 7. 0. 03, 8. 0. 01.

inhibition caused by malathion decreased. Flies treated with 3, 1 and 0. 3μg of UC-10854 per fly were knocked down at the time of malathion treatment, while those treated with 0. 1μg of UC-10854 became hyperactive, and those treated with 0. 03 and 0. 01μg remained normal. These results suggested that the decreased esterase inhibition by malathion with UC-10854 was associated with symptoms produced by UC-10854. Thus, inefficacy of malathion on the esterases like E_2, E_4 and ChE in the mixture or pretreatment with UC-10854 may be a cause of non-increase in insecticidal action in the mixture.

c. *The mechanism of joint action of organophosphates and carbamates.*

It is assumed that the mechanisms for interactions between carbamate and organophosphate insecticides may be as follows : (1) penetration of organophosphate into the insect body is inhibited by carbamates, (2) carbamates inhibit oxidation of organophosphorous compounds into oxygen analogs which have more powerful inhibitory action than their original thiono compounds, and (3) carbamates have more powerful affinity for esterases than organophosphates, so that organophosphates are unable to combine with esterase in the presence of carbamates.

Sun and Johnson[10] (1960) have presented that synergistic and antagonistic action of pyrethrin synergists such as sesamex, piperonyl butoxide, and sulfoxide in combination with many organophosphorus and chlorinated insecticides appear to be mainly due to the inhibition of certain biological oxidations which either activate or detoxify the compounds. In the present study, the failure of malathion in esterase inhibition caused by UC-10854 may be due to the inhibition of certain biological oxidations which activate malathion to a potent esterase inhibitor, i. e., malaoxon. When DDVP, an oxide type organophosphate was employed instead of malathion, it was observed that house flies treated with UC-10854 and then with DDVP showed more active E_2, E_4 and ChE activities than those treated with DDVP alone (Fig. 10). Moreover, parathion and its oxide analogue, paraoxon were tested for house flies with or without pretreatment of UC-10854 (Fig. 11). The ChE activity of house flies pretreated with UC-10854 was active following parathion treatment as well as following paraoxon treatment, in spite of a complete inhibition produced by the use of either parathion or paraoxon. These results suggest that ineffecacy of malathion on esterase activity in combination with UC-10854 is not due to the inhibition of certain biological oxidations which activate malathion to malaoxon.

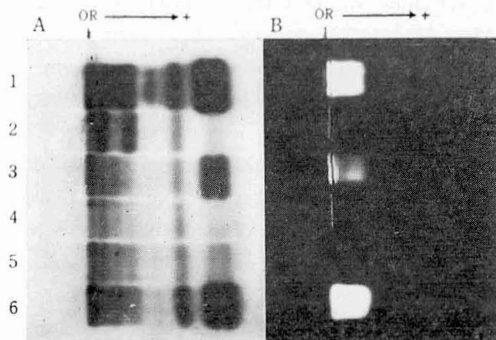

Fig. 10. Effect of DDVP on esterase activity of house flies suffered pre-treatment of UC-10854.

A : Non-specific esterases.

B : Acetylcholinesterases.

1. Control, 2. DDVP 0. 2μg, 3. DDVP 0. 2μg was applied to flies which have been pre-treated with 0. 5μg of UC-10854. 4. DDVP 0. 2μg was firstly applied and then post-treated with 0. 5μg of UC-10854. 5. A mixture of DDVP 0. 2μg and UC-10854 0. 5μg. 6. UC-10854 0. 5μg.

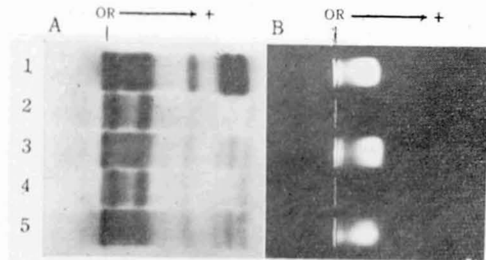

Fig. 11. Effect of parathion and paraoxon on esterases of house flies with or without pretreatment of UC-10854.

A : Non-specific esterases.

B : Acetylcholinesterases.

1. Control 2. Parathion 3. UC+Parathion 4. Paraoxon 5. UC+Paraoxon

Augustinsson and Nachmansohn[34] (1949) reported that, if esterase is first incubated with a reversible inhibitor such as prostigmine and then with an alkylphosphate, it is found that little or no irreversible inhibition occurs. In the present study, it is assumed that a similar phenomenon may have occurred in carbamate insecticides and organophosphates. To ascertain this *in vitro*, esterase was pre-inhibited with carbamate and then treated with organophosphate (Fig. 12). As malathion is a weak inhibitor of esterases, DDVP was employed for the experiment as an example of oxide type organophosphate. E_{18}, E_{19} and E_{23} bands were markedly inhibited with UC-10854, while E_2, E_4 bands were markedly inhibited with DDVP, and all these bands were inhibited in combination of UC-10854 and DDVP. These results indicate that the decreased degree of esterase inhibition by malathion is not due to the formation of carbamate-

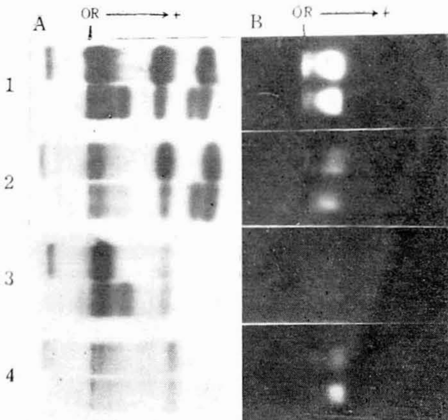

Fig. 12. *In vitro* inhibition of esterases of *ro*; *ct*;*cm* (upper) and *RP* (lower) strains of the house fly treated with UC-10854 or DDVP.

A : Non-specific esterases.

B : Acetylcholinesterases.

1. Control, 2. UC-10854(10^{-5}M) incorporated into agar-gel medium for electrophoresis. 3. The agar-gel plate after electrophoresis was immersed into DDVP (10^{-5}M) solution, then esterase activity was detected. 4. UC-10854 was incorporated into medium, and the agar-gel plate after electrophoresis was immersed into DDVP solution.

esterase complex as far as E_2, E_4 bands are concerned. Therefore, it is considered that permeability of malathion may be affected by UC-10854. However, ChE zymograms (Fig. 12B) indicated that at the presence of UC-10854, organophosphate failed to inhibit ChE in *in vitro* experiments.

It is indicated that non-increase in insecticidal action in the mixture consisting of carbamates and organophosphates against the *RP* strain of house flies may be caused by the two mechanisms. One of the mechanisms is that the inhibition of some esterases such as ChE by organophosphates is protected by carbamates, and the other is that, when carbamate-insecticides were affecting house flies, the permeability of organophosphates into the insect body was prevented.

4. *Joint action produced in a heterogeneous constitution of insect population.*

A combined use of two insecticides having different physiological action may be useful for the control of an insect population with varying resistant levels, for one insecticide may be influential in affecting part of the population, leaving the rest of the population under the influence of the other. In a preceding paper, a genetic analysis of the house fly made by Kasai and Ogita[35] (1965) showed that the main factor responsible for γ-BHC resistance is located on the 2nd chromosome, while the most important factor responsible for sevin-resistance is located on the 5th chromosome. They reported that a combined use of γ-BHC and sevin resulted in an increased insecticidal action, and that two factors might be responsible for these increased insecticidal action, i. e., a joint action for the genetic heterogeneity of insect population viewed on an insecticideresistance basis, and a *dissimilar joint action* reported by Metcalf[5].

Ogita[36] (1961) reported that a mixture of insecticide with negatively correlated substances[37)~41] had an effective insecticidal action for a mixed population consisted of insecticide resistant and susceptible flies, and that, though this finding appears to be the same as the so-called *joint toxic action*, it was essentially different from the usual *joint toxic action*. This joint action was shown only for the mixed population consisted of insecticide-resistant and susceptible flies. The phenomenon does not take place for the pure population consisted of equal resistant levels because the flies are killed by either of component of the mixture.

5. *Type of joint action.*

The formalized treatment of the joint action of insecticides was described by many investigators. Metcalf[5] (1955) reviewed the joint action of insecticides, and one of the typical scheme was presented by Plackett and Hawlett[42] (1948) as follows:

1) *Dissimilar joint action*, where toxicants *A* and *B* applied separately produce a common response yet when applied jointly produce the same response by action on separate and distinct physiological systems (*A*) and (*B*).

a. *Independent joint action*, where in addition to (1), *A* affects neither the amount of *B* reacting, nor the reaction with (*B*), as well as the converse.

b. *Dependent joint action*, where in addition to (1), *A* affects the amount of *B* reacting and/or the reaction with (*B*), and/or the converse.

2) *Simmilar joint action*, where *A* and *B* applied separately produce a common response and when applied jointly produce the same response by

independent action on the same physiological system.

3) *Synergistic action*, where A and B applied jointly produce a total response greater than the sum of their independent effects.

a. *Activation*, a special case of synergism where a substance with no toxicity at the dosage employed increases the effect of a toxicant.

4) *Antagonistic action*, where A and B jointly produce a total response smaller than the sum of the independent effects of either applied separately.

Later, these hypotheses or models of joint action were revised and extended (Plackett and Hawlett, 1952[43]). They proposed a two-way classification, with the joint action of insecticides described as ***similar*** or ***dissimilar*** according as they act at the same site or different sites in the insect to produce the response, and as ***noninteractive*** or ***interactive*** as one (or each) does not or does modify the physiological action of the other. The terminology for the four resultant types of joint action can be shown as follows:

	Similar	Dissimilar
Noninteractive	Simple similar	Independent
Interactive	Complex similar	Dependent

Sakai[4] (1960) proposed a new type of joint action including *pseudo joint toxic action* which was defined as the phenomenon exhibited by the alternate application of two or more components or by the altimate compound effect of them used separately at different times, and also as the phenomenon in which the living matter is influenced by the phenomenon produced by one or more compounds formed by the chemical reactions before penetrating into the living body.

These authors, however, did not take into consideration heterogeneous constitutions of an insect population subjected to insecticides. Thus, a new classification for joint action is to be required considering the above mentioned factors such as, a synergistic action due to an increased degree of permeability of insecticides, or due to environmental factors. Another factor to be mentioned is joint action caused either by genetic heterogeneity of an insect population viewed from resistance levels or by mixing negatively correlated substances. Referring to the present data it is proposed to offer a new scheme for joint action of insecticides as follows:

I. Synergistic action (joint action of insecticides with non-insecticidal chemicals).
 1. positive synergistic action (including activation).
  Ex. pyrethrin+piperonyl butoxide
   BHC+glycerol α,γ-dichlorohydrin (house fly larvae)
   BHC+glycerol (house fly adults)
 2. negative synergistic action (antagonistic action).
  Ex. Malathion+butoxide

II. Homo joint action (joint action produced in a homogeneous constitution as to insecticide resistance in insect population).
 1. positive joint action.
  Ex. γ-BHC+sevin
 2. unchanged joint action.
 3. negative joint action.
  Ex. Malathion+UC-10854

III. Hetero joint action (joint action produced in a heterogeneous constitution as to insecticide resistance in insect population).
 1. positive joint action.
  Ex. mixture of insecticides with negatively correlated substances.
   mixture of insecticides having different physiological action.
 2. unchanged joint action.
 3. negative joint action.

Summary

Joint action of insecticides has been studied in the house fly and *D. melanogaster* from genetical and biochemical viewpoint. Various factors which cause a synergistic or antagonistic action to insecticides, and interactions having different physiological properties were analyzed. The results obtained were as follows:

1. Relations between the chemical structure and synergistic activity of glycerol-derivatives to γ-BHC have been studied against larvae of the house fly by the immersion method. The following compounds were found to be synergistic, in descending order; glycerol α,γ-dibromohydrin>glycerol α,γ-dichlorohydrin>glycerol α-monochlorohydrin>glycerol (no activity). Ethylene chlorohydrin had a synergistic activity which was denied to ethyleneglycol.

Chlorals also showed a synergistic activity.

The succinic dehydrogenase activity of the house fly and of *D. melanogaster* was inhibited by these chemicals which had a synergistic activity. Addition of the above synergistic chemicals to γ-BHC resulted in an increased permeability into the larval body. It was suggested that one of the factors of synergists might be an increase of permeability of insecticides.

2. Synergistic activity of glycerol-derivatives on some insecticides against adult house flies and *D. melanogaster* was examined by the contact method. The following compounds were found to be synergistic, in descending] order ; glycerol≫glycerol α-monochlorohydrin>glycerol α,γ-dichlorohydrin (no activity). Ethyleneglycol and propyleneglycol had also a synergistic activity. The synergistic activity might be due to an increase in voratilization due to the above synergistic chemicals.

3. Esterase inhibition by organophosphates or carbamates has been studied with the Ogita's thin layer electrophoresis. It was found that esterases susceptible to organophosphates were different from those susceptible to carbamate insecticides. A combined use of organophosphates and carbamates did not increase the insecticidal action in strains of house flies. Studies on possible mechanisms for interaction between two chemicals indicated that two factors might be responsible for non-increase in insecticidal action in the mixtures. One is that the inhibition of esterases by organophosphate is protected by carbamates, and the other is that carbamates might prevent the penetration of organophosphates into the insect body.

4. A joint action produced by a genetic heterogeneity of insect population viewed on an insecticide-resistance basis was discussed.

The present data lead to a new classification of joint action covering the genetic constitution of insect population subjected to insecticides. Thus a new concept of joint action was proposed.

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Sterilizing Effect of Dowco-186 on the Azuki Bean Weevil, *Callosobruchus chinensis* L., with Special Reference to the Hatchability of the Eggs Deposited by Treated Weevils. Studies on the Chemosterilants of Insects. VI. Sumio NAGASAWA, Hiroshi SHINOHARA and Michiyo SHIBA (Ihara Agricultural Chemicals Institute, Shimizu, Shizuoka) Received July, 2, 1965. *Botyu-Kagaku*, 30, 91, 1965 (with English summary, 95)

16. Dowco-186のアズキゾウムシに対する不妊作用，とくに処理された成虫が産下した卵のふ化率．昆虫の化学的不妊剤に関する研究．第VI報

長沢純夫・篠原　寛・柴三千代（イハラ農薬研究所）40. 7. 2 受理

Dowco-186 (triphenyl tin hydroxide) のアセトン溶液を，アズキゾウムシの成虫に滴下処理し，産下した卵のふ化率と処理薬量の関係を，Wadley, Finney の方法で解析した結果，中央ふ化阻害薬量 0.201μg/♀，♂ (0.151～0.269μg/♀，♂) の値をえた．

Apholate および metepa によってひきおこされる，アズキゾウムシの不妊性については，主に処理個体によって産下された卵のふ化率を中心に，筆者ら[5,6,8]はその作用性を論じた．そして実験結果の整理にあたっては，Finney[1]によってとかれたプロビットをもちいる，quantitative response data の解析方法を適用した．しかし今までにしめした様な実験記録に対して，すべての観測値がおなじ variance をもつと考えて，この解析方法をそのままもちいることには，厳密に言って異論があり，期待ふ化数に比例した variance を考慮にいれた解析がなされるべきことを附記した[7]．本文においてはそうした見地から，Dowco-186 をもちいてアズキゾウムシについてえられた実験記録を解析した結果をのべる．本文に入るに先立ち解析の方法について種々御教示を賜った英国Aberdeen大学のD. J. Finney博士と，試料の御提供を戴いたDow Chemical Co. の E. Kenaga 博士に謝意を表する．

実験材料および方法

Dowco-186 の試料番号を有する薬物は，Dow Chemical Co. の Bioproducts Department から提供をうけた technical grade の錫化合物 triphenyl tin hydroxide である．アズキゾウムシは当所の累代飼育系統で試験には含水量約 15.3% のアズキでその幼虫