Mechanism of the Selective Toxicity of Organophosphorus Compounds in the Armyworm, *Leucania separata* Walker. Part I. Topical Toxicity and Anticholinesterase Activity of Certain Organophosphorus Compounds. Neungpanich SINCHAISRI, Tadashi MIYATA and Tetsuo SAITO (Laboratory of Applied Entomology and Nematology, Faculty of Agriculture, Nagoya University, Chikusa-Ku, Furo-Cho, Nagoya, 461) Received May 9, 1977. *Botyu-Kagaku*, 42, 125, 1977.

19. アワヨトウにおける有機リン殺虫剤の選択毒性に関する研究 I. 数種有機リン殺虫剤の殺虫力とコリンエステラーゼ阻害作用 ヌアンパニッチ シンチャイシイ，宮田正，斎藤哲夫 (464名古屋市千種区不老町 名古屋大学農学部害虫学教室) 52. 5. 9 受理

4種有機リン殺虫剤のアワヨトウ，*Leucania separata* Walker 幼虫に対する殺虫力を局所施用により調べるとともに *in vitro* におけるアワヨトウのコリンエステラーゼ (ChE) 阻害作用を ^{14}C-acetylcholine chloride を用いて調べた．LD$_{50}$ 値は methyl parathion が最も小さく，ついで fenitrothion, phenthoate, diazinon の順であった．アワヨトウの ChE は真性 ChE の特徴を示した．*In vitro* における ChE 阻害作用は phenthoate-oxon が最も強く，ついで methyl paraoxon, diazoxon, fenitroxon の順であった．

4種殺虫剤の殺虫力と ChE 阻害作用の相関を検討したところ，methyl parathion, diazinon, fenitrothion の3種殺虫剤での相関は高かった．一方，phenthoate の場合 methyl parathion の5倍のChE阻害作用を示したが殺虫力は methyl parathion の約 1/3.8 であった．これらの事実は phenthoate の虫体内への透過，あるいは虫体内で代謝等の要因が他の3種殺虫剤と異なっているという可能性を暗示している．

Armyworm, *Leucania separata* Walker is one of the important pests of corn, *Zea mays* L. and rice, *Oryza sativa* L.[1,2] Many ecological and physiological works have been reported so far but it seems that publications concerning with the toxicological study on this species are still lack. The toxicity test with 31 insecticides to the armyworm larvae indicated that most of organophosphorus (OP) insecticides were highly toxic whereas carbamates were not and endrin and dieldrin, the only two organochlorine compounds gave relatively high effect[3].

It is generally acknowledged that OP insecticides kill animals by inhibiting cholinesterase (ChE). Most of them are rather poor inhibitors of ChE *in vitro*. They owe their potency *in vivo* to the fact that they are converted in the body to give compounds which are presumed to be the direct inhibitors[5~9].

Thus, as the first approach to clarify the mechanism of selective toxicity of four OP compounds, methyl parathion, phenthoate, diazinon and fenitrothion against the armyworm, topical toxicity of them and anticholinesterase activity of their oxygen analogs *in vitro* were investigated.

Despite the measurement of ChE activity by the method described Hestrin[10] showed the unsatisfied result in the preliminary procedure due to the strong interference of melanization effect in the armyworm enzyme, the radioisotopic assay, exercised in this study, offered much advantages and quite desirable for the micro-volume of solution in the measurement of ChE activity.

Materials and Methods

Insect

The colony of the armyworm larvae was obtained from the University Farm, Nagoya University, Togo, Aichi prefecture in 1974. The larvae were reared at 25°C in the plastic box, 24×30×5cm, fed with fresh corn leaves as their basic food (partly fed with the leaves of Italian Rye grass to keep the successive generations during winter season). The mass rearing technics were essentially the same as the previous described[3,4]. The 5th-instar, 2-3-day larvae were provided for experiments.

Chemicals

Over 97% purity of four organophosphorus compounds were used. Methyl parathion and

fenitrothion were received from Sumitomo Chemical Co., Ltd., phenthoate was obtained from Nissan Chemical Industry Ltd., diazinon was obtained from Nihon Kagaku Co., Ltd.

Cholinesterase inhibitors, P=O analogs of these 4 OP compounds were synthesized by an oxidation of P=S compounds in bromine solution and purified by thin layer chromatography (tlc).

Acetyl [1- ^{14}C]-choline chloride (^{14}C-AChCl), 13.7 mCi/mmole, was purchased from Amersham Co., England. The Amberlite CG-120, type 2, resin sodium salt was a product of Organo Co., Tokyo. Chemicals for the preparation a scintillation solution, for instances, naphthalene (special grade), 2,5-diphenyloxazole (PPO), dimethyl POPOP and 1,4-dioxane were products of Katayama Chemical Co., Ltd., Osaka.

Toxicity test

The evaluation of contact toxicity of 4 OP compounds was accomplished by topical treating the 5th-instar larvae of the armyworm with 0.7 μl of acetone solution containing amount of OP compounds. In control, insects were treated with the same volume of acetone. Insects were fed with fresh corn leaves and kept in a constant temperature chamber of 25°C. After 72hr, mortality counts were taken. Badly affected or moribund larvae were justified as dead insects according to the method of Ando and Sherman[11]. At least 4 replicates of 10 insects at 5-8 dosage levels were provided for each insecticide in this test. The values of LD$_{50}$ and LD$_{95}$ were calculated by the method of Finney[12] and toxic index was evaluated by the method of Sun[13].

Radioisotopic assay of ChE activity

Substrate solution.-Stock solution of ^{14}C-AChCl, 8×10^{-3}M, containing approximate 3,300,000 cpm/ml was prepared by dissolving the contents of AChCl from a ampoule in distilled water. Stock solution of non-labelled AChCl, 0.55M, was simultaneously prepared. The mixture of labelled and non-labelled AChCl was provided to obtain higher concentrations than 8×10^{-3}M of AChCl with highly enough radioactivity for the experiments. The mixture were always kept under −20°C.

Source of enzyme.-Three kinds of homogenate, head (H), whole body (WB) and brain & ventral nerve cord (B & VNC), from the 5th-instar larvae of the armyworm were prepared separately. Homogenates of H and WB were centrifuged at 900g for 5 min. The supernatants were used as enzyme sources throughout the study. B & VNC homogenate was directly applied as the enzyme source without centrifugation.

Buffer's solution.-Phosphate buffer's solution, 1/15M, pH 7.4, was mainly used in the routine works. In the experiment of an effect of pH on the ChE activity, buffer of Chadwick et al.[8] was used: NaCl, 26.30g; KH$_2$PO$_4$, 3.85g; NaOH, 1.00g; water to make one litre. With all suspensions, pH was adjusted to the desired value by addtion of 0.1N NaOH or 0.1N HCl. The range of pH values adjusted would cover enough in the acidic and alkaline conditions from pH 6.1-9.2.

Amberlite resin-dioxane mixture.-Preparation was essential the same as the method described by Siakotos et al.[14]. Ten grams of dried resin was put into 100ml of dioxane.

Scintillation solution.-It was a modified Bray's Cocktail[14] containing 100g naphthalene, 4g; PPO, 200mg POPOP and 100ml absolute methanol, and then made to 1 litre with 1,4-dioxane.

Assay procedure

With an equal volume of 0.05ml, the mixture of enzyme and ^{14}C-AChCl solution was put in a 20ml-tube, and the content of the tube was well mixed by shaking and incubated for 10 min at 37°C. Non-enzymatic hydrolysis of the substrate was measured with the solution containing of 0.05ml of buffer instead of enzyme. ChE activity was expressed as μ mole of the substrate hydrolyzed/hr/g.

The proceeding steps of reaction were assayed as previously described by Siakotos et al.[14]. In order to stop the reaction, an approximately 5ml of the suspended resin-dioxane mixture was added. Then, the enzyme-resin-dioxane solution was brought to an exact volume of 10ml with dioxane. The tube was tightly capped, and mixed by inversion or shaking and allowed it to stand. The resin was allowed to settle by gravity for at least 2 hr. Five millilitres of the supernatant solution was transferred to a scintilltion counting vial, and 10ml of the modified Bray's scintilltion solution was added. Special care must be taken

to avoid transferring resin particles into the vial. The samples, total volume of 15ml, were counted the radioactivity of the hydrolysis product in a liquid scintillation spectrometer (Tricarb Model 3320 Liquid Scintillation Spectrometer) which has an effeciency about 60%at the optimized ^{14}C setting.

For the inhibition study, inhibitors (P=O analogs of OP compounds) were dissolved in acetone in widely different concentrations. With the volume of 0.01ml, an inhibitor solution was delivered by means of a microsyringe into the 20ml tube and allowed acetone to evaporate absolutely, 0.05ml of enzyme solution, thenafter, was added and pre-incubated at 37°C for 30 min and then, 0.05ml of ^{14}C-substrate was added, incubated at 37°C for 20 min. The next procedure for ChE activity measurement was carried out by the same manner as described above.

Results

Toxicity test

LD_{50} and LD_{95} values of 4 OP compounds were shown in Table 1. Methyl parathion gave the highest toxicity to the 5th instar larvae of the armyworm. The relative toxicities of fenitrothion, phenthoate and diazinon were lower than methyl parathion, a standard, as expressed by toxic index value of 297, 383 and 1080 respectively.

Measurement of ChE activity

Substrate concentration.- The ChE activities of three homogenates were shown in Fig. 1. From the bell shaped curves in Fig. 1, it is likely to
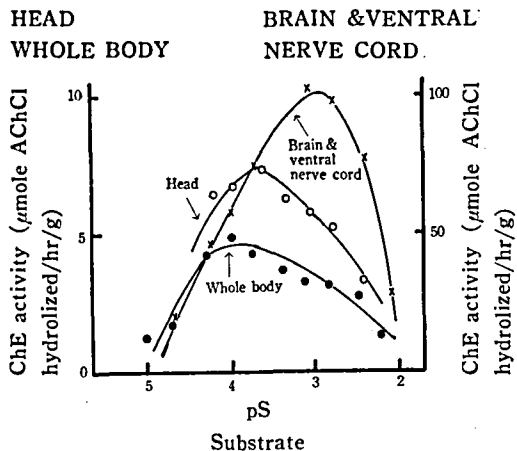

HEAD WHOLE BODY   BRAIN &VENTRAL NERVE CORD

Fig. 1. Activity-pS (−log mole of substrate) curves for the enzymatic hydrolysis of acetylcholine chloride in three sources of armyworm enzyme. Reaction conditions; enzyme concentrations, head, 4%, whole body, 10% and brain & ventral nerve cord, 1%, pH adjusted at 7.4 with phosphate buffer. Incubation time, 10 min at 37°C.

presume that the maximum enzymatic activities of H and WB are quite similar to each other at $2.5 \times 10^{-4}M$ final concentration of the substrate, but it needs higher concentration of $1.0 \times 10^{-3}M$ to produce the maximum activity for tissue homogenate of B & VNC. The data of this experiment provided an additional evidence that ChE in the armyworm is true ChE.

Enzyme concentration.- From Fig. 2, enzymatic

Table 1. LD_{50}, LD_{95}, regression equation of dosage-mortality and the relative toxicity of 4 organophosphorus insecticides topically treated on the 5th instar larvae of the armyworm.

Insecticides	Regression equation	LD_{50} μg/g	LD_{95} μg/g	Toxic index (LD_{50})
Methyl parathion	Y=5+3.9893 (x−0.9916)	0.97	2.51	100
Phenthoate	Y=5+2.5934 (x−1.5729)	3.72	15.85	383
Diazinon	Y=5+3.1467 (x−2.0263)	10.48	34.75	1080
Fenitrothion	Y=5+1.7571 (x−1.4684)	2.88	25.20	297

Y=mortality in probit, x=log dosage (μg x 10)

activity was in the proportional rate to enzyme concentration up to 10% in H, 20% in WB and 4% in B & VNC enzymes. Higher enzyme concentrations yielded non-linear results.

Time course of the reaction.-As illustrated in Fig. 3, the time course of the substrate hydrolysis by enzymes was demonstrated. Absolute linearity was found in ChE activities of all homogenates

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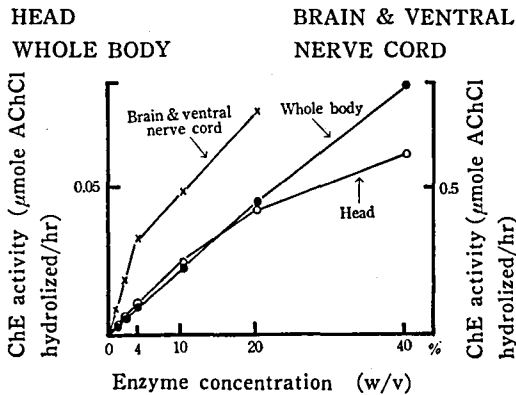
BRAIN & VENTRAL
NERVE CORD

Fig. 2. Linearity of enzyme activity as a function of enzyme concentration. Activities were measured after 10 min incubation at 37°C. Substrate concentrations: 2.5×10^{-4}M final for head and whole body and 1.0×10^{-3}M for brain & ventral nerve cord homogenates, pH adjusted to 7.4 with phosphate buffer.

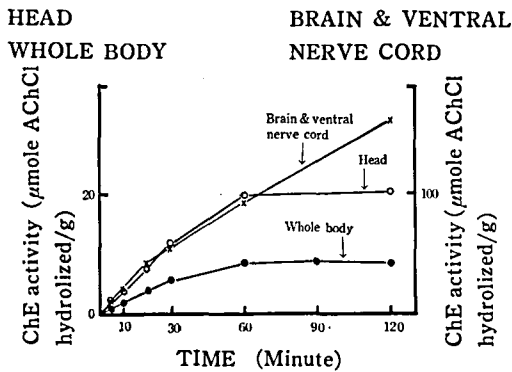HEAD
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NERVE CORD

Fig. 3. Proportionality of substrate hydrolized with time of incubation at 37°C. Enzyme concentrations: head, 4 %, whole body, 10%, brain & ventral nerve cord, 1%. Substrate concentrations and pH were the same conditions as Fig. 2.

up to 20 min incubation. The hydrolysing rates decrease with the time thereafter. The sharp decrease occurred from 60 min in H and WB but slow decrease was found in B & VNC homogenates.

Optimum pH.- Based on the suitable condition obtained from the previous experiments, the measurement of ChE activity as a function of pH was carried out and the obtained results were illustrated in Fig. 4. The effect of pH in the reaction of enzyme and substrate caused different enzymatic activity. The optimum pH of H and B & VNC homogenates were obtained in slight alkaline condition at the range of 8.0-8.6 while of WB homogenate was almost neutral at the range of 7.2-7.4 (Fig.4 and Table 2).

The peaks of the curves in Fig. 4 would represent the maximum ChE activity of the armyworm from different enzyme sources which were

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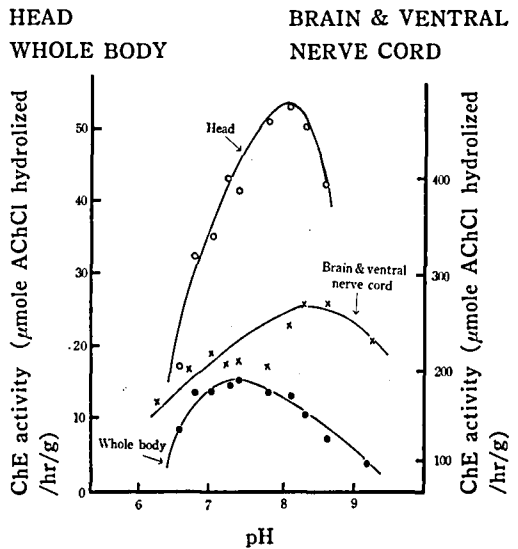
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Fig. 4. Variation in ChE activity of 3 sources of armyworm enzyme as a function of pH. The reaction conditions were: substrate concentration for head and whole body homogenates, 2.5×10^{-4}M final, brain & ventral nerve cord, 1.0×10^{-3}M final, enzyme concentrations: head, 4%, whole body, 10%, brain & ventral nerve cord, 1%. Incubation time, 10 min at 37°C. For full explanation see Text.

Table 2. The different properties among the sources of enzyme in the armyworm larvae

Source of enzyme	Optimum	
	AChCl	pH
Head	2.5×10^{-4}M	8.0-8.3
Whole body	2.5×10^{-4}M	7.2-7.4
Brain & ventral nerve cord	1.0×10^{-3}M	8.3-8.6

Table 3. Cholinesterase activity in the armyworm larvae

Source of enzyme	ChE activity (μmole AChCl hydrolized/hr/g)
Head	53
Whole body	15
Brain & ventral nerve cord	265

shown in Table 3. ChE activities were 53 μmoles AChCl hydrolyzed/hr/g in H, 15 μmoles in WB, and the highest activity was found in B & VNC at 265 μmoles AChCl hydrolyzed/hr/g.

Anticholinesterase activity

P=O analogs or oxon isomers of 4 OP compounds were applied as ChE inhibitors in the ChE inhibition test. The results were tabulated in Table 4. Methyl paraoxon was given as a standard compound in the comparative inhibitory activities. The activity was expressed as 50% inhibition (IN_{50}) and 80% inhibition (IN_{80}) by the molar concentrations of the inhibitors.

Diazoxon and fenitroxon gave lower inhibitory activities than methyl paraoxon which, by all cases, were well corresponding with their P=S toxicities. But phenthoate-oxon showed an exceptional result where it exhibited its anti ChE activity about 5 times higher than methyl paraoxon while its corresponding P=S toxiciy was 3.8 times lower.

Discussion

The basic properties of ChE in the armyworm enzymes from H, WB and B & VNC homogenates seemed to be the typical characteristic of true

Table 4. Molar concentration on 4 OP-compounds for 50 and 80 percent inhibition of ChE in the armyworm.

Sources of enzyme	Head				Whole body				Brain & veutral nerve cord			
Inhibitors	IN_{50} (M)	Ratio	IN_{80} (M)	Ratio	IN_{50} (M)	Ratio	IN_{80} (M)	Ratio	IN_{50} (M)	Ratio	IN_{80} (M)	Ratio
M. paraoxon	4.8×10^{-8}	1.00	1.6×10^{-7}	1.00	1.4×10^{-7}	1.00	3.3×10^{-8}	1.00	4.0×10^{-8}	1.00	3.3×10^{-7}	1.00
Phenthoate-oxon	7.1×10^{-9}	0.15	4.7×10^{-8}	0.29	1.7×10^{-8}	0.23	7.7×10^{-9}	0.12	1.8×10^{-8}	0.37	7.1×10^{-8}	0.21
Diazoxon	1.3×10^{-7}	2.71	4.8×10^{-7}	3.00	3.7×10^{-7}	3.64	1.2×10^{-7}	2.64	2.1×10^{-7}	5.25	7.9×10^{-7}	2.40
Fenitroxon	1.4×10^{-7}	2.92	7.9×10^{-7}	4.94	2.5×10^{-7}	3.04	1.0×10^{-7}	1.78	2.8×10^{-7}	7.00	8.9×10^{-7}	2.70

ChE but it needs much lower concentration of substrate (2.5×10^{-4}M) to produce a maximum enzymatic activity, particularly, in head and whole body homogenates than the other insects tissue homogenates (5×10^{-3}M-1.0×10^{-2}M)[14]. This obtained optimum substrate concentration strongly confirms the failure in the measurement of

ChE activity by the colorimetric method described by Hestrin[10]. As well as the interference of melanization effect of enzyme solution, too low concentration will gives a narrow range in activity variation and subsequently much larger error appears causing a difficulty in enzymatic activity measurement. The optimum pH in H and B & VNC homogenates were obtained at the range of 8.0-8.6 which was also found in the enzyme of developing grasshopper eggs[16], house fly head[8] and cat brain[17]. Stegwee[18], working with central nerve tissue of beetle, *Hydrophyllus* and roach, *Periplaneta*, recorded rather sharp optima at pH 7.4 which is quite similar to the result with whole body homogenate in this experiment.

It is evident from previous works[9,19~22], that interpreting the selective toxicity of different insecticides is difficult because the toxicity data are the end-product of the complex interaction of many factors. According to Saito[19] and others [6,20~22], factors responsible for selectivity of insecticides were divided into 3 steps ; differences of distribution of toxicants in insect body, differ-

ences of susceptibilities of insect ChE to toxicants and differences of activation and detoxification metabolism of toxicants. Metcalf and March [23] demonstrated the strong relationship between the *in vitro* antiChE activity and the *in vivo* toxicity of diisopropyl paraoxon in mouse, honey bee, and cockroach. The similar relationship between house fly and mouse was reported in the diethyl substituted phenyl phosphate compounds[24].

The correlation coefficient (r) of toxicity and antiChE activity among 4 OP compounds were calculated as shown in Table 5. From r values, seemingly, there is a weak correlation among all four compounds but positive correlation is obtained among three compounds except phenthoate. Considering the toxic index of phenthoate is 383 and antiChE activity ratio of phenthoate-oxon is 0.12-0.37 in IN_{50} and IN_{80} of all three sources of enzyme, the mechanism of selective toxicity of phenthoate might associate with other factors being different from those of other 3 OP compounds.

In some cases two factors may cancel each other out. This has been shown for OP compound, famphur which is degraded eight times faster by mice than by milkweed bugs, yet is 20 times better against mouse than against milkweed bug ChE *in vitro*. The combination of two factors led to a very small selective toxicity, famphur is only 1.5 times more toxic to milkweed bugs than to mice[9]. The other work[25] supports this evident

Table 5. The correlation of the toxicity of methyl parathion, phenthoate, diazinon and fenitrothion and the anticholinesterase activity of their oxygen analogs in the armyworm.

Sources of enzyme	Correlation coefficient (r)			
	LD_{50}-IN_{50}	LD_{50}-IN_{80}	LD_{93}-IN_{50}	LD_{93}-IN_{80}
Head	r =0.1844	r =0.2509	r =0.2611	r =0.4109
	r_1=0.8036	r_1=0.6367	r_1=0.9855	r_1=0.9158
Whole body	r =0.3114	r =0.1933	r =0.3680	r =0.1991
	r_1=0.9019	r_1=0.9846	r_1=0.9997	r_1=0.9578
Brain and ventral nerve cord	r =0.4087	r =0.1903	r =0.5605	r =0.2828
	r_1=0.7508	r_1=0.7595	r_1=0.9678	r_1=0.9711

r values were calculated from the logarithmic values of $LD_{50} \times 10$, $LD_{93} \times 10$ $IN_{50} \times 10^9$, and $IN_{80} \times 10^9$; r_1 is the values of correlation coefficient calculated without phenthoate

as the data are enable an explanation of unusual features of the toxic action of dimethoxon on *Periplaneta americana* L. comparing with diazoxon. From a small restriction of both compounds to penetrate into the central nervous system(CNS), dimethoxon was found 200 times less active than diazoxon (both against cockroach ChE and in electrophysiological test againstthe CNS of cockroach). Yet, this weak neurotoxicity, however, is compensated for by relatively high stability to detoxication and allowing sufficient to accumulate in insect to render it almost as toxic to cockroaches as a more active antiChE but more rapidly being detoxified like diazoxon.

In order to clarify the mechanism of selective toxicity of these 4 OP compounds, other factors (metabolism, penetration and etc.) will be contributed to the further outline of experiments.

Summary

As the first step to clarify the selective toxicity of 4 OP compounds in the armyworm, *Leucania separata* Walker, the bioassay and *in vitro* antiChE activity were studied. The compounds were treated topically to the 5th-instar, 2-3-day larvae. Comparing their relative toxicities by LD_{50} values, it was found that the toxic index of fenitrothion, phenthoate and diazinon to methyl parathion were 297, 383 and 1080 respectively.

Cholinesterase (ChE) activity was measured by radioisotopic method using ^{14}C-acetylcholine chloride (AChCl) as a substrate. ChE of the armyworm shows the typical characteristic of true ChE having an optimum substrate concentrations : $2.5 \times 10^{-4}M$ with tissue homogenates of head and whole body and $1.0 \times 10^{-3}M$ with brain and ventral nerve cord homogenate.

The results of *in vitro* antiChE activity with oxon isomers of the compounds were orderly accomplished; phenthoate-oxon>methyl paraoxon >diazoxon>fenitroxon.

The correlation between the toxicity of the OP compounds and the antiChE activities of their oxygen analogs was examined. Statistically, basing on the correlation coefficient values (r), it seems that there is poor correlation between toxicity and antiChE activity data among 4 compounds but positive correlation among 3 compounds, methyl parathion, fenitrothion and diazinon. Unexpectedly, phenthoate was an exception where its antiChE activity was the highest, exhibiting about 5 times higher inhibitory activity whereas it was 3.8 times less toxic than that of methyl parathion, a standard compound. This might indicate that the mechanism of selective toxicity of phenthoate against the armyworm is attributable to the different feature from those of other three OP ompounds.

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抄　　録

成虫間の交信撹乱によるワタアカミムシの防除，

Controlling the Pink Bollworm by Disrupting Sex Pheromone Communication Between Adult Moths. L. K. GASTON, R. S. KAAE, H. H. SHOREY, D. SELLERS: *Science*, 196, 904 (1977).

ワタアカミムシは，世界中の棉畠の害虫であるが，1シーズン中に，数世代が重なって繰り返して発生する．ワタアカミムシの性フェロモンは，すでに化学構造も解明され，合成されていて "gossyplure" と呼ばれている．

フェロモンは，ポリオキシメチレン製のチューブに吸い上げ，その一方の端を封じておく．このチューブには内径 0.22mm の孔があいていて，一本の長さは 10.4cm なので1巻半の輪ができる．これを棉の小枝に手でかけていく．

それぞれ5ヘクタール，6ヘクタール，12ヘクタールの合計23ヘクタールの試験区には1m毎の格子状に輪を配置し合計230,000使用した．1シーズン3週毎に5回これをとりかえた．

3試験区の他に，同じような地形の10区を比較区として調査し，これらの区は，フェロモン処理せずに普通の栽培上の管理を行なっている．

3つのフェロモン区には，おのおの4つのフェロモントラップを設置して週に2回雄の誘引数を調べたが，各区平均7頭のトラップ数であった．10比較区では，各区平均356頭もあるので，概算98%の減少とみなされる．しかし，実用面からいえば，幼虫の食害の減少を調べなければならない．棉実を採取し，幼虫の食入の有無を調べた．7月初旬のシーズン最初の採集棉実では，フェロモン区の方に食害されたものが多かった．その後，7月中旬から8月下旬まで，フェロモン区の幼虫数が低く経過し，9月中旬では，ヘクタール当り 22,000（フェロモン区）32,000（比較区）であった．

このシーズン中，必要と認めた時には，殺虫剤の散布も行なった．フェロモン区は，3区のうちで1区だけ1回使用したので，ヘクタール当りの使用は0.3回で，比較区は，2.6回であった．単純に計算すると1ヘクタール当りの使用数は $\frac{1}{9}$ に減少したといえる．この実験で，1シーズンに使用したフェロモンの総量は，33g/ヘクタールで，現在 0.8$/g である．従って1シーズン 26$/ヘクタールとなり，殺虫剤2回散布の費用に相当する．大量生産するようになれば，gossyplure の価格も下り，要する費用も少なくなるであろう．

フェロモン使用に基づいた害虫防除を害虫管理プログラムに導入することは，ワタアカミムシのような重要害虫の駆除に種々の利点をもたらすことであろう．

（髙橋正三）