Original Articles

Mechanism of the Selective Toxicity of Organophosphorus Compounds in the Armyworm, *Leucania separata* WALKER*

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Cuticular penetration of ^{14}C -labelled methyl parathion, phenthoate, diazinon and fenitrothion in the armyworm, *Leucania separata* WALKER was examined. These compounds showed variable rate of penetration. Regarding the comparison based on half-time (t_{50}) value, the rates of penetration were, methyl parathion > fenitrothion = diazinon > phenthoate. Particular differences in penetration were observed between methyl parathion and phenthoate with the ratio of 6 : 1. Additional investigation on the excretion rate of these ^{14}C -compounds was performed. Recovery of radioactive compounds in excreta was low in methyl parathion but high in phenthoate. Diazinon and fenitrothion, did not showed any marked difference in the excretion rate. Results obtained here, however, seemed to indicate that cuticular penetration might be the possible factor accounting for the mechanisms of selective toxicity of phenthoate and the other three compounds.

INTRODUCTION

The topical toxicities and anticholinesterases of methyl parathion, phenthoate, diazinon and fenitrothion against the larvae of the armyworm, *Leucania separata* WALKER, were previously reported.¹⁾ As the second step to clarify the mechanism of the selective toxicity of these compounds in the armyworm, cuticular penetration of four ^{14}C -labelled organophosphorus (OP) insecticides was studied.

MATERIALS AND METHODS

1. Insect

Fifth instar armyworm larvae were obtained from the same colony of the laboratory mass-rearing as described in the previous study.¹⁾ Average body weight was 0.2 g. Two to ten

larvae were used for each experiment with 3 replicates. The number of larvae used in each experiment varied with specific activities of radiolabelled chemicals applied.

2. ^{14}C -labelled Compounds

^{14}C -Alkyl-labelled methyl parathion, phenthoate and diazinon were synthesized according to the methods described by Fletcher *et al.*,²⁾ Hirose *et al.*³⁾ and Gasser.⁴⁾

^{14}C -Methyl parathion: one mmol of anhydrous methanol containing 2 mCi of ^{14}C -methanol (60 mCi/mmol) and 0.25 mmol of phosphorus pentasulfide were refluxed in benzene for the preparation of ^{14}C -O,O-dimethyl dithiophosphoric acid and followed by chlorination at 20°C by bubbling chlorine gas into benzene solution. The product and 0.5 mmol of *p*-nitrophenol were refluxed with potassium carbonate for 20 hr at 80–90°C. The solvent layer was filtrated and then evaporated at 40°C, and oily residue was dissolved in 30 ml of chloroform. The chloroform solution was

* Cuticular Penetration of ^{14}C -Labelled Certain Organophosphorus Compounds (Part II)

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washed with an equal volume of 10% sodium carbonate and then with distilled water.

The chloroform fraction was dried over anhydrous sodium sulfate overnight. The chloroform solution was condensed to approximately 2 ml at 40°C and was purified by thin layer chromatography (*tlc*, Silica gel, F 254) with a solvent mixture of *n*-hexane : acetone (4 : 1, v/v) as a mobile phase. The portion of ¹⁴C-methyl parathion on the plate was scraped out, and was extracted with acetone. The acetone was evaporated at 40°C. ¹⁴C-Methyl parathion was dissolved in acetone as a stock solution and was kept at -20°C. The chemical purity of the synthesized ¹⁴C-methyl parathion was shown to exceed 98% with the specific radioactivity of 3,000 dpm/μg determined by Packard Tricarb, liquid scintillation spectrometer, Model 2311. The yield was 13%.

¹⁴C-Phenthoate: ¹⁴C-*O,O*-dimethyl dithiophosphoric acid was obtained by the similar procedure to that of ¹⁴C-methyl parathion preparation. Subsequently, the preparation was achieved according to the method of Hirose *et al.*⁸⁾ ¹⁴C-*O,O*-Dimethyl dithiophosphoric acid in benzene was reacted with ammonia gas. The ammonium ¹⁴C-*O,O*-dimethyl phosphorodithioate was dried up at 40°C and was dissolved in acetone. This acetone solution was refluxed with ethyl α-bromophenylacetate at room temperature for 3 hr and additional 1 hr at 45°C. The solution was dried up at 40°C, and 50 ml of benzene was added. The benzene solution was washed twice with an equal volume of distilled water and was dehydrated with sodium sulfate overnight. The solution was filtrated, concentrated to about 2 ml and was purified by the *tlc* with a solvent mixture of *n*-hexane : acetone (4 : 1, v/v). The chemical purity of synthesized ¹⁴C-phenthoate was over 98% with specific activity of 4,400 dpm/μg. The yield was 42.8%. It was dissolved in acetone and was kept at -20°C as a stock solution.

¹⁴C-Diazinon: as described by Fletcher *et al.*²⁾ ¹⁴C-*O,O*-diethyl dithiophosphate was prepared by refluxing one mmol of ethanol containing 1.5 mCi of 1-¹⁴C-ethanol (57 mCi/mmol) with 0.25 mmol of phosphorus pentasulfide for 7 hr in 2 ml anhydrous chloroform, ethanol was added and refluxed for further

2 hr. To the reaction mixture, 0.5 mmol (45 μl) of sulfuryl chloride was added, and ¹⁴C-*O,O*-diethyl chlorothiophosphate was obtained, which was purified by active carbon column chromatography. The further step was followed by the method of Gasser⁴⁾ that is, 2-isopropyl-4-methyl-6-hydroxy pyrimidine was reacted with sodium methoxide in benzene to obtain its sodium salt. The product of this step was further refluxed with ¹⁴C-*O,O*-diethyl chlorothiophosphate in benzene for 20 hr. The reaction mixture was filtrated and washed twice with an equal volume of distilled water. The benzene solution was dehydrated with sodium sulfate overnight, and the benzene was evaporated at 40°C. The product was purified by the *tlc* with the solvent system, *n*-hexane : ethyl acetate (5 : 1, v/v). The portion of ¹⁴C-diazinon was scraped and was extracted with acetone. The purified ¹⁴C-diazinon which showed more than 98% of radiochemical purity with a specific activity of 850 dpm/μg, was kept in acetone solution at -20°C. The yield was 14.1%.

¹⁴C-Fenitrothion: ¹⁴C-*m*-methyl-labelled fenitrothion (6.7 mCi/mmol) of more than 99% purity was provided by Sumitomo Chemical Company, Osaka, Japan.

Scintillation vials containing sample solution and toluene-based scintillation solution [5 g of PPO (2,5-diphenyloxazole) and 0.3 g of dimethyl POPOP (1,4-bis-2(4-methyl-5-phenyloxazolyl) benzene) in 1 litre of toluene] were counted in the Packard Tricarb, liquid scintillation spectrometer, Model 2311.

3. Procedure of Cuticular Penetration Studies

The rate of cuticular penetration was determined by topical applying or 0.7 μl of acetone solutions of the 4 labelled OP compounds to the dorsal part (5-7th segments) of the insect body using a 100 μl Terumo microsyringe (MS-100). Three dosages of LD₁₀ (methyl parathion, 0.5 μg/g; phenthoate, 1.0 μg/g; diazinon, 1.6 μg/g; fenitrothion, 0.7 μg/g), LD₅₀ and LD₉₅ were adopted for this study. The toxicity data of latter two dosages were previously reported.¹⁾ It might be possible to determine the nature of penetrability of compounds at equitoxicity and equidose simultaneously by the experiment in 3 levels of

dosage. Treated insects were kept in a 15 ml Erlenmeyer flask at 25°C, and the flask was connected with the carbon dioxide trap. At various time intervals, 1/4, 1/2, 1, 4, and 24 hr after treatment, the radioactivities were analyzed. With 24 hr experiment, the LD₅₀ dose treatments of methyl parathion and fenitrothion resulted in complete mortality. The additional treatments, therefore, at the shorter time intervals at 7.5 min for fenitrothion and 3.0 and 7.5 min for methyl parathion were performed.

The amount of cuticular penetration was determined by rinsing the body surface of treated insects with 2 ml of toluene (guaranteed grade) per insect. Radioactivity of rinsing solution was counted in 10 ml of toluene-based scintillator. The difference of radioactivity between the recovery from the body surface rinsing and the total recovery was assumed to be the amount of penetrated into or through the cuticle.

Internal content was evaluated by combusting the dried body in a Sample Oxidizer apparatus (Packard Tricarb, Model 306) after rinsing out the body surface.

For the measurement of radioactivity in the excreta, fecal pellets were removed into a small (3×6 cm) paper bag, and the flask was rinsed with 2.5 ml of toluene and 2.5 ml of water. The volume of these rinsing solutions was reduced to about 0.5 ml by evaporation on hot plate and was absorbed by pieces of filter paper, which was burnt in combination with fecal pellets using Sample Oxidizer.

Expired carbon dioxide gas: carbon dioxide trapping reagent was prepared according to Crout *et al.* (1966)⁵⁾. Carbon dioxide-free air was passed through the apparatus at a flow rate of 20 ml/min. Effluent air from the flask, containing respiring worms, was passed through a stopcock and into a trapping reagent containing 6 ml (3 ml/vial) of 2-methoxyethanol: 2-ethanolamine (11 : 1, v/v). At the end of each experimental period, 12 ml of toluene based scintillation solution was added to the whole volume of trapping solution in each vial, and radioactivity was measured.

Total recovery was obtained from the combination of radioactivity from four sources.

RESULTS

The penetration rates with these radio-labelled OP compounds are given in Figs. 1 and 2. The data are plotted as a logarithmic function of the percentage of radioactivity recovered from the surface against the time. Comparing the data of three equitoxic dosages (LD₁₀, LD₅₀ and LD₉₅) and considering the slope of the line from 0.05–24 hr. It was found that the time for half penetration⁸⁾ of methyl parathion was the shortest among four compounds while phenthoate gave the slowest penetrability (Table 1). With diazinon and fenitrothion, they were almost identical but showed lower penetration rate than that of methyl parathion. To confirm these evidences, the comparative evaluation was made with available equidoses applied. At 0.2 µg/insect (1 µg/g), the penetration of phenthoate was estimated to be 6 times as slower as that of methyl parathion. In addition, at 0.5–0.74 µg/insect (2.5–3.7 µg/g), methyl parathion exhibited the fastest penetrability. With diazinon and fenitrothion, there were no significant differences. At 0.5–0.74 µg/insect phenthoate was substantially similar to the ratio obtained in 1.0 µg/g dose as comparing with methyl parathion. In the case of fenitrothion and methyl parathion, it might be assumed that the rate of penetration was almost equal at 0.5–1.0 µg/g dose but somewhat different in higher dose applied with 2.5–2.9 µg/g.

Simultaneously, times when radioactivity of internal content and excretion reach 10(*t*₁₀) to 50 percent (*t*₅₀) of the dosages were calculated as shown in Tables 2 and 3. The values of *t*₁₀ and *t*₅₀ were shown marked differences, methyl parathion has smaller value than other insecticides both in the cases of internal and excretion.

For carbon dioxide gas trapping, the detectable radioactivity was too low to be estimated.

DISCUSSION

Most of the data published during the past two decades support the view point that contact insecticides, including organophosphorus compounds, penetrate into the cuticle of insects in order to reach the site of action via haemolymph.^{6–16)}

Fig. 1 Unpenetrated (○-○), internal (●-●), and excreted (□-□) amounts of topically applied methyl parathion and phenthoate in the 5th-instar armyworm, represents the total recovery.

Table 1 Half-time (t_{50})⁸⁾ in minutes for four OP compounds to penetrate into the cuticle of the 5th-instar armyworm.

OP-compound	t_{50} (min)		
	(doses)	LD ₁₀	LD ₅₀ LD ₉₅
Methyl parathion		7	10 15
Phenthoate		60	100 50
Diazinon		25	30 60
Fenitrothion		10	35 65

The nonlinear forms of the kinetics of penetration were obtained as shown in Figs. 1 and 2. Similar forms have been shown for the penetration of organophosphates into insect cuticle,^{11,13,16)} but not by Olson and

O'Brien⁸⁾ or Bull *et al.*¹⁷⁾ The resolution of this discrepancy probably lies in the time scales used. Where one examines the first hour of penetration, one may see apparently only monophasic fast penetration and miss the later slow phase.⁸⁾ In the case of malathion penetration into cockroaches, the phase change occurred unusually soon, 2 min after application. The first rapid phase could be an artifact cause by the solvent in which the solute is dissolved carrying in a portion of solute. In other words, it could be caused by fast penetration into a zone which saturated after which a slower penetration into other areas is found.¹⁶⁾ The feature of penetration as a resistant factor, with diazinon, malathion and methyl parathion, demonstrated that the penetration rate into the house fly cuticle is

Fig. 2 Unpenetrated (○—○), internal (●—●), and excreted (□—□) amounts of topically applied diazinon and fenitrothion in the 5th-instar armyworm, represents the total recovery.

not a major factor contributing to the resistance, based on the study concerning tolerance of the resistant flies to the toxicants which were either topically applied or injected.¹⁰⁾ It is not in agreement with the other report that the rate difference in cuticle penetration of diazinon and malathion may be a contributable factors in resistance, since a somewhat slower penetration of these two compounds was observed with the resistant flies than with the susceptible ones.¹⁸⁾ More intensive studies were performed on the site and mechanism of malathion penetration in the house fly, *Musca domestica* (L.) by auto radiographic technique. It was observed that the localized application of toxicants was an important factor for studying penetration.¹⁹⁾ This finding was quite similar to the early works on malathion in the desert locust, *Schistocerca gregaria* Forskal,²⁰⁾ and on DDT in the house fly.^{21,22)}

Regards to the comparative study in this paper, with four OP compounds, the rate of penetration could be evaluated by the t_{50} value. Presumably, the results, based on the equidoses, demonstrated the selectivity between methyl parathion and phenthoate which was canceled out by the evidence of anti-cholinesterase activity.¹⁾

The amount of internally existing compound and its excretion rate (Tables 2 and 3) probably supported the further consideration of its selective toxicity. The results shown in Table 2, seems to have a relationship with the fact that faster penetration compound such as methyl parathion can reach 50 percents of internal content in earlier time. (Fig. 1) Yet, methyl parathion is slowly excreted from the body (Table 3). It is necessary to bear a current idea that variable solubility of chemicals, both lipophilic and hydrophilic

Table 2 Estimated initial times for ten (t_{10}) and fifty (t_{50}) percents recovery of radio-labelled OP-compounds found internally.

OP-compound	(doses)	Time in minutes (t)					
		LD ₁₀		LD ₅₀		LD ₉₅	
		t_{10}	t_{50}	t_{10}	t_{50}	t_{10}	t_{50}
Methyl parathion		1.5	12.0	2.0	24.0	2.5	48.0
Phenthoate		5.0	*	8.0	240.0	24.0	*
Diazinon		6.0	190.0	7.0	138.0	11.0	*
Fenitrothion		3.0	27.0	14.0	*	60.0	*

* is unable to estimate during the course of the experiment.

Table 3 Estimated initial times for ten (t_{10}) and fifty (t_{50}) percents of recovery of radio-labelled OP-compounds found in the excretion.

OP-comound	(doses)	Time in minutes (t)					
		LD ₁₀		LD ₅₀		LD ₉₅	
		t_{10}	t_{50}	t_{10}	t_{50}	t_{10}	t_{50}
Methyl parathion		4	240-1440	3	240-1440	3	*
Phenthoate		20	260	110	>1440	16	>1440
Diazinon		12	>1440	36	140-1440	13	>1440
Fenitrothion		9	255	3	*	5	*

* is unable to estimate during the course of the experiment.

properties, may influence the penetrability. It may, therefore, be much complicated to have conclusion as to the differentiation mechanism of the selective toxicity since the information on degradation or activation of these compounds are still lacking.

Attention should be paid, however, on this results as it seems to be a rare case on which the rate of cuticular penetration acts as a promising factor to contribute to the mechanism of the selective toxicity.

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要 約

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Ⅱ, ^{14}C -標識数種有機リン殺虫剤の表皮透過性

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^{14}C -Methyl parathion, phenthoate, diazinon および fenitrothion のアワヨトウ幼虫における皮ふ透過性をしらべた。透過性は化合物により異なり, 50% 透過時間 (t_{50}) で比較すると methyl parathion > fenitrothion = diazinon > phenthoate の順に透過性が大きかった。methyl parathion と phenthoate との平均 t_{50} 値の比は大きく 6:1 であった。排泄速度については排泄物の放射能から比較すると methyl parathion が最も低く phenthoate が最も高かった。diazinon と fenitrothion は大きな差はなかった。phenthoate と他の3種殺虫剤の選択毒性の差異について皮ふ透過性が関係するようである。