Studies on Metabolism of Parathion in Parathion Susceptible and Resistant Larvae of the Rice Stem Borer¹

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INTRODUCTION

It is well known that a number of organophosphorus insecticides and chlorinated hydrocarbon compounds are metabolized by insects, and the type of metabolic pathways depends on the chemical structure of compounds and species of insects. For example, DDT is metabolized by some species of insects and the metabolism follows at least five different pathways (PERRY, 1960a, b). According to recent studies on insect resistance to organophosphorus insecticides, it is apparent that the resistance has an intimate relation with detoxication mechanism (BROWN, 1960; MARCH, 1959, 1960).

In Japan, parathion was tested at first on the pests of rice plants in 1951. It has shown excellent control against several species of rice pests, especially against larvae of the rice stem borer, *Chilo suppressalis* WALKER. So it has been widely used by rice growers since 1953. However, in summer of 1960, some rice growers at Zentsuji district of Kagawa Prefecture, in Western part of Japan, complained about the much lowered effect on larvae of rice stem borer. As a result, the area was inspected. And the authorities of Kagawa Prefecture reported that several other insecticides such as methyl parathion, EPN, dipterex and BHC were still effective on the larvae of rice stem borer in that district. Therefore, the situation looked that there were no failures in the spray timing, the application methods, and the amount of active ingredients used, but rather the rice stem borer built some resistance to

parathion.

According to our preliminary test, parathion resistant larvae in Kagawa (collected from rice field of Zentsuji and Sakaide Cities, Kagawa Prefecture; hereafter abbreviated R) showed over twice resistance in the first brood and over 5 times resistance in the second brood to parathion, in the LD₅₀ value by the topical application or in the LC₅₀ value by the spray method on pot, as compared with parathion susceptible larvae in Shizuoka (collected from rice fields of Shizuoka City, Shizuoka Prefecture; hereafter abbreviated S). The R larvae were also resistant to paraoxon, but there was no significant differences in susceptibility to parathion and paraoxon between S and R larvae in diapausing stage, because the resistant factors to parathion and paraoxon in the R larvae were not retained during diapause.

As shown Table 1, it is apparent that R larvae degraded paraoxon more rapidly than S larvae *in vivo* by enzyme assay using human plasma cholinesterase and *in vitro* by Warburg manometric determination. But there was no differences between S and R in (1) the penetration of parathion or paraoxon through cuticle, (2) the oxidation of parathion to paraoxon, (3) the activities of cholinesterase, A-esterase (aromatic- or aryl-esterase) and B-esterase (aliphatic- or ali-esterase), (4) sensibilities of above three enzymes to paraoxon (KOJIMA *et al.*, unpublished data).

The present paper gives further informations on the metabolic detoxication mechanism of parathion by R larvae as well as S larvae of rice stem borer ap-

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Table 1. Physiological and biochemical aspect of insect resistance to organophosphorus insecticides

Factor	House fly*	Rice stem borer**
Penetration of insecticide through the cuticle	—	—
Enzyme activity		
Cholinesterase	—	—
A-esterase	—	—
B-esterase	±	—
Sensitivity to inhibitor of enzymes		
Cholinesterase	—	—
A-esterase	—	—
B-esterase	—	—
Activation	—	—
Detoxication	+	+

— No significant difference.

± Lower activity in resistant strain than susceptible strain.

+ Higher activity in resistant strain than susceptible strain.

* Data published by March (1959).

** Our unpublished data.

plied with parathion topically.

EXPERIMENTAL

Insect: The experiment materials in diapausing and developing stages were obtained by the following way. Diapausing larvae were collected from rice straws which were reaped in Zentsuji City and Shizuoka City in December, 1960 respectively, and these larvae were kept in a laboratory without being exposed to any insecticides. We obtained continuously the developing larvae of the next generation.

Insecticide: Parathion was purified by a elution column chromatography using ether as a solvent.

Treatment, Extraction and Analysis: The larvae were treated topically with 1 μ l of 0.1% acetone solution of parathion on dorsal surface with a "Agla" micro-meter syringe attached to a capillary tube. In this experiment, sex discrimination was neglected. After the treatment,

ten treated larvae were kept in a petri dish under the room temperature of 25°C and 50% relative humidity without feeding. About one thousand larvae were used from each of S and R larvae. Mortality was observed at 5 or 24 hours after the treatment, and then larvae were homogenated in a mortar with 300 ml of acetone. The homogenate was filtered and acetone layer was separated. After acetone was evaporated to dryness on a steam bath, it was partitioned with a mixture of 100 ml of *n*-hexane and 100 ml of water. *n*-Hexane was discarded, and aqueous layer was partitioned with 30 ml of chloroform, and then aqueous layer was separated by centrifugation and was subjected to the following ion exchange chromatography.

Ion exchange chromatography technics were used according to the method described by PLAPP and CASIDA (1958a). Ion exchange resin was Dowex 1-X8 (100-200 mesh, Cl type). The eluting solvents were as follows:

- I. pH 2 to pH 1 HCl.
- II. pH 1 HCl plus methanol (1:3) to 1 N-HCl plus methanol (1:3)
- III. 1 N-HCl plus methanol (1:3) to concentrated HCl, water, and methanol (1:1:6).

Fractions (10 ml) were collected at an interval of about 3.5 minutes. The aliquots (1-5 ml) were analyzed for total phosphorus by ALLEN's method modified by NAKAMURA (1950).

Components contained in each fraction were identified by chromatography comparing with chromatograph of known samples. Known samples were obtained as follows: Parathion and paraoxon were hydrolyzed with alcoholic potassium hydroxide (PLAPP and CASIDA, 1958b, c), and chromatographed by ion exchange column chromatography and then extracted from the elute with amyl alcohol or chloroform (PLAPP and CASIDA, 1958a), and identified by infrared absorption spectra. The elution chromatograms and infrared spectra of the authentic samples are given in Figs. 1 and 2.

Fig. 1. Ion exchange separation on Dowex 1 of the derivatives of parathion and paraoxon. Solvent systems I, II and III are described in the text in detail.

Fig. 2. Infrared spectra.

RESULTS AND DISCUSSION

Table 2 shows the results of the water soluble metabolites of S and R larvae of the rice stem borer applied with parathion topically.

As shown in Table 2, *O,O*-diethyl phosphorothioic acid, *O,O*-diethyl phosphoric acid, *des*-ethyl parathion, *des*-ethyl paraoxon and monomethyl phosphoric acid (or phosphoric acid) were found in both S and R larvae, but there were differences in quantities of each metabolite.

In both diapausing and developing stages, approximately same amount of *des*-ethyl parathion, *O,O*-diethyl phosphorothioic acid and monoethyl phosphoric acid (or phosphoric acid) were found. However, *O,O*-diethyl phosphoric acid was the predominant metabolite in the S larvae while *des*-ethyl paraoxon was predominant in the R larvae. The percentage of hydrolytic metabolite formed by cleavage of the aryl-phosphate bond of paraoxon showed no difference between the diapausing and developing stages among the S and R larvae, but alkyl-phosphate hydrolysis tends to increase in developing stage rather than diapausing stage, that is, the quantity of *des*-ethyl paraoxon was elevated from 25% in 24 hours to 41% in 5 hours in the S larvae and from 58% to 77% in the R larvae.

PLAPP and CASIDA (1958c) demonstrated that certain dialkyl aryl phosphorothioates such as parathion, methyl parathion, diazinon, Dow ET-57, chlorthion and dicapthion were hydrolyzed at the alkyl-phosphate and the aryl-phosphate bonds. According to the studies by several workers on the hydrolysis of compounds of the type having the general formula of $(\text{RO})_2\text{P}(\text{Y})\text{OX}$ ($\text{Y}=\text{O}, \text{S}$), the common $\text{P}-\text{O}-\text{X}$ bond is cleaved by mammals, insects and plants. PLAPP and CASIDA (1958c) also suggested that the alkyl-phosphate hydrolysis in mammals seemed to be an alternate mechanism for detoxication when the toxicant can not be detoxified through hydrolysis at aryl-phosphate bond, and that the detoxication by the cleavage of alkyl-phosphate bond was of minor importance in insects as compared with mammals. Nevertheless, it is noted that the major metabolic detoxication process in the R larvae was primarily due to the cleavage of alkyl-phosphate bond of paraoxon.

As shown above, organophosphorus insecticides were generally metabolized rapidly by insects. However, a little information has been obtained about the enzymes which are responsible for the

Table 2. Hydrolytic metabolites of the parathion susceptible and

Stage	Larvae	Average body weight (mg)	Number of larvae used	Dosage (μ g/larva)	Mortality (%)
Diapausing ^a	Susceptible	84.9	1077	1	37.7
	Resistant	57.6	934	1	33.5
Developing ^b	Susceptible	12.3	584	1	100
	Resistant	13.8	970	1	93.7

^a Results at 24 hours after treatment, tested in February, 1961.

^b Results at 5 hours after treatment, tested in July, 1961.

metabolic detoxication. From the several studies *in vivo*, the metabolism of paraoxon in insects and mammals follows at least three different metabolic detoxication pathways, as shown in Fig. 3.

At present, the cleavage sites of almost of all organophosphorus compounds by enzymes *in vitro* have not been confirmed except for DFP by DFPase (MAZUR, 1946; MOUNTER and DIEN, 1954), tabun by tabunase (AUGUSTINSSON and HEIMBÜRGER, 1954), and sarin by sarinase (HOSKIN, 1956), in mammals. ALDRIDGE (1953a, b) has reported an enzyme which hydrolyzes paraoxon to *O*, *O*-diethyl phosphoric acid and *p*-nitrophenol, in mammalian sera and other tissues. He concluded that paraoxon-hydrolyzing enzyme in serum identical with A-esterase which was known to hydrolyze *p*-nitrophenyl acetate but not to be inhibited by paraoxon. MOUNTER and WHITTAKER (1953) also suggested that serum aryl-esterase was identical with paraoxon-hydrolyzing A-esterase. Later, MOUNTER (1954) and AUGUSTINSSON (1953) confirmed the observation of ALDRIDGE (1953a, b) that one enzyme in mammalian sera hydrolyzed *p*-nitrophenyl acetate and paraoxon. And MOUNTER (1954) cleared that TEPP and DFP were also hydrolyzed by A-esterase. Recently, however, MAIN (1960a, b) purified an enzyme that hydrolyzed paraoxon in sheep serum, and he showed that paraoxon-hydrolyzing enzyme was different

from A-esterase. Thus he called the enzyme paraoxonase to distinguish from other A-type-esterase. ERDÖS and BOGGS (1961) also indicated that in addition to A-esterase, mammalian serum albumin hydrolyzed paraoxon at an appreciable rate.

On the other hand, METCALF *et al.* (1956) presented that A-esterase in insect tissues hydrolyzed papaoxon and parathion, liberating *p*-nitrophenol. Contrary to the result of METCALF *et al.*, LORD and SOLLY (1956) reported that A-esterase preparation of house flies did not hydrolyzed paraoxon. KRUEGER and CASIDÀ (1961) demonstrated that the enzymes hydrolyzing dialkyl phosphoric anhydrides in house flies could be at least partially separated from the cholinesterase, A-esterase and B-esterase, and more than one esterase of the house fly acted on the phosphates. We found that the activities of cholinesterase, A-esterase and B-esterase in the larvae of the rice stem borer were not related to the hydrolyzing activity of paraoxon and parathion (KOJIMA *et al.*, unpublished data).

Although the enzyme activity, which is responsible for cleavage of the alkyl-phosphate bond of paraoxon, is not demonstrated yet for any animals *in vitro*, the enzymatic hydrolysis of paraoxon by the R larvae which we observed manometrically is considered to be attributed to a new enzyme which cleaves alkyl-phosphate

resistant larvae of the rice stem borer applied with parathion topically

Per cent of hydrolytic metabolites				
$\begin{array}{c} \text{C}_2\text{H}_5\text{O} \\ \text{C}_2\text{H}_5\text{O} \end{array} \text{P}(\text{S})\text{OH}$	$\begin{array}{c} \text{C}_2\text{H}_5\text{O} \\ \text{HO} \end{array} \text{P}(\text{S})\text{O}-\text{C}_6\text{H}_4-\text{NO}_2$	$\begin{array}{c} \text{C}_2\text{H}_5\text{O} \\ \text{C}_2\text{H}_5\text{O} \end{array} \text{P}(\text{O})\text{OH}$	$\begin{array}{c} \text{C}_2\text{H}_5\text{O} \\ \text{HO} \end{array} \text{P}(\text{O})\text{O}-\text{C}_6\text{H}_4-\text{NO}_2$	$\begin{array}{c} \text{C}_2\text{H}_5\text{O} \\ \text{OH} \end{array} \text{P}(\text{O})\text{OH}$ or $\begin{array}{c} \text{HO} \\ \text{HO} \end{array} \text{P}(\text{O})\text{OH}$
1.8 2.8	8.7 10.8	58.1 21.4	25.0 58.1	6.4 6.9
— 4.1	— —	59.4 19.1	40.6 76.8	— —

Fig. 3. Possible pathways and responsible enzymes in paraoxon metabolism of mammals and insects.

bond of paraoxon. We have been working on purification and characterization of the enzyme in the S and R larvae of rice stem borer. In case of the rice stem borer, it seems that the development of parathion resistance is originated in change of the enzyme responsible for the detoxica-

tion of paraoxon. According to O'BRIEN's hypothesis (1959), in which "weak link" is presented as a responsible factor for the selective toxicity of organophosphorus insecticides, it seems that the alkyl-phosphate bond of paraoxon is related to the mechanism of parathion resistance in rice

stem borer.

Moreover, in the ruminant, it has been reported that the reduction of parathion and paraoxon is a major metabolic detoxication pathway, by DAHM *et al.* (1950), PANKASKIE *et al.* (1952) and AHMED *et al.* (1958). On the other hand, it has been shown that the enzyme system which reduces aromatic nitro compounds to amino compounds exists in organisms, by WILLIAMS (1947), BUEDING and JOLIFFE (1946), WESTFALL (1943), SMITH and WORRELL (1950), EGAMI *et al.* (1951) and PARKER (1952), but this reduction is prevented by cyanides (EGAMI *et al.*, 1951; PARKER, 1952). However, it is not yet proved *in vitro* and *in vivo* whether this enzyme system is responsible for the reduction of parathion and paraoxon in animals.

SUMMARY

The mechanism of metabolic detoxication of parathion in the rice stem borer was examined in relation to parathion resistance.

From each of susceptible and resistant larvae, *O,O*-diethyl phosphorothioic acid, *O,O*-diethyl phosphoric acid, *des*-ethyl parathion, *des*-ethyl paraoxon and monoethyl phosphoric acid (or phosphoric acid) were detected, but there were differences in quantities of each metabolite. But there were almost equal amount of *des*-ethyl parathion, *O,O*-diethyl phosphorothioic acid and monoethyl phosphoric acid (or phosphoric acid) in the susceptible and resistant larvae. However, *O,O*-diethyl phosphoric acid was the predominant metabolite in the susceptible larvae while *des*-ethyl paraoxon was predominant in the resistant larvae. It seems that alkyl-phosphate bond of paraoxon is a "weak link" which is responsible for selective toxicity between parathion susceptible and resistant larvae of the rice stem borer.

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摘 要

パラチオン感受性および抵抗性ニカメイガ幼虫におけるパラチオンの代謝

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最近, MARCH や BROWN は有機燐殺虫剤に対して抵抗性のイエバエの系統が有機塩素殺虫剤に対して抵抗性のものと種々の点で著しく異なることを報告し, イエバエの有機燐殺虫剤感受性, 抵抗性の両系統のいずれの系統においてもそのコリンエステラーゼ活性度やコリンエステラーゼ阻害剤に対する感受性には有意な差異がなく, また体内における投与薬剤の活性化作用も両系統間に差がないこと, そして抵抗性はむしろ解毒機構に関係するところがで, 体内の作用点に有機燐剤が薬効発現の臨界濃度に蓄積される速さに対し, その殺虫剤が体内で分解されて無毒物質に変化する速さとの関係が抵抗性機構に大きく関与することを指摘した。

ところで, 有機燐殺虫剤および有機塩素殺虫剤の代謝的分解は薬剤の化学構造や生物の種類によって異なるこ

とが知られている。我々はニカメイガ幼虫によるパラチオンの代謝を明らかにすると共に, パラチオンに対する抵抗性と解毒機構との関係を解明するため, パラチオン感受性および抵抗性ニカメイガ幼虫にパラチオンを局所施用し, その分解物をイオン交換樹脂クロマトグラフによって分離した。その結果, 体内における分解解毒は感受性および抵抗性幼虫共にパラオクソンで行われることがわかった。しかし, 感受性幼虫ではパラオクソンの aryl-phosphate 結合の分解によって代謝されるが, 抵抗性幼虫は昆虫では重要な代謝経路でないといわれている alkyl-phosphate 結合の分解によって代謝されることを示した。さらにパラオクソンの分解を触媒する酵素について論じた。