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# クルマエビにおける有機燐剤フェニトロチオンの硫酸抱合およびグルコシド抱合代謝物質の生成


| 誌名 | 日本水産學會誌 |
| --- | --- |
| ISSN | 00215392 |
| 巻/号 | 5112 |
| 掲載ページ | p. 2013-2017 |
| 発行年月 | 1985年12月 |


農林水産省 農林水産技術会議事務局筑波産学連携支援センター
Tsukuba Business-Academia Cooperation Support Center, Agriculture, Forestry and Fisheries Research Council Secretariat

Bulletin of the Japanese Society of Scientific Fisheries 51(12), 2013–2017 (1985)

# Formation of the Sulfate and Glucoside Metabolites of Fenitrothion in Tiger Shrimp *Penaeus japonicus**1

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(Accepted May 15, 1985)

A study was made of the identification of the conjugated metabolites of fenitrothion [*O*,*O*-dimethyl *O*-(3-methyl-4-nitrophenyl)phosphorothioate; Sumithion] formed by tiger shrimp *Penaeus japonicus*, and also the accumulation of the metabolites in the shrimp with exposure time.

After benzene-extraction from the homogenate of shrimp exposed to 0.5 ppb [$^{14}$C]fenitrothion, some conjugated [$^{14}$C]metabolites remaining in the residues were extracted with ethyl ether. The identification and determination of the conjugated [$^{14}$C]metabolites were performed by an enzymatic analysis, using $\beta$-glucuronidase, $\beta$-glucosidase and arylsulfatase. The conjugated [$^{14}$C]metabolites were identified as the sulfate and $\beta$-glucoside of [$^{14}$C]3-methyl-4-nitrophenol. The amounts of both the conjugates of sulfate and $\beta$-glucoside accumulated in the shrimp increased with exposure time and reached 60 and 36 pmol/g body weight in 24-h exposure period, corresponding to 58 and 38% of the total conjugates, respectively. However, glucuronide was not detected in the shrimp.

It is very interesting from the view point of comparative biochemistry that the shrimp forms both conjugates of sulfate and $\beta$-glucoside, instead of the glucuronide, as well as insects which belong to the same phylum of Arthropoda.

In the preceding paper,[1] we reported that the amounts of the metabolites of [$^{14}$C]fenitrothion [*O,O*-dimethyl *O*-(3-methyl-4-nitrophenyl)phosphorothioate; Sumithion] extracted with ethyl ether after benzene-extraction from tiger shrimp *Penaeus japonicus* exposed to [$^{14}$C]fenitrothion-sea water increased with exposure time, reaching rather higher levels than the total amounts of [$^{14}$C]fenitrothion and its benzene-extractable metabolites such as fenitrooxon, desmethylfenitrothion, desmethylfenitrooxon and 3-methyl-4-nitrophenol.

The fenitrothion-metabolites in the ethyl ether-extract are water-soluble and non-extractable with benzene from their acidified aqueous solutions. This suggests that the metabolites are some conjugates of 3-methyl-4-nitrophenol which is a hydrolizate of fenitrothion, such as its glucuronide which was isolated by a similar separation procedure for fenitrothion-metabolites in rainbow trout.[2] The present study was performed on the identification of the conjugated metabolites of fenitrothion formed by tiger shrimp during exposure to [$^{14}$C]fenitrothion-sea water, and also on the accumulation of the metabolites in the shrimp with exposure time.

## Materials and Methods

As demonstrated in the preceding paper,[1] very small amounts of fenitrothion and its metabolites less than 1 nmol/g body weight are accumulated in tiger shrimp even at 24-h exposure to 0.5 ppb fenitrothion which is nearly at its lethal level. Therefore, an enzymatic analysis was employed for the identification and determination of the conjugated [$^{14}$C]metabolites.

### *Enzymes*

The three hydrolases were used in this experiment as follows:

(1) $\beta$-Glucuronidase (EC 3.2.1.31) (Sigma, Type IX from *Escherichia coli*)
(2) $\beta$-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*)

### *Special Chemicals*

As the respective substrates for the above en-

*1 Studies on the Relation Between Toxicity and Metabolism of Organophosphorus Insecticides in Shrimp—II. The outline of this report was presented at the annual meeting of the Japanese Society of Scientific Fisheries (April 4, 1982 in Tokyo) and also at the 5th International Congress of Pesticide Chemistry (Aug. 29–Sept. 4, 1982 in Kyoto).

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zymes, p-nitrophenyl-β-glucuronide, p-nitrophenyl-β-glucopyranoside and p-nitrophenyl sulfate potassium salt were purchased from Sigma Chemical Co.

Radioactive [ring-U-$^{14}$C]fenitrothion (specific activity: 20.4 μCi/μmol; 75 μCi/mg) and 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 the Institute for Biological Science, Sumitomo Chemical Co., Ltd.

*Extraction of Conjugated Metabolites of [$^{14}$C]-Fenitrothion Formed by Shrimp*

Five shrimps (average body weight, 2.65 g each) were placed in each of ten tanks containing 8 *l* of [$^{14}$C]FS-sea water (FS, 0.5 ppb=1.8 nmol/*l*; radioactivity, 37.5 nCi/*l*=83 dpm/m*l*; $Cl^-$, 18.3‰) dispersed with 1 ppm Tween 80. Air was supplied by pump and the temperature was kept at 25±1°C. At each of 0.5-, 1-, 2-, 4-, 8-, 12- and 24-h exposure, five shrimps were taken out and homogenized with the same amount of distilled water by a Waring blender.

At first, the homogenates were subjected to extraction of [$^{14}$C]FS and its metabolites with a benzene-ethanol mixture (9:1, v/v), and then the conjugated [$^{14}$C]metabolites remaining in the residues were extracted with an ethyl ether-ethanol mixture (2:1, v/v), according to the procedure as shown in our preceding paper.[1]

*Enzymatic Analysis of Conjugated [$^{14}$C]Metabolites*

1. Purity test for enzymes: To confirm the purity of the enzymes employed in this experiment, their hydrolytic activities on the respective substrates (p-nitrophenyl-β-glucuronide, p-nitrophenyl-β-glucopyranoside and p-nitrophenyl sulfate) were assayed as shown in Fig. 1.

2. Determination of [$^{14}$C]conjugates accumlated in shrimp: The conjugated [$^{14}$C]metabolites extracted with ethyl ether from each group of five shrimps exposed to 0.5 ppb [$^{14}$C]FS-media for 4, 8, 12 and 24 h were subjected to an enzymatic analysis, using β-glucuronidase, β-glucosidase and arylsulfatase which also contained the considerable amounts of β-glucuronidase and β-glucosidase, as mentioned subsequently.

In the hydrolysis of the respective p-nitrophenyl compounds added to the conjugate solutions, a slight inhibition was observed in β-glucuronidase among the above enzymes. Therefore, the enzymes were employed in large excess to complete the hydrolysis of the respective [$^{14}$C]conjugates within 1 or 2 h.

0.3 m*l* of 1 mM p-nitrophenyl-β-glucuronide, p-nitrophenyl-β-glucopyranoside or p-nitrophenyl sulfate
- Add 1 m*l* of 0.2 M acetate buffer (pH 5.0) and 0.2 m*l* of distilled water.
- Stand for 5 min at 35°C.
- Add 0.5 m*l* of each enzyme (β-glucuronidase, β-glucosidase or arylsulfatase) solution with appropriate activity.
- Incubate for 1 h at 35°C.

1 m*l* of reaction medium
- Add 5 m*l* of 0.12 M $Na_2CO_3$.

Absorbance at 400 nm

**Fig. 1.** Procedure for purity test of the enzymes employed for hydrolysis of the conjugated [$^{14}$C]-metabolites.

0.5 m*l* aliquots of [$^{14}$C]conjugates solution
- Add 2.5 m*l* of 0.2 M acetate buffer (pH 5.0) and 0.5 m*l* of distilled water.
- Stand for 5 min at 35°C.
- Add 1.5 m*l* of each enzyme (β-glucuronidase, β-glucosidase or arylsulfatase) solution with adequate activity.
- Incubate for 2 h at 35°C.
- Acidify with HCl and extract 3 times with 10 m*l* each of benzene.

Benzene layer | Aqueous layer
- Benzene layer: Concentrate under reduced pressure at 40°C.

Measurement of radioactivity by liquid scintillation counter

**Fig. 2.** Enzymatic determination of the conjugated metabolites of [$^{14}$C]fenitrothion accumulated in tiger shrimp.

The activities of the enzyme solutions employed for the hydrolysis were as follows: β-glucuronidase, 0.17 U/m*l* for p-nitrophenyl-β-glucuronide; β-glucosidase, 0.17 U/m*l* for p-nitrophenyl-β-glucopyranoside; arylsulfatase, 1.25, 0.75 and 0.15 U/m*l* for p-nitrophenyl sulfate, p-nitrophenyl-β-glucopyranoside and glucuronide, respectively.

Three aliquots (0.5 m*l* each) of each aqueous solution containing the conjugated [$^{14}$C]metabolites were placed in three test tubes, and 2.5 m*l* of 0.2 M acetate buffer (pH 5.0) and 0.5 m*l* of distilled water were added to each tube. After standing for 5 min, 1.5 m*l* of the enzyme solutions were added to the respective tubes and incubated for 2 h at 35°C.

After acidifying the media with hydrochloric

**Table 1.** Changes in radioactivity and amount of [$^{14}$C]fenitrothion (FS) and its metabolites accumulated in tiger shrimp, during exposure to 0.5 ppb [$^{14}$C]FS-sea water

| Metabolites | | Exposure time (h) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 0.5 | 1 | 2 | 4 | 8 | 12 | 24 |
| FS+benzene-extractable | (dpm/g) | 285 | 394 | 720 | 1,173 | 2,374 | 2,088 | 4,728 |
| | (pmol/g) | 6.2 | 8.5 | 15.6 | 25.4 | 51.5 | 45.3 | 102.5 |
| Conjugated | (dpm/g) | 100 | 240 | 420 | 1,202 | 1,471 | 3,423 | 4,798 |
| | (pmol/g) | 2.4 | 5.2 | 9.1 | 26.1 | 31.9 | 74.2 | 104.0 |
| Residual | (dpm/g) | 252 | 300 | 421 | 651 | 597 | 2,075 | 2,024 |
| | (pmol/g) | 5.5 | 6.5 | 9.1 | 14.1 | 20.8 | 45.0 | 43.9 |

**Table 2.** Purities of the enzymes employed for hydrolysis of the conjugated metabolites of [$^{14}$C]-fenitrothion

Values are expressed as their relative activities (%) on the respective *p*-nitrophenyl compounds.

| Enzymes | | Relative activity (%) | | |
|---|---|---|---|---|
| | | Sulfatase | β-Glucosidase | β-Glucuronidase |
| Arylsulfatase | (Sigma, Type H-1) | 100 | 60 | 12 |
| β-Glucosidase | (P-L Biochem.) | ca. 0.2 | 100 | ND |
| β-Glucuronidase | (Sigma, Type IX) | ca. 0.14 | ca. 0.21 | 100 |

ND: Not detected.

acid, the [$^{14}$C]hydrolyzate liberated from [$^{14}$C]-conjugates by each enzyme was extracted three times with 10 m*l* each of benzene. The benzene-extracts were evaporated under reduced pressure at 40°C and subjected to the measurement of the radioactivity by a liquid scintillation counter (Aloka LSC-900; LSC), using ACS-II aqueous counting scintillant (Amersham), as shown in Fig. 2. The radioactivity of [$^{14}$C]metabolites remaining in the aqueous layer was also counted.

The amounts of the respective conjugates were calculated from their radioactivities on the basis of the specific radioactivity of [$^{14}$C]FS (46 dpm/pmol) used in this experiment.

3. Identification of the hydrolyzate of [$^{14}$C]-conjugates: To identify the hydrolyzate liberated from [$^{14}$C]conjugates by sulfatase which hydrolyzed more than 90% of the total [$^{14}$C]conjugates as mentioned subsequently, the benzene-extract from the reaction medium was subjected to the thin-layer co-chromatography (TLC) on silicagel plates (Merck 60 $F_{254}$) with non-radioactive FS and its authentic metabolites, using two solvent systems: (I) *n*-hexane-acetone (1:1, v/v) and (II) toluene-ethyl formate- formic acid (5:7:1, v/v).

After removal of the solvents, the TLC plates were subjected to autoradiography, allowing to stand for two weeks in contact with X-ray films (Sakura QS), and finally the spots of FS and its metabolites and also other parts were scraped off from the plates and subjected to the measurement of the radioactivity by LSC.

## Results and Discussion

*Accumulation of Conjugated Metabolites of [$^{14}$C]-Fenitrothion in Shrimp*

The loss of [$^{14}$C]FS in the exposure-media was approximately 10% of the initial concentration at 24-h exposure and no mortality was observed in the tested shrimps except one which died at 24-h.

Table 1 shows the changes in the radioactivity and amount of [$^{14}$C]FS and its benzene-extractable, conjugated and residual metabolites in shrimp, during exposure to 0.5 ppb [$^{14}$C]FS-sea water for 0.5 to 24 h.

The accumulation of [$^{14}$C]FS and its benzene-extractable metabolites in the shrimp has been demonstrated in detail in our preceding paper.[1] The amounts of the conjugated metabolites extracted with ethyl ether increased with exposure time, especially after 4-h, reaching almost the same amounts of FS and its benzene-extractable metabolites.

*Purity of Enzymes*

As shown in Table 2, arylsulfatase (Sigma, Type H-1) showed also β-glucosidase and β-glucuronidase activities, corresponding to 60 and 12% of

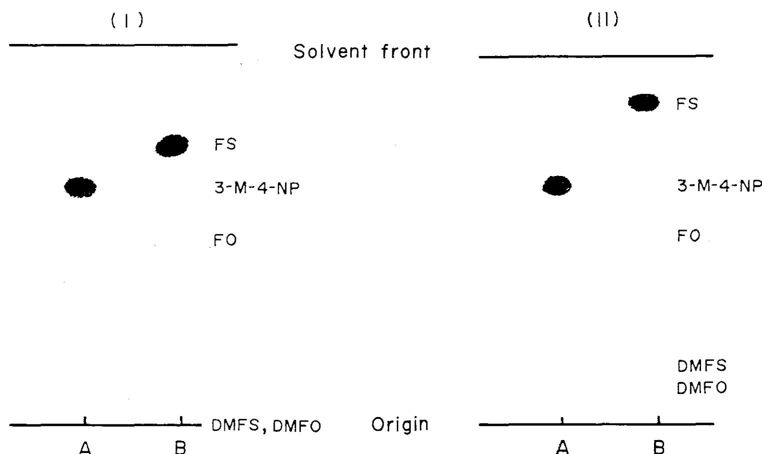


**Fig. 3.** Autoradiographs of [$^{14}$C]fenitrothion and [$^{14}$C]hydrolyzate liberated from the conjugated [$^{14}$C]metabolites by arylsulfatase.

The abbreviations indicate the positions of the respective spots detected under UV-light on the TLC plates.

Abbreviations: FS, fenitrothion; FO, fenitrooxon; DMFS, desmethylfenitrothion; DMFO, desmethylfenitrooxon; 3-M-4-NP, 3-methyl-4-nitrophenol.

Samples: A, [$^{14}$C]hydrolyzate+non-radioactive FS and its metabolites;
B, [$^{14}$C]FS +non-radioactive FS and its metabolites.

TLC plate: Silicagel 60F$_{254}$ (Merck; 10×20 cm, 0.25 mm thick).

Solvent systems: (I) *n*-hexane-acetone (1: 1, v/v);
(II) toluene-ethyl formate- formic acid (5: 7: 1, v/v).

sulfatase activity on the respective *p*-nitrophenyl compounds, whereas β-glucosidase (P-L Biochem.) and β-glucuronidase (Sigma, Type IX) showed only slight activities of the other enzymes used in this experiment.

*Identification of Hydrolyzate of* [$^{14}$C]*Conjugates*

The abbreviations of fenitrothion and its metabolites shown in Fig. 3 indicate the positions of the respective spots detected under UV-light on the TLC plates.

The [$^{14}$C]hydrolyzate liberated from the conjugated [$^{14}$C]metabolites by sulfatase showed only one spot on the autoradiographs, which completely coincided with 3-M-4-NP in position. Even in the counting by LSC, no radioactivity higher than the background level was detected from any parts of the TLC plates except the spot of 3-M-4-NP.

The result indicates that the conjugated [$^{14}$C]-metabolites formed by tiger shrimp are some conjugates with [$^{14}$C]3-M-4-NP.

*Enzymatic Determination of* [$^{14}$C]*Conjugates Accumulated in Shrimp*

Sulfatase (Sigma, Type H-1) hydrolyzed more than 90% of the total [$^{14}$C]conjugates under the conditions shown in Fig. 2. The [$^{14}$C]3-M-4-NP liberated from the [$^{14}$C]conjugates by sulfatase, however, included also that from both of the glucoside and glucuronide, because the sulfatase contained β-glucosidase and β-glucuronidase in relatively high ratios, as shown in Table 2. Therefore, the amount of the sulfate-conjugate was obtained by deduction of the amount of [$^{14}$C]3-M-4-NP liberated by the latter enzymes from that by the sulfatase.

As shown in Table 3, the amounts of both conjugates of sulfate and β-glucoside accumulated in the shrimp increased with exposure time and reached 60 and 36 pmol/g body weight at 24-h exposure, corresponding to 58 and 35% of the total conjugates, respectively. The glucuronide of 3-M-4-NP which has been found in rainbow trout[2] exposed to FS was not detected in the shrimp.

The major metabolic pathway of FS in tiger shrimp is summarized in Fig. 4, combining the result of our preceding report.[1] The pathway includes main biotransformation reactions such as oxidation, demethylation, hydrolysis and conjugation.

The result of this experiment suggests that both the sulfate and β-glucoside conjugations must be the general detoxication mechanisms for phenolic

Table 3. Change in amount of [$^{14}$C]3-methyl-4-nitrophenyl conjugates accumulated in tiger shrimp, during exposure to 0.5 ppb [$^{14}$C]fenitrothion

| Enzymes | Hydrolyzed conjugates | Exposure time (h) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 4 | | 8 | | 12 | | 24 | |
| | | (pmol/g) | (%) | (pmol/g) | (%) | (pmol/g) | (%) | (pmol/g) | (%) |
| Arylsulfatase (A) | Sulfate<br>β-Glucoside<br>β-Glucuronide | 24.3 | 93.3 | 29.5 | 92.4 | 69.0 | 92.9 | 96.5 | 92.8 |
| β-Glucosidase (B) | β-Glucoside | 9.5 | 36.4 | 10.5 | 33.0 | 23.5 | 31.6 | 36.0 | 34.7 |
| β-Glucuronidase | β-Glucuronide | ND | | ND | | ND | | ND | |
| (A)-(B) | Sulfate | 14.8 | 56.9 | 19.0 | 59.4 | 45.5 | 61.3 | 60.5 | 58.1 |
| | Unhydrolyzed | 1.7 | 6.6 | 2.4 | 7.6 | 5.3 | 7.1 | 7.5 | 7.2 |

ND: Not detected.

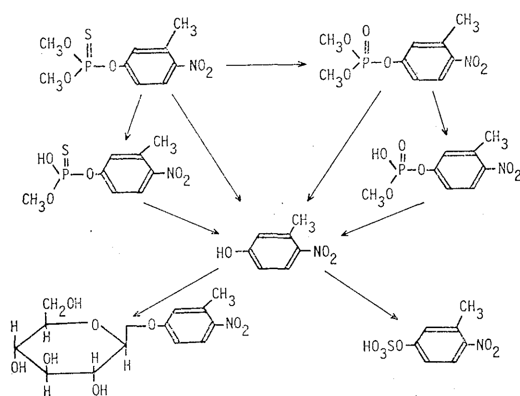

Fig. 4. The major metabolic pathway of fenitrothion in tiger shrimp.

compounds in shrimp. The conversion of phenols to glycosides in crustaceans had been assumed by BRODIE and MAICKEL,[3] because of the formation of some conjugates with phenolic compounds by crayfish and lobsters, which were hydrolyzed by treatment with 3 N HCl at 100°C, but not by treatment with β-glucuronidase and arylsulfatase.

As the detoxication mechanisms for phenolic compounds in fishes, both the sulfate[4-8] and β-glucuronide[9-12] conjugations have been reported, while as that in a clam the sulfate conjugation only.[13] It is very interesting from the view point of comparative biochemistry that the shrimp forms both conjugates of sulfate and glucoside, especially the glucoside instead of the glucuronide, as well as insects[14] which belong to the same phylum of Arthropoda.

## Acknowledgements

We wish to express our gratitude to the generosity of the Institute for Biological Science, Sumitomo Chemical Co., Ltd., for the gift of $^{14}$C-labeled and non-labeled fenitrothion and its authentic metabolites.

This work was supported in part by Grant-in-Aid for Scientific Research (56560277) from the Ministry of Education.

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