クルマエビ幼生の成長に伴う有機リン殺虫剤フェニトロチオン代謝能の変化

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Changes in Metabolic Activity of Tiger Shrimp Larvae at Different Stages to Fenitrothion, An Organophosphorus Insecticide*1

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A study was performed on the metabolism of fenitrothion (FS) in tiger shrimp *Penaeus japonicus* at different larval stages to elucidate the cause of the remarkable increase in the toxicity of organophosphorothionates to the shrimp larvae with the progress of the stages.

The shrimp larvae (zoea, mysis and postlarvae) were exposed to 0.5 ppb [^{14}C]FS. After 1.5–12 h exposure, [^{14}C]FS and its metabolites accumulated in the larvae were extracted with benzene and then with ethyl ether. The benzene-extracts were determined by TLC and the ethyl ether-extracts were subjected to an enzymatic analysis. Zoea and mysis showed very low FS metabolic activity, and most of the FS absorbed by the larvae was present as FS itself in their bodies. However, their FS metabolic activity increased abruptly with their growth and resulted in an increase in the amount of fenitrooxon which displays extremely high toxicity as compared with FS, accompanying the simultaneous increase in the FS detoxication activity such as demethylation, hydrolysis and conjugation. The occurrence of the remarkable increase in the toxicity of organophosphorothionates to tiger shrimp larvae with the progress of the stages is probably due to the increase in the oxidative desulfuration activity, beyond the effect of the detoxication activity.

In the preceding paper,[1] it has been demonstrated that the toxicity of thiono-form organophosphorus insecticides to tiger shrimp *Penaeus japonicus* at different larval stages abruptly increased with the progress of the stages, whereas that of oxo-form phosphates was almost unchanged from nauplius to postlarva. Then, the changes in the susceptibility of larval acetylcholinesterase (AChE) was conjectured to be due to the increase in the toxicity of organophosphorothionates to the larvae. However, the susceptibility of the larval AChE to both of the thiono- and oxo-forms was almost unchanged throughout the stages.

To elucidate the cause for the occurrence of the remarkable increase in the toxicity of organophosphorothionate insecticides to tiger shrimp larvae with the progress of the stages, a further study was performed on the metabolism of fenitrothion (dimethyl 3-methyl-4-nitropheyl phosphorothionate) in the shrimp larvae at different stages, which has been produced in a large quantity in Japan amounting to *ca.* 9,000 t a year[2] and displays high toxicity to tiger shrimp after metamorphosis into the postlarva.[1,3]

Materials and Methods

Special Chemicals

Radioactive [ring-U-^{14}C] fenitrothion ([^{14}C]FS; specific activity, 20.4 μCi/μmol=75 μCi/mg), non-radioactive fenitrothion (FS) and its authentic metabolites (fenitrooxon, FO; desmethylfenitrothion, DMFS; desmethylfenitrooxon, DMFO and 3-methyl-4-nitrophenol, 3-M-4-NP) were offered by Takarazuka Research Center, Sumitomo Chemical Co., Ltd.

Enzymes

The three hydrolases used for the determination of the conjugated metabolites of [^{14}C]FS were as follows:

(1) β-Glucuronidase (EC 3.2.1.31) (Sigma, Type IX from *Escherichia coli*)
(2) β-Glucosidase (EC 3.2.1.21) (P-L Biochem., from sweet almonds)
(3) Arylsulfatase (EC 3.1.6.1) (Sigma, Type H-1 from *Helix pomatia*)

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Table 1. Exposure condition of tiger shrimp larvae to [^{14}C]fenitrothion

Stage	Body length (mm)	Individual weight (mg)	Approx. number	Total weight (g)
Zoea	2.4	0.16	6,000	1.0
Mysis	4.3	0.46	6,000	2.8
Postlarva				
P_1*	5.0	0.65	5,000	3.3
P_5*	6.6	1.35	2,750	3.7
P_{20}*	10.5	9.1	375	3.4
P_{30}*	15.2	15.0	310	4.7

The larvae at each stage were exposed to eight aquaria respectively containing 10 *l* of 0.5 ppb [^{14}C]fenitrothion-sea water for 1.5, 3, 6 and 12 h at 25–26°C. The values of number and total weight express those of the larvae at each stage exposed to an aquarium.
* Postlarvae on 1st, 5th, 20th and 30th day after metamorphosis into postlarva.

Shrimp Larvae

Tiger shrimp larvae at different stages: zoea, mysis and postlarvae on the 1st, 5th, 20th and 30th day after metamorphosis into postlarva (P_1, P_5, P_{20} and P_{30}) were supplied from the Fukuoka Prefectural Marine Culture Center, Kanesaki, Fukuoka Prefecture. The average body length and weight of the larvae used in this experiment are shown in Table 1.

Exposure of Larvae to [^{14}C] Fenitrothion

Appropriate amounts (1–4.5 g) of tiger shrimp larvae at each stage (zoea, mysis, P_1, P_5, P_{20} and P_{30}) were placed in each of eight aquaria respectively containing 10 *l* of [^{14}C]FS-sea water (FS, 0.5 ppb=1.84 nM; radioactivity, 37.5 nCi/*l*=83 dpm/m*l*; chlorinity, 18.3‰). [^{14}C]FS was dispersed with 1 ppm Tween 80 into sea water. Air was supplied to the media by an air pump, and the temperature of media was kept at 25–26°C throughout the experiment.

At each of 1.5-, 3-, 6- and 12-h exposure, the larvae (2–9 g) in two of the respective eight [^{14}C] FS-aquaria were taken out, rinsed with FS-free sea water, weighed, and frozen by dipping in dry ice-ethanol at −65°C, and then stored in a deep-freezer at −80°C until subjected to an analysis.

Extraction of [^{14}C] Fenitrothion and Its Metabolites Accumulated in Larvae

The frozen specimens were homogenized with the same amount of distilled water by a Waring blender. The homogenates were subjected to extraction of [^{14}C]FS and its metabolites with a benzene-ethanol mixture (9: 1, v/v) and then with a ethyl ether-ethanol mixture (2: 1, v/v), according to the procedure as shown in our previous paper.[3]

Determination of [^{14}C] Fenitrothion and Its Metabolites

1. Benzene- and ethyl ether-extracts: Appropriate aliquots of both extracts with benzene and ethyl ether were subjected to the measurement of their radioactivities by a liquid scintillation counter (Aloka LSC-900; LSC), using toluene scintillator (PPO, 5 g+POPOP, 0.25 g/*l*) for the benzene-extract and ACS-II aqueous counting scintillant (Amersham) for the ethyl ether-extract.

2. Residue: After benzene- and ethyl ether-extraction, [^{14}C]residues remaining in shrimp larvae were dissolved with Hyamine 10-X (Packard) and subjected to the measurement of radioactivity by LSC, using toluene scintillator.

3. [^{14}C]FS and its benzene-extractable metabolites: [^{14}C] FS and its benzene-extractable metabolites were separated by thin-layer co-chromatography (TLC) on silica gel plates (Merck 60 F_{254}) with carriers such as non-radioactive FS and its authentic metabolites, using two solvent systems: (I) toluene-ethyl formate-formic acid (5: 7: 1, v/v) and (II) toluene-ethyl acetate-isopropanol-acetic acid (8: 12: 5: 3, v/v). The Rf values of FS, 3-M-4-NP, FO, DMFS and DMFO in the solvent system (I) were 0.91, 0.76, 0.65, 0.22 and 0.11, and those in (II) 0.95, 0.91, 0.83, 0.58 and 0.27, respectively.

Before being subjected to TLC, the benzene-extract was treated by the partition between acetonitrile and *n*-hexane to remove lipid substances. An aliquot of the benzene-extract was dissolved in 30 m*l* of *n*-hexane saturated with acetonitrile and shaken with the same amount of acetonitrile saturated with *n*-hexane. [^{14}C]FS and its metabolites were transferred to the acetonitrile layer. The *n*-hexane layer was washed twice with an additional 20 m*l* of acetonitrile. The acetonitrile layer was evaporated under reduced pressure at

40°C, and the residue was dissolved in 2 m*l* of benzene, and an appropriate amount (usually 200 μl) of the benzene solution was mixed with 100 μl of the benzene solution containing non-radioactive FS and its authentic metabolites (*ca.* 100 μg each) and subjected to TLC. The spots of FS, FO, DMFS, DMFO and 3-M-4-NP were detected under UV-light (254 nm), scraped off from the TLC plates, put into glass vials with ACS-II scintillator, and then subjected to the measurement of their radioactivities by LSC.

4. Ethyl ether-extractable [^{14}C]metabolites: The [^{14}C] metabolites extracted with ethyl ether from the larvae after benzene-extraction were water-soluble and non-extractable with benzene from their acidified aqueous solution. This suggests that the [^{14}C] metabolites are some conjugates of [^{14}C]3-M-4-NP, as well as shown in our previous paper[4] on the metabolism of [^{14}C]FS in young tiger shrimp.

An enzymatic analysis was employed for the identification and determination of the conjugated [^{14}C] metabolites, using three hydrolases described previously.[4]

Prior to the enzymatic analysis, an appropriate amount of the conjugated [^{14}C]metabolites in aqueous solution was shaken with benzene to remove the benzene-extractable [^{14}C]substances such as [^{14}C]3-M-4-NP, which might be present. After separating the benzene layer and then removing the benzene remaining in the aqueous layer with a jet of nitrogen gas, 1 m*l* of β-glucuronidase solution with an adequate enzymatic activity was added to the aqueous layer and incubated for 1 h at 35°C. To the incubated medium, 10 m*l* of benzene was added and shaken for 5 min, and then centrifuged at 3,000 rpm for 4 min to separate the benzene layer. The benzene layer containing [^{14}C]3-M-4-NP liberated by β-glucuronidase from the corresponding conjugate was evaporated under reduced pressure at 40°C and subjected to the measurement of the radioactivity by LSC, using ACS-II scintillator.

The adequate amounts of β-glucosidase and arylsulfatase were stepwisely added to the remaining aqueous layer to hydrolyze the respective con-

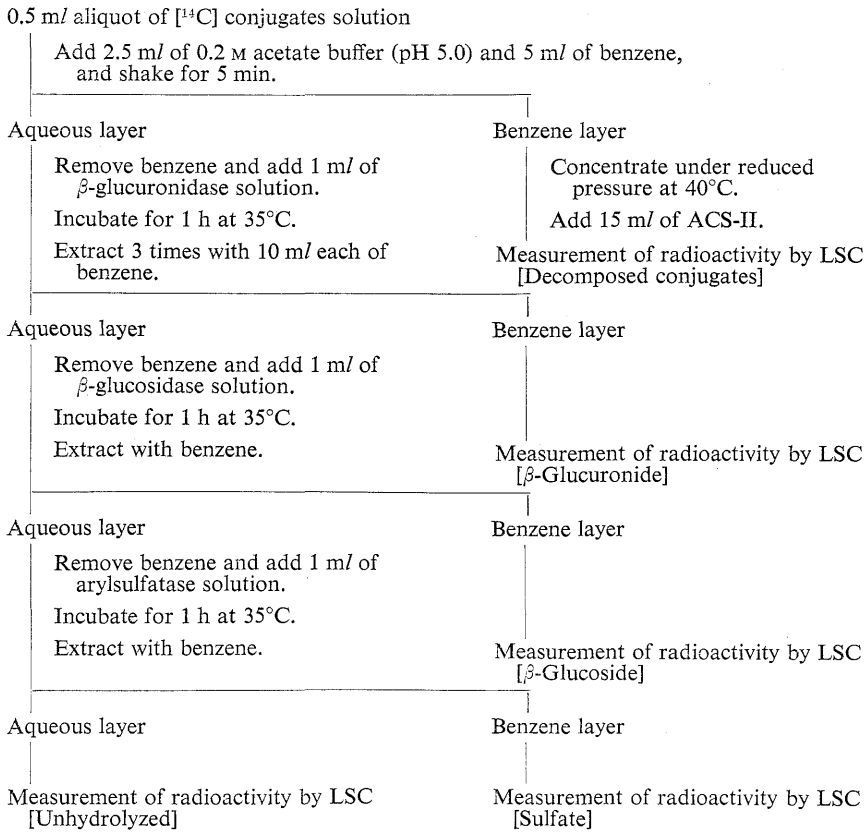

Fig. 1. Procedure for stepwise hydrolysis of the conjugated [^{14}C] metabolites with β-glucuronidase, β-glucosidase and arylsulfatase.

Table 2. Accumulation of [^{14}C] fenitrothion and its metabolites in tiger shrimp larvae at different stages, during exposure to 0.5 ppb [^{14}C] fenitrothion-sea water

	Exposure time (h)	Zoea	Mysis	P_1	P_5	P_{20}	P_{30}
Benzene-extract	1.5	2,033 (45.2)	3,367 (74.8)	3,381 ((75.1)	2,539 (56.4)	1,086 (24.1)	865 (19.2)
	3	2,305 (51.2)	3,657 (81.3)	3,284 (73.0)	2,880 (64.0)	1,334 (29.7)	1,159 (25.8)
	6	2,447 (54.4)	3,298 (73.3)	3,422 (76.0)	3,956 (87.9)	3,133 (69.6)	1,812 (40.3)
	12	1,854 (41.2)	2,732 (60.7)	3,243 (72.1)	3,560 (79.0)	2,544 (56.5)	3,501 (77.8)
Ethyl ether-extract	1.5	373 (8.3)	1,003 (22.3)	2,806 (62.4)	2,098 (46.6)	1,113 (24.7)	851 (18.9)
	3	322 (7.2)	994 (22.1)	2,985 (66.3)	3,432 (76.3)	1,748 (38.8)	1,702 (37.8)
	6	313 (7.0)	488 (10.8)	2,789 (62.0)	6,564 (145.9)	5,111 (113.6)	3,698 (82.2)
	12	189 (4.2)	386 (8.6)	2,199 (48.9)	5,492 (122.0)	4,568 (101.5)	7,595 (168.8)
Residue	1.5	83 (1.8)	138 (3.1)	55 (1.2)	83 (1.8)	133 (3.0)	106 (2.3)
	3	110 (2.4)	124 (2.8)	101 (2.2)	74 (1.6)	166 (3.7)	129 (2.9)
	6	37 (0.8)	60 (1.3)	92 (2.0)	170 (3.8)	46 (1.0)	207 (4.6)
	12	110 (2.4)	115 (2.6)	92 (2.0)	106 (2.4)	166 (3.7)	152 (3.4)
Total	1.5	2,489 (55.3)	4,508 (100.2)	6,242 (138.7)	4,720 (104.8)	2,332 (51.8)	1,817 (40.4)
	3	2,737 (60.8)	4,775 (106.2)	6,371 (141.5)	6,385 (141.9)	3,248 (72.2)	2,990 (66.4)
	6	2,797 (62.2)	3,846 (85.4)	6,294 (140.0)	10,690 (237.6)	8,289 (184.2)	5,718 (127.1)
	12	2,153 (47.8)	3,234 (71.9)	5,534 (123.0)	9,159 (203.4)	7,277 (161.7)	11,247 (250.0)

The values are expressed as dpm (pmol) per gram of larva.

jugates, as shown in Fig. 1.

The amounts of [^{14}C]FS and its metabolites were calculated from their radioactivities on the basis of the specific activity of [^{14}C]FS (45 dpm/pmol) used in this experiment.

Results and Disccusion

The losses of [^{14}C]FS in the media during exposure were within a range of 2–14% of the initial concentration even at 12-h exposure except the P_1-medium which lost 24%.

[^{14}C]*Fenitrothion and Its Metabolites Extracted with Benzene and Ethyl Ether*

Table 2 shows the accumulation of [^{14}C]FS and its metabolites in the larvae at different stages, during exposure to [^{14}C]FS-sea water. The time-course of the total radioactivity in the larvae showed that the rapid absorption of [^{14}C]FS at the early stages led their total radioactivities to the respective maximum levels within *ca.* 3-h, whereas the absorption of [^{14}C]FS by postlarvae except P_1 was relatively slow, but their total radioactivity increased with exposure time. In comparison with the total radioactivity at 12-h exposure, P_{30} showed about 5 times to that of zoea.

In zoea and mysis, almost all of the total radioactivities at the respective exposure times were due to [^{14}C]FS and its benzene-extractable metabolites, accompanying with the less amounts of [^{14}C]conjugates extracted with ethyl ether. The radioactivity of ethyl ether-extracts in postlarvae (P_5, P_{20} and P_{30}), however, increased with exposure time, and after 6-h exposure it reached approximately twice to that of the respective benzene-extracts.

Fig. 2 shows the relation between both radioactivities of [^{14}C]benzene- and ethyl ether-extracts in the larva at each stage, as percent of the total radioactivity. The [^{14}C]benzene-extract amounting *ca.* 80% at 1.5-h in zoea and mysis increased further with the exposure time, whereas that in the postlarvae except P_1 decreased with exposure time from *ca.* 50% at 1.5-h to 35% at 12-h, as the result in the increase of [^{14}C]ethyl ether-extract. In P_1-larva, both the levels of [^{14}C]benzene- and ethyl ether-extracts were almost constant during the exposure for 12 h.

Fig. 3 shows the bioconcentration ratios of [^{14}C] FS based on the total radioactivity of larvae to the radioactivity of [^{14}C]FS-medium (83 dpm/m*l*=1.84 pmol/m*l*). The ratios in zoea, mysis and P_1 reached the respective maximum levels in short periods (3–6 h), whereas those in the postlarvae increased with exposure time except P_5 and P_{20} at 12-h, reaching the highest value of 135 in P_{30} at 12-h.

Determination of [^{14}C]*Fenitrothion and Its Metabolites by TLC*

Fig. 4 shows the changes with exposure time in the amounts of FS, 3-M-4-NP, FO, DMFS+DMFO and other benzene-extractable metabolites from the respective larvae, comparing with the

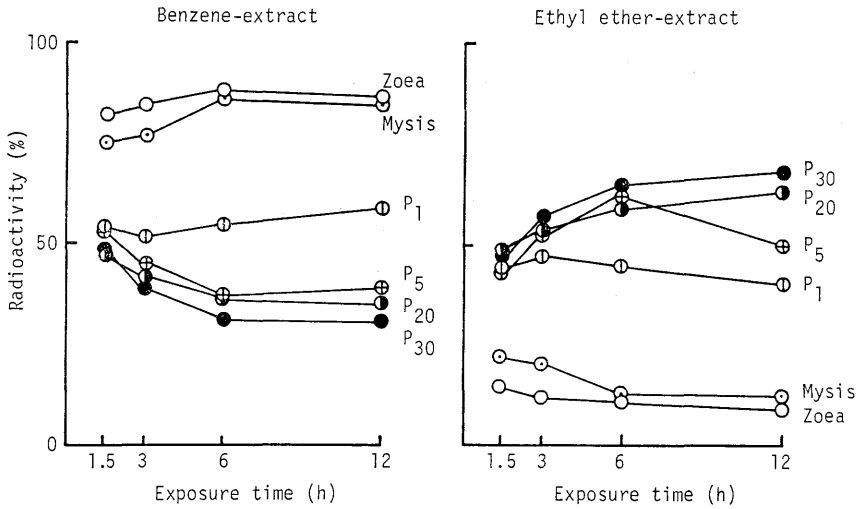

Fig. 2. Changes in the radioactivity of ^{14}C extracted with benzene and ethyl ether from tiger shrimp larvae, during exposure to 0.5 ppb [^{14}C] fenitrothion.
The values are expressed as percent of total ^{14}C in larvae.

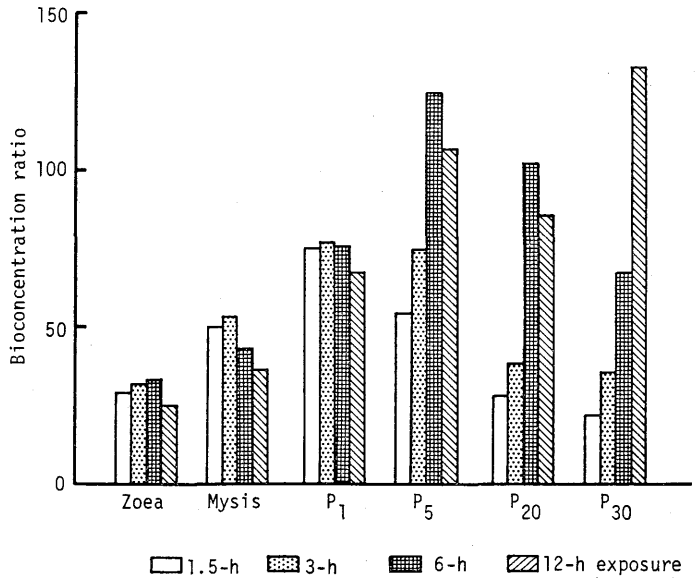

Fig. 3. Changes in the bioconcentration ratio of [^{14}C]fenitrothion and its metabolities in tiger shrimp larvae, during exposure to 0.5 ppb [^{14}C] fenitrothion.

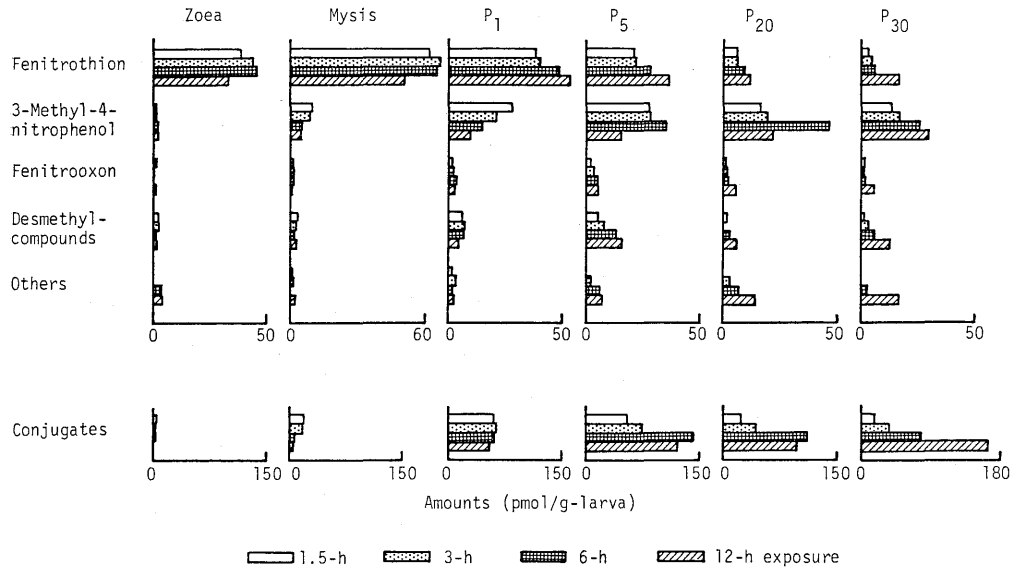

Fig. 4. Changes in the amount of [^{14}C] fenitrothion and its metabloites found in tiger shrimp larvae, during exposure to 0.5 ppb [^{14}C] fenitrothion.

total amounts of [^{14}C] conjugates.

In zoea and mysis, the radioactivities of their benzene-extracts were dominated by the parent compound [^{14}C]FS corresponding to *ca.* 80 to 87% of the total ^{14}C and accompanied with the small amounts of its metabolites, as well as the accumulation of FS and its metabolites in fish exposed to FS.[5]

On the other hand, the degradation of FS in the postlarvae increased with their growth and resulted in the increase in the amounts of [^{14}C] metabolites such as 3-M-4-NP, FO, DMFS+DMFO and others, corresponding to the high percentage of 55 to 85 of the total ^{14}C.

The amount of 3-M-4-NP among the above metabolites increased remarkably with the progress of larval stages. The increase in the amounts of 3-M-4-NP and its conjugates in the larvae with

the progress of stages must be due to the increase in the esterase activity of larvae, which hydrolyzes FS, FO, DMFS and DMFO, resulting the liberation of 3-M-4-NP.

Although the amount of FO found in the postlarvae was very small as compared with that of 3-M-4-NP, it increased with exposure time and reached 2.7–4.3 pmol/g-larva at 12-h exposure, whereas that in zoea and mysis was almost undetectable during the FS-exposure for 12 h.

It is well known that the conversion of phosphorothionate insecticides to the respective oxoforms results usually in an increase of their toxicity.[6] In our previous report[7] on the relation between the mortality and accumulation of FS and FO in young tiger shrimp during the exposure to FS at a lethal level (2 ppb), it was demonstrated that the major cause for the occurrence of high mortality was due to FO rather than it parent compound FS, and the estimated lethal concentration of FO was *ca.* 14 pmol/g-body weight, comparing with the amounts of FS and FO found in the surviving and dead shrimps. In the same report,[7] the minimum lethal concentrations of FO and FS *in vivo* in the shrimp were also estimated to be 8–20 pmol/g and *ca.* 200 pmol/g-body weight by intramuscular administration, respectively.

Enzymatic Determination of Conjugated [^{14}C] Metabolites

The conjugated [^{14}C]metabolites found in tiger shrimp larvae were identified as the sulfate and β-glucoside of [^{14}C]3-M-4-NP, as well as those in young tiger shrimp.[4] The glucuronide which has been found in fish[5] was not detected in the larvae.

Fig. 5 shows the changes in the amounts of the respective conjugates found in the larvae during exposure to 0.5 ppb [^{14}C]FS-sea water. The amounts of the conjugated metabolites were very little at zoea, and most of the conjugates was β-glucoside, as well as that in insects.[8] The ratio of sulfate to glucoside in zoea was 1 : 4. However, the amounts of the conjugates and also the formation ratio of sulfate to glucoside increased with the progress of larval stages, especially in the postlarva. The total amount of the conjugates in P_{30} reached 169 pmol/g-larva at 12-h exposure, in which the sulfate and glucoside were 116 and 33 pmol/g-larva respectively, *i.e.*, the ratio of the sulfate to the glucoside was 3.5 : 1, on the contrary to that in zoea.

It is very interesting from the view point of comparative biochemistry that tiger shrimp form both conjugates of sulfate and β-glucoside, instaed

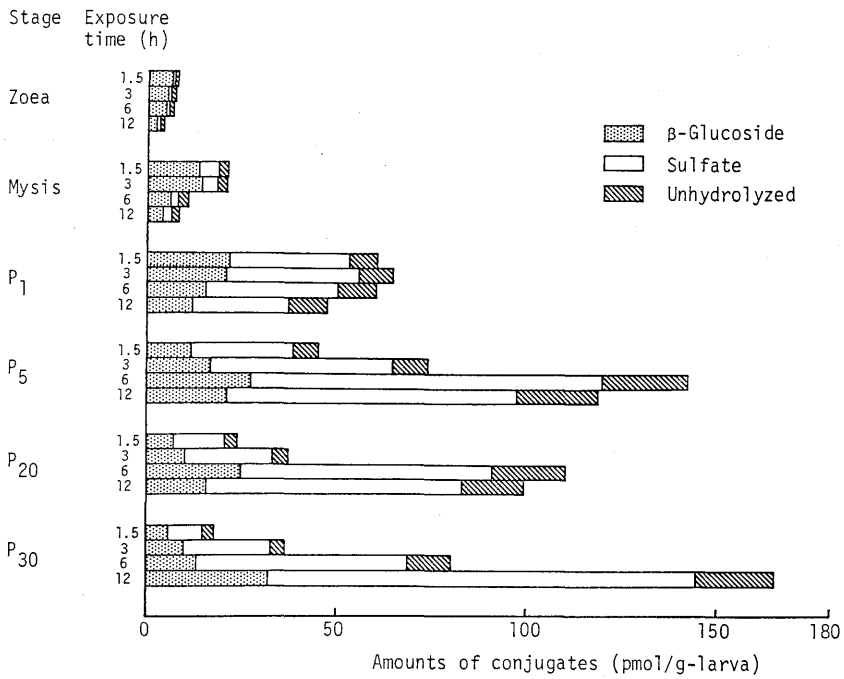

Fig. 5. Changes in the amount of the respective conjugates of [^{14}C] 3-methyl-4-nitrophenol in tiger shrimp larvae, during exposure to 0.5 ppb [^{14}C] fenitrothion.

of the glucuronide, as well as in insects which belong to the same phylum of Arthropoda, and that the conjugation activity and the formation ratio of sulfate to glucoside, which are respectively very low in the larvae at the early stages, increase abruptly with the progress of the stages.

As demonstrated in our preceding paper,[1] the remarkable increase in the toxicity of organophosphorus insecticides to tiger shrimp larvae with the progress of the stages occurred when they were exposed to thiono-forms, whereas the high toxicity of oxo-forms to the larvae at early stages was almost unchanged throughout the larval stages, and the susceptibility of larval acetylcholinesterase (AChE) to both of the thiono- and oxo-forms was almost unchanged from zoea to postlarvae, although the oxo-forms showed the extremely high AChE inhibition compared with the respective thiono-forms.

The results of this experiment demonstrate that the high resistance of the shrimp larvae at early stages to organophosphorothionates is due to their low conversion activity to the respective oxo-form phosphates, whereas the occurrence of the remarkable increase in their susceptibility to the thiono-forms with the progress of larval stages is probably due to the increase in their oxidative desulfuration activity, which displays more toxicity beyond the simultaneous increase in the detoxication activities such as demethylation, hydrolysis and conjugation.

Acknowledgements

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