High Paraoxon-Hydrolyzing Activity in Organophosphorous Insecticide-Resistant Mosquitoes

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We found a strong paraoxon-hydrolyzing activity (23.4±8.50 nmol/h/individual and 137±86.2 nmol/h/mg protein) in the crude extract from larvae of *Culex tritaeniorhynchus* Toyama 89, which is markedly resistant to organophosphorous insecticides. The activity was higher than those from *Cx. tritaeniorhynchus re-e-ae* (0.175±0.0336 and 1.83±0.651), *Anopheles omorii* (0.112±0.0301 and 1.86±0.746) and *An. stephensi* (0.0651±0.0713 and 0.789±0.910), which are susceptible to organophosphorous insecticides. These facts suggest that the high paraoxon-hydrolyzing activity plays a role in the development of organophosphorous resistance in *Cx. tritaeniorhynchus*. The enzyme preparation obtained from Toyama 89 showed higher activity in the alkaline pH range and its K_m values to paraoxon were 0.67 mM in larvae and 0.50 mM in adults. A calcium ion was strictly required for the hydrolysis of paraoxon. Fenitroxon was also hydrolyzed, in addition to paraoxon. However, it did not degradate parathion and fenitrothion at all. Dichlorvos and phenyl acetate competitively inhibited the enzyme. The phenyl acetate-hydrolyzing activity in the preparation of Toyama 89 was significantly ($p<0.01$) lower than those in susceptible strains, and was irreversibly inhibited by paraoxon. Therefore, the paraoxon-hydrolyzing activity belongs to the class of organophosphate compound hydrolases; it must be thus distinguished from bacterial phosphotriesterase.

Keywords *Culex tritaeniorhynchus*; organophosphorous insecticide-resistance; paraoxon; paraoxonase; arylesterase; phenyl acetate

Introduction

Several mechanisms concerning the development of pesticide-resistance in insects have been reported as follows[1]; an alteration of acetylcholinesterase (AChE: the enzyme targeted by insecticides), an activation and induction of esterase, gene amplification of esterase and/or alteration of mixed-function oxidase *etc.* The *Culex tritaeniorhynchus* Toyama 89 strain was extremely resistant to both organophosphorous and carbamate insecticides.[2] Lethal concentration 50% (LC_{50}) values of dichlorvos, parathion or fenitrothion against the mosquito were 5875-, 2712- or 2541-fold higher than those against the *Cx. tritaeniorhynchus re-e-ae* strain, respectively. The great difference in these LC_{50} values between Toyama 89 and *re-e-ae* has never been reported in any insect of medical importance, and therefore it is very interesting to clarify the mechanism of its development of resistance to organophosphorous insecticides. Acetylcholinesterase from the Toyama strain has a markedly poor affinity to organophosphorous compounds,[3] and is altered from a true- to a pseudo-type.[4] Especially, the weak inhibition of AChE from Toyama with organophosphorous compounds roughly corresponds to an increase of the concentrations for lethality. We therefore suggest that in the Toyama strain, the AChE plays the major role in the development of the acquired resistance to organophosphorous insecticides. Moreover, we[2] and Takahashi and Yasutomi[5] showed that the naphthylacetate-hydrolyzing activity from the resistant strain was higher as compared to that from the susceptible strain, and that a zymogram for the naphthylacetate-hydrolyzing activity in Toyama differed from that in *re-e-ae*. Matsumura and Hogendijk[6] first reported the presence of parathion-hydrolyzing activity *in vitro* from houseflies, but that partially purified enzyme hydrolyzed little paraoxon. Welling *et al.*,[7] and Oppenoorth and Voerman[8] detected a low paraoxon-hydrolyzing activity from parathion-resistant houseflies and aphides, respectively. Kao *et al.*[9] reported that the purified esterase from insecticide-resistant houseflies had low paraoxon-hydrolyzing activity. Konno and Shishido[10] reported that the metabolites produced *in vivo* by hydrolysis of fenitroxon in an organophosphorus-resistant strain of rice stem borers were considerably detectable. In this paper, therefore, we measured a phosphotriester-hydrolyzing activity in a crude extract of the larvae, as well as the adults of *Cx. tritaeniorhynchus* Toyama 89 and *re-e-ae* strain.

Materials and Methods

Mosquitoes *Cx. tritaeniorhynchus* Toyama 89, *re-e-ae*, *Anopheles omorii* and *An. stephensi* were reared in our insectarium at 23±1 °C and a 16:8 (L:D) photoperiod. Lethal concentration 50% of various insecticides on these mosquitoes was reported previously[2] in detail. Larvae were fed on a powdered mixture containing insect food (Oriental Yeast Co., Tokyo, Japan) and dry beer yeast (Asahi Brewery Ltd., Tokyo, Japan) (ratio 1:1). Larvae (final stage) and adult mosquitoes (2 d after emergence) were stored at −30 °C until use.

Chemicals Paraoxon was purchased from Aldrich Chemical Co. Inc., WI, U.S.A., and parathion, fenitrothion, *O,O*-dimethyl-*O*-(2,2-dichlorovinyl)phosphate(dichlorvos) and phenyl acetate were purchased from Wako Pure Chemical Industries Ltd., Osaka, Japan. Fenitroxon was kindly supplied by Sumitomo Chemical Co. Ltd., Takarazuka, Japan. Phenylphosphorodiamidate was synthesized in our laboratory.

Preparation of Crude Extract from Mosquitoes In the case of Toyama 89, five larvae or ten adults were homogenized in 2 ml of 106 mM Tris HCl buffer, pH 8.5, containing 2.1 M sodium chloride, 50 mM calcium chloride and 0.1% Triton X-100 (buffer A) in a glass homogenizer with a Teflon pestle in an ice-bath. The homogenates were centrifuged at 20000×*g* for 30 min and the supernatants were used as a crude extract. In the cases of *re-e-ae*, *An. omorii* and *An. stephensi*, one hundred larvae or twenty adults were used to prepare a crude extract according to the method described above. In the experiment concerning the effect of salts, one hundred larvae of Toyama 89 were homogenized in 10 ml of 10 mM Tris HCl buffer, pH 8.5, containing 0.1% Triton X-100. The supernatants after the centrifugation at 20000×*g* for 30 min were used as a crude extract.

Separation of Hydrolyzed Products with Thin Layer Chromatography (TLC) Fifty larvae were homogenized in 10 ml of buffer A adjusted pH at 7.5 to prepare a crude extract. Paraoxon was incubated with 1.0 ml of the crude extract at 30 °C for the indicated period of time as shown in Table II. Eight hundred μl of the reaction mixture was extracted repeatedly three times with the same volume of ethyl acetate, then the organic layer was evaporated at 45 °C to dryness. The sediment was dissolved in 50 μl of acetone. Five μl of the sample was applied to a Silica gel F_{254} plate and was developed with the solvent system, benzene, ethyl acetate and hexane (ratio 2:2:1) at room temperature until a solvent front was

developed to 7 cm. Ultraviolet (UV) light was used for the detection of the products, and 2% sodium carbonate was sprayed for the coloring of 4-nitrophenol and 3-methyl-4-nitrophenol.

Measurement of Hydrolysis of Paraoxon and Fenitroxon Hydrolysis of paraoxon was measured by the method of Furlong *et al.*[11] with minor modification. Eight hundred μl of the crude extract was incubated at 30 °C for 1 h with 200 μl of 6 mM paraoxon in a 50 mM Tris HCl buffer, pH 8.5, containing 5% acetone. The absorbance at 405 nm was measured after centrifugation at 15000 × *g* for 5 min. The amount of hydrolyzed paraoxon or fenitroxon was calculated based on the molecular extinction coefficient of 4-nitrophenol (18050) or 3-methyl-4-nitrophenol (15100), respectively.

Hydrolysis of phenyl acetate was measured by the method of Lorenz *et al.*[12] with minor modification. One hundred μl of 18.8 mM phenyl acetate was added into 500 μl of buffer A preincubated at 30 °C for 5 min. The crude extract was added within 1 min after adding the substrate. After incubation for a further 10 min, 160 μl of 0.5 M ethylenediaminetetraacetic acid (EDTA) was added to terminate the reaction, then 60 μl of 8.9 mM aminoantipyrine and 30 μl of 213 mM potassium ferricyanide were added into the reaction mixture to measure the amount of produced phenol. The amount of hydrolyzed phenyl acetate was calculated based on the molecular coefficient of (3-benzoquinone-monoimino)-phenazone (8770) at 550 nm in buffer A. The two blanks for hydrolysis of paraoxon, fenitroxon and phenyl acetate were performed as follows: blank 1 omitting the substrate, and blank 2 omitting the crude extract.

The protein of the crude extract was determined with a BCA Protein Assay Reagent (Pierce Chemical Co., IL, U.S.A.) using bovine serum albumin as a standard.

Results

Hydrolysis of Paraoxon by Crude Extracts from Mosquitoes Paraoxon-hydrolyzing activity is found in crude preparations from larvae and adults of *Cx. tritaeniorhynchus* extracted with a buffer containing high concentrations of calcium chloride and sodium chloride. Their activity was extremely elevated as compared with the paraoxon-hydrolyzing activities from houseflies[7] and aphides.[8] The great difference among species of insects may be due to the presence of calcium chloride in a buffer used for homogenizing insects, since the buffer without calcium chloride could extract only a little activity from Toyama 89, and they[7,8] did not use a calcium-containing buffer for the preparation of insect extracts.

As shown in Fig. 1 and Table I, the activities per individual and those per mg protein from Toyama 89 larvae were significantly ($p<0.01$) higher (130- or 75-fold) than those from *re-e-ae* larvae, respectively. The values from Toyama 89 larvae were significantly ($p<0.01$) higher than those from adults of the same strain as shown in Figs. 1 and 2. The average activity per individual from female adults of Toyama 89 was significantly ($p<0.01$) 2.4-fold higher than from the males. Total protein amounts from the whole body of male mosquitoes were significantly ($p<0.01$) less than those of female ones, but there is a significant difference ($p<0.05$) between female and male Toyama 89 in the activity per mg protein. These results show that female mosquitoes possess higher paraoxon-hydrolyzing activity than males. However, no sexual difference in the activity was found in *re-e-ae*, and the activities from adults in a susceptible strain were significantly ($p<0.01$) higher than those from the larvae. These observations in the susceptible strain were inverse to those in the resistant strain.

An. omorii and *An. stephensi* were susceptible to organophosphorous insecticides as well as *Cx. tritaeniorhynchus re-e-ae*. The paraoxon-hydrolyzing activities of these two *Anopheles* and of *re-e-ae* resembled each other in both adults

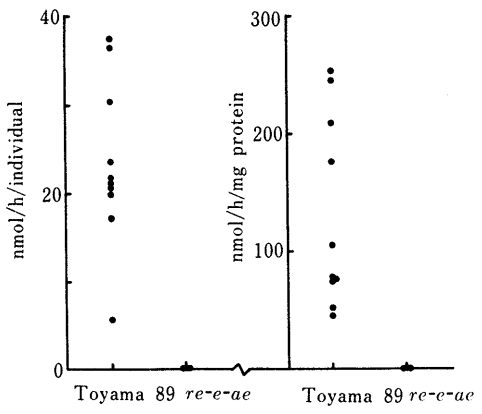

Fig. 1. Hydrolysis of Paraoxon with Larvae of *Cx. tritaeniorhynchus*

One point represents the activity from 5 larvae in Toyama 89 and from 100 larvae in *re-e-ae*.

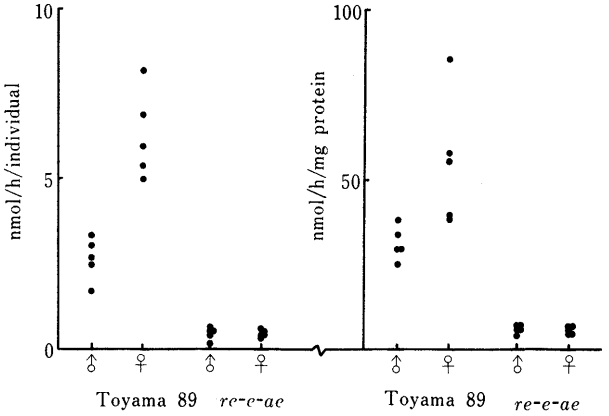

Fig. 2. Hydrolysis of Paraoxon with Adults of *Cx. tritaeniorhynchus*

One point represents the activity from 10 mosquitoes in Toyama 89 and 20 mosquitoes in *re-e-ae*.

TABLE I. Hydrolysis of Paraoxon with Some Mosquitoes

	Produced 4-nitrophenol (nmol/h/individual)				Produced 4-nitrophenol (nmol/h/mg protein)			
	Cx. tritaeniorhynchus		*An. omorii*	*An. stephensi*	*Cx. tritaeniorhynchus*		*An. omorii*	*An. stephensi*
	Toyama 89	*re-e-ae*			Toyama 89	*re-e-ae*		
Larvae	23.4 ±8.50[a]	0.175±0.0336[c]	0.112±0.0301[c]	0.0651±0.0713[c]	137 ±86.2[a]	1.83±0.651[c]	1.86±0.746[c]	0.789±0.910[c]
Adults								
Male	2.65±0.62[b]	0.481±0.131[c]	0.105±0.067[b]	0.240 ±0.153[b]	31.6± 4.85[b]	6.35±1.19[c]	1.03±0.702[b]	2.33 ±1.570[b]
Female	6.30±1.28[b]	0.492±0.080[c]	0.598±0.081[b]	0.418 ±0.197[b]	55.8±19.3[b]	6.23±0.932[c]	1.48±0.268[b]	0.745±0.357[b]

a) Average±S.D. of 10 experiments. *b*) Average±S.D. of 5 experiments. *c*) Average±S.D. of 3 experiments.

and larvae as shown in Table I, and they were significantly ($p<0.01$) lower than those of Toyama 89.

Hydrolysis of Fenitroxon by Crude Extract from Mosquitoes Fenitroxon was also hydrolyzed with the crude extract from both larvae and adults resistant to organophosphorous insecticides. The results shown in Table II were obtained by measuring hydrolysis at pH 7.5, because spontaneous hydrolysis of the substrates occurred at pH 8.5 during prolonged incubation time. The fenitroxon-hydrolyzing activity level in larvae or adults was 72% of the paraoxon-hydrolyzing activity level at the same concentration of substrates. However, parathion and fenitrothion were not hydrolyzed under the assay condition at all. Even though the concentrations of these thiono-compounds and the incubation pH were elevated to 0.5 mM and 8.5, respectively, no hydrolysis was detected. Strain *re-e-ae* also did not exhibit any parathion- or fenitrothion-hydrolyzing activity.

TABLE II. Hydrolysis of Oxo- and Thiono-Organophosphorous Insecticides by *Cx. tritaeniorhynchus*

	mM	Hydrolysis			
		nmol/4 h/larva		nmol/5 d/adult	
		Toyama 89	*re-e-ae*	Toyama 89	*re-e-ae*
Paraoxon	1	26.8	0.153	59.0	1.46
	0.1	4.15	<0.102	3.56	<0.0966
Parathion	0.1	<0.102	<0.102	<0.0966	<0.0966
Fenitroxon	0.1	3.00	<0.125	2.56	<0.115
Fenitrothion	0.1	<0.125	<0.125	<0.115	<0.115

Organophosphorous compounds (10 μl) were incubated with the crude extract (1.0 ml) for the indicated time. The values were calculated based on the molecular extinction coefficients of 4-nitrophenol and 3-methyl-4-nitrophenol at pH 7.5.

Identification of Products of Hydrolysis by TLC The reaction mixture (Table II) was extracted with ethyl acetate and the concentrated organic layer was then analyzed by TLC. The results obtained by using larvae extract were shown in Fig. 3. Three distinct quenching spots were observed: at the origin, at a substrate position and at an upper position in each lane under UV light. The third quenching spot corresponded to authentic 4-nitrophenol or 3-methyl-4-nitrophenol, and these spots were colored in alkaline. The third spot was therefore identified to be 4-nitrophenol or 3-methyl-4-nitrophenol. By incubating either paraoxon or fenitroxon with the crude extract from Toyama 89, the density of the third spot increased

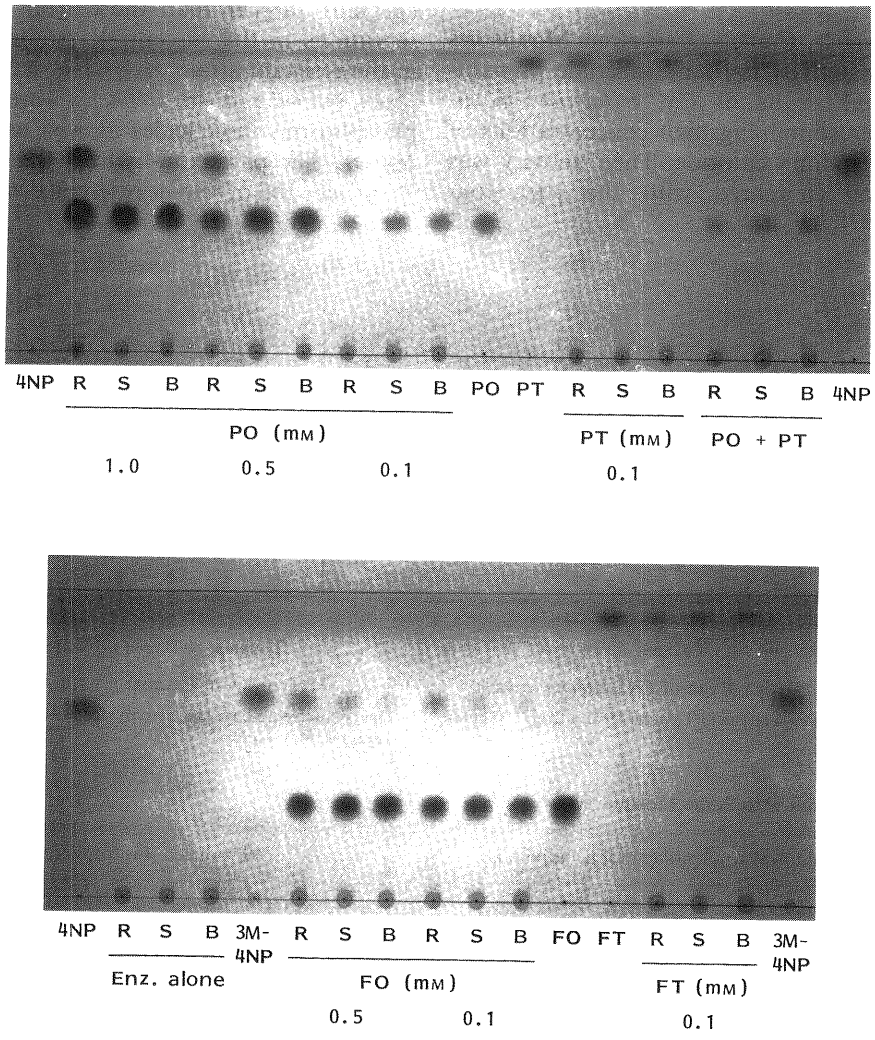

Fig. 3. Detection of 4-Nitrophenol (4NP) and 3-Methyl-4-Nitrophenol (3M4NP) Produced from Paraoxon (PO), Parathion (PT), Fenitroxon (FO) and Fenitrothion (FT)

Details of the method are described in the legend to Table II. R, S and B represent resistant strain extract (Toyama 89 larvae), susceptible strain extract (*re-e-ae* larvae), and a buffer as a control, respectively.

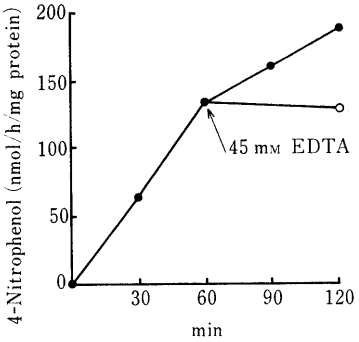

Fig. 4. Time Course of Paraoxon Hydrolysis

The crude extract of Toyama 89 larvae was prepared and the activity was measured at the indicated time according to Materials and Methods. After 60 min incubation, 100 μl of 500 mM EDTA in 50 mM Tris HCl buffer, pH 8.5, was added to the reaction mixture. The open circle represents the activity after a further 60 min incubation with EDTA.

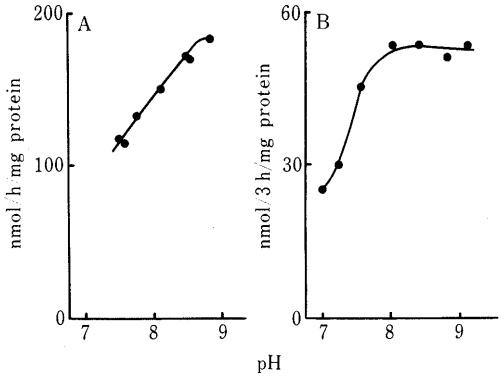

Fig. 5. Paraoxon Hydrolysis at Various pHs by Crude Extract of Toyama 89

A, larvae; B, adults.

The crude extract (170 μl) was incubated with buffer A (830 μl) at various pHs and 1.45 mM paraoxon. The amount of the hydrolyzed paraoxon was calculated based on the molecular extinction coefficient of 4-nitrophenol corrected at the corresponding pHs.

TABLE III. Effect of Divalent Ions on Paraoxon Hydrolysis

Ion	Produced 4-nitrophenol (nmol/h/mg protein)					
	With 2 M NaCl			Without 2 M NaCl		
	1 mM	10 mM	50 mM	1 mM	10 mM	50 mM
Mg^{2+}	9.20	10.9	12.6	13.2	13.5	23.9
Ca^{2+}	17.5	45.2	38.3	39.7	49.6	48.4
Mn^{2+}	7.67	18.9	31.5	19.7	20.4	35.6
Co^{2+}	7.11	16.4	24.5	14.5	14.9	9.29
Ni^{2+}	7.94	7.43	7.23	13.6	20.4	12.1
Zn^{2+}	9.96	30.1	3.83	11.8	18.9	6.68
None		6.01			11.8	

Crude extract (200 μl) from Toyama 89 larvae were incubated with 600 μl of the salt solutions at final concentrations described in the table.

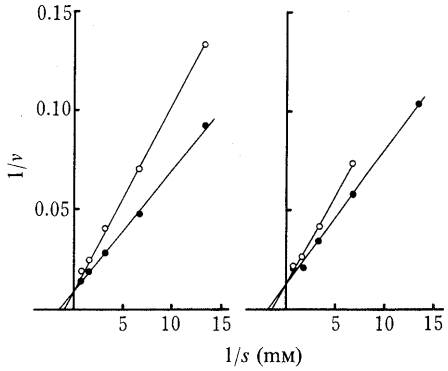

Fig. 6. Competitive Inhibition of Paraoxon Hydrolysis by Dichlorvos and Phenyl Acetate

The crude extract was incubated with 10 μM dichlorvos in the left figure or with 100 μM phenyl acetate in the right figure. The open and closed circles represent the activities in the presence and the absence of test compounds, respectively.

TABLE IV. Effect of Ca^{2+} on Paraoxon Hydrolysis

	Produced 4-nitrophenol (nmol/h/mg protein)
None	6.96
18 mM EDTA	1.21
Ca^{2+} 0.1 mM	12.7
1	35.4
10	89.2
50	114
90	91.0

Six larvae of Toyama 89 were extracted with 1.0 ml of the buffer A without calcium chloride.

dependently on the concentration of the substrate. In this condition, no other spot colored in alkaline was detected except spots of 4-nitrophenol, 3-methyl-4-nitrophenol, paraoxon and fenitroxon, with the result that no derivatives of 4-nitrophenol or 3-methyl-4-nitrophenol were produced. At the position of 3-aminophenol, which was produced by the reduction of 4-nitrophenol, no quenching spot was observed under UV light. These results, obtained from TLC analysis, evidently proved that paraoxon and fenitroxon were hydrolyzed to 4-nitrophenol and 3-methyl-4-nitrophenol, respectively and also that the enzyme(s) could not hydrolyze parathion and fenitrothion at all.

General Properties of Paraoxon-Hydrolyzing Activity When paraoxon was incubated with the crude extract obtained from the larvae of the resistant strain, 4-nitrophenol was progressively produced with time until 1 h, then the rate of production of 4-nitrophenol decreased (Fig. 4). 4-Nitrophenol increased for 1 h of incubation to 20 μM in a final concentration, but the product inhibition at the concentration was not observed. Therefore, the inactivation gradually occurred during long-term incubation at 30 °C. Inactivation was also observed during preincubation for 15 min at 30 °C (88% of the original activity) and at 45 °C (complete loss of activity).

Hydrolysis of paraoxon was terminated by the addition of EDTA (Fig. 4) because little activity was exhibited in the presence of EDTA. Table III showed the effects of sodium chloride and divalent cations on paraoxon-hydrolyzing activity. The highest activity was observed in the presence of Ca^{2+} without sodium chloride. As shown in Table IV, 50 mM Ca^{2+} was required for the maximal activity. Zn^{2+}, Mn^{2+} and Co^{2+} at 10 mM activated the paraoxon hydrolysis as well as Ca^{2+} with sodium chloride. Higher activities with sodium chloride than without the salt were observed with 50 mM Co^{2+} as well as 10 mM Zn^{2+}, but a contrary effect of sodium chloride was shown with 1 mM Ca^{2+}, 10 mM Ni^{2+} and 50 mM Mg^{2+}. We have no explanation for the various effects of sodi-

TABLE V. Hydrolysis of Phenyl Acetate by Crude Extract from Mosquitoes

	Hydrolysis of phenyl acetate	
	μmol/h/individual	μmol/h/mg protein
Cx. tritaeniorhynchus		
Toyama 89	0.368±0.0536	6.39±0.192
re-e-ae	0.666±0.118	6.23±1.46
An. omorii	2.20 ±0.188	8.69±1.24
An. stephensi	5.79 ±1.07	28.3 ±3.60

Twenty larvae were extracted with 2 ml of the buffer A, and the preparation method of the crude extract was described in Materials and Methods. The value is the average±S.D. of 5 experiments.

um chloride on the activation by divalent ions.

The effect of pH on paraoxon hydrolysis as shown in Fig. 5 indicated that the maximal activity in larvae was at around pH 9.0 but that in adults, maximal activity ranged from pH 8 to 9. The K_m values of paraoxon were 0.67 mM in larvae and 0.50 mM in adults. Dichlorvos (10 μM) and phenyl acetate (0.1 mM) competitively inhibited the paraoxon-hydrolyzing activity as shown in Fig. 6. However, 0.1 mM each of parathion, phenylphosphorodiamidate and *O*-cumenyl methylcarbamate did not inhibit this activity at all. One % 1-butanol, 1-propanol or dioxan in the assay mixture for the measurement of paraoxon-hydrolyzing activity decreased it to 46, 81 or 73% of the activity measured in 1% acetone, in that order. The activity observed in the presence of 1% methanol, ethanol or dimethyl sulfoxide was the same value as that in 1% acetone.

Hydrolysis of Phenyl Acetate by Crude Extract of Mosquitoes Phenyl acetate was hydrolyzed with four species of larvae under the same condition as for paraoxon-hydrolyzing activity, as shown in Table V. The highest activity was detected with *An. stephensi*, whose activities per individual and mg protein were 39000- and 89000-fold higher than those of its paraoxon hydrolysis, respectively. The activity per individual from Toyama 89 larvae was significantly ($p<0.01$) lower than those from three species of larvae which were susceptible to organophosphorous insecticides. The phenyl acetate-hydrolyzing activity from Toyama 89 larvae was almost completely inhibited by paraoxon at 1.2 mM, which was the same concentration as a substrate concentration for the measurement of paraoxon-hydrolyzing activity. The inhibited preparation was gelfiltrated though Sephadex G-25 to remove free-paraoxon, but phenyl acetate-hydrolyzing activity was not restored any more even after 1 h of incubation. After this procedure, however, the paraoxon-hydrolyzing activity was kept at half of the original activity, independently, whether the crude extract was preincubated with paraoxon or not. These results therefore suggest that the paraoxon-hydrolyzing enzyme should be distinguished from the phenyl acetate-hydrolyzing enzyme.

Discussion

Cx. tritaeniorhynchus Toyama 89 was a unique strain, having the highest resistance to organophosphorus insecticides among insects of medical importance as reported previously.[2,13] Our results indicated that the larvae of Toyama 89 had a higher phosphotriesterase activity, which was measured by using paraoxon and fenitroxon as substrates. This activity was at least 130-fold higher than that of organophosphate-susceptible strains such as *re-e-ae*, *An. omorii* and *An. stephensi*. A small correlation between the degree of resistance and paraoxon-hydrolyzing activity in houseflies[7] and aphides[8] might be due to a low degree of resistance in these insects. On the other hand, LC_{50} of paraoxon to *Cx. tritaeniorhynchus* was 4.25 ppm, which was similar to that of parathion (5.46 ppm). This similarity agreed with LD_{50} values of fenitrothion and fenitroxon to rice stem borers[14] and planthoppers.[15] Therefore, our results suggested that a rate of conversion of parathion to paraoxon did not participate in the development of resistance to organophosphorous compounds.

The crude extract from Toyama 89 did not hydrolyze parathion and fenitrothion, although Matsumura and Hogendijk[6] reported that the partially purified enzyme from resistant houseflies showed parathion-hydrolyzing activity. It has not been reported whether the homogenates of insecticide-resistant insects showed parathion-hydrolyzing activity, except for their report.[6] Their enzyme might have been contaminated with bacteria, since parathion-hydrolyzing activity has been reported to be present in some bacteria.[16] The paraoxon-hydrolyzing activity shown in insects must be different from phosphotriesterases detected in bacteria, since the purified phosphotriesterases from bacteria hydrolyze parathion as well as paraoxon.[17]

Paraoxon-hydrolyzing activity from the sera of humans was stimulated by adding sodium chloride,[18] while the salt did not affect the activity from Toyama 89 in the presence of calcium chloride. In our experiment, the stimulation of the activity by salt was observed in the presence of 10 mM zinc ion. The requirement for a zinc ion was reported in bacterial phosphotriesterase,[19] which contains one atom per subunit and is irreversibly inactivated by the loss of a zinc ion. In this study, we found that the paraoxon-hydrolyzing enzyme required a calcium ion, but it is not clear how the calcium ion participates in the enzyme–substrate interaction. This problem will be clarified by using the enzyme purified from Toyama 89 in the future.

K_m values of paraoxon were found to be several hundred μM, and these values were extremely higher than those reported in houseflies[7] and aphides,[8] which were several hundred pM and several nM, respectively. Poor affinity of the Toyama 89 enzyme to paraoxon may indicate a low correlation between the expression of resistance and paraoxon-hydrolyzing activities. However, if paraoxon is condensed in some cells or the subcellular particles of insects, it is possible that paraoxon is degraded with the enzyme. Our assumption is supported by the high solubility of parathion and paraoxon in lipid layers.

Phenyl acetate-hydrolyzing activity, classified as arylesterase EC 3.1.1.2 by the Nomenclature Committee, includes A-esterase, namely paraoxonase. The "International Meeting on Esterases Hydrolyzing Organophosphorus Compounds"[20] recommended to the NC-IUB that the paraoxonase is segregated from arylesterases to "Organophosphorus Compound Hydrolase," which is a new category. Phenyl acetate-hydrolyzing activity in our preparation was observed in all four species tested, while the degrees of their activities did not correlate with the

expression of resistance to organophosphorous insecticides. On the contrary, the resistant strain showed lower activity than the susceptible strains. Moreover, phenyl acetate hardly inhibited the paraoxon-hydrolyzing activity, but the phenyl acetate-hydrolyzing activity was irreversibly inhibited by paraoxon. We therefore propose that these two compounds are degraded in insects by different enzymes.

Consequently, we suggest that higher paraoxon-hydrolyzing activity and lower affinity of organophosphorus-insecticides to acetylcholinesterase[3] are related to the development of organophosphorus-resistance in *Cx. tritaeniorhynchus* Toyama 89. But we have not tested the permeability of insecticides into the insect or the conversion from parathion to paraoxon in the present experiment. Further investigations on these points of view will be required in order to prove the mechanism of development of resistance in *Cx. tritaeniorhyncus*.

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