魚類におけるクロロフェノール類の代謝に関する研究 III

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Studies on the Metabolism of Chlorophenols in Fish—III Isolation and Identification of a Conjugated PCP Excreted by Goldfish

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A study has been made of the isolation and identification of a conjugated PCP occurring in goldfish, *Carassius auratus*. The amount of PCP in the fish body was less than that lost from the PCP-medium during culture. This loss of PCP was caused, not by decomposition, but by transformation of the PCP by the fish to some bound-form which was easily hydrolyzed on heating in acidic solution to release free-PCP.

Most of the PCP excreted by the fish was in a conjugated form accompanied with a small amount of free-form. Conjugated PCP excreted by goldfish into the culture medium was isolated by treating the medium with activated charcoal, followed by elution with an ammonia-acetone mixture, and finally by passing the concentrated eluate, under reduced pressure, through a Sephadex G-10 column.

The isolated conjugate was identified as pentachlorophenylsulfate which was identical to that found in the short-necked clam by KOBAYASHI *et al.* (1970). The PCP conjugate was identified by precipitation with $BaCl_2$, extraction with xylene, thin layer chromatography, UV-absorption spectra and the molar ratio of PCP to SO_4. Glucuronide which is another typical conjugate of phenols in mammals was not detected.

The present study shows that fish possess a detoxication mechanism for drugs, at least chlorophenols, which involves sulfate conjugation. This is contrary to the conclusion of BRODIE *et al.* (1962) that fish dispose of drugs only by passive diffusion through the gills without detoxication by conjugation or oxidation.

Both of sulfate and glucuronide conjugations are well-known as typical detoxication mechanisms for phenolic compounds in mammals. SATO *et al.*[1] reported that *o*-, *m*- and *p*-monochlorophenols were conjugated with sulfuric acid in liver slices of rat, dog and cat. TASHIRO *et al.*[2] reported that in rabbit a part of the orally administered sodium pentachlorophenolate (PCP-Na) was recovered in urine as pentachlorophenyl β-glucuronide.

Few studies, however, have been made of the conjugation of drugs by fish. Although there are some discussions on the presence in fish of uridine diphosphate glucose (UDPG), UDPG dehydrogenase, UDP glucuronic acid (UDPGA), UDP glucuronyltransferase, etc. which are concerned with the glucuronide conjugation, there is no established theory, as reviewed by DUTTON.[3] According to NAGAYAMA *et al.*[4], the glucuronide conjugation undetectable in fish may be attributed to the weak activity of UDP glucuronyltransferase rather than the lack of UDPG dehydrogenase as reported by BRODIE *et al.*[5]

On the other hand, there is no study of sulfate conjugation by fish. Sulfate conjuga-

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tion is well-known as an important mechanism of detoxication for phenolic compounds in mammals. In any case, it has been believed that fish cannot dispose of lipid-soluble substances by detoxication, but only by passive diffusion through the gills, according to BRODIE *et al.*[5]

In previous papers[6-8], it was found that the rapid decrease of free-PCP in the medium during the culture of the short-necked clam was due to the transformation of PCP to pentachlorophenylsulfate by the shell-fish rather than accumulation in the body.

It has also been reported that the PCP concentration gradually decreased in the media during the culture of fish, such as rainbow trout[9] and carp[10], but the amount of PCP accumulated by the fish was smaller than that lost from the media. Although a similar decrease in media was observed in goldfish, the decomposition of PCP by the fish was small, as reported in our preceding paper.[11] The present paper deals with confirmation of the excretion of a conjugated PCP by goldfish, and isoits lation and identification.

Materials and Methods

Excretion of a bound-PCP by goldfish One hundred goldfish (each approx. 1 g in body weight) were placed in 20 *l* of 0.25 ppm PCP. After 24 hr exposure, 30 fish were removed and the amount of PCP was immediately determined. The remainder were divided into 7 groups of 10 fish each. Each group was transferred to 10 *l* of PCP-free water. At selected times during the course of the experiment (4 to 62 hr), the amount of PCP retained in the fish of each group and the amounts of free- and bound-PCP excreted in the medium were determined by the 4-aminoantipyrine method, as described in a previous paper.[7] The temperature of the media was kept at 20±1°C.

Isolation of a conjugated PCP excreted by goldfish It is difficult to collect directly a

Scheme 1. Isolation of conjugated PCP excreted by goldfish

240 fish (each 30–40 g in body weight) which had accumulated a total of 168 mg PCP by exposure to 560 *l* of 0.5 ppm PCP for 15 hr at 20±1°C

↓ Place in 560 *l* of PCP-free water for 24 hr at 20±1°C.

Medium containing conjugated PCP excreted by the fish

↓ Filter with Toyo No. 2 filter paper and adsorbed on activated charcoal columns at 5°C.

Activated charcoal columns (charcoal, 30 g; column size, 5.3 $cm^2 \times 30$ cm; two columns)

↓ Wash columns with water and elute with acetone-NH_4OH (100: 5).

Eluate

↓ Concentrate under reduced pressure at 50°C and pass through a Sephadex G-10 column.

Sephadex G-10 column (G-10, 100 g; column size, 2.5 $cm^2 \times 70$ cm)

↓ Elute with water at 5°C.

Conjugated PCP in water

large quantity of fish excreta. Therefore, bound-PCP excreted by goldfish was isolated as follows: Two hundred and forty goldfish having an average body weight of 35 g were placed in 560 *l* of 0.5 ppm PCP for 15 hr. After exposure, the fish were transferred to 560 *l* of PCP-free water and cultured for 24 hr. The temperature of both media was kept at 20±1°C.

A conjugate containing 96.6 mg PCP was excreted in the PCP-free water during the 24 hr culture period. The conjugated PCP was isolated by treating the medium as shown in Scheme 1. The isolated conjugate was stored in aqueous solution at 5°C, because it was easily decomposed in organic solvents, such as acetone and ethanol.

Identification of the conjugated PCP excreted by goldfish The isolated conjugate in acidic solution was easily decomposed on heating. The precipitation obtained on addition of $BaCl_2$ indicated the presence of sulfate as a constituent of the conjugate. Further identification of the isolated conjugate was carried out by comparing it with PCP–Na and synthesized potassium pentachlorophenylsulfate (PCP–SO_3K) as follows.

1. Extraction with xylene from acidic solutions: The amount of PCP in the xylene extracts from the acidic solutions (0.02 N HCl) of the conjugate, PCP–Na and PCP–SO_3K was determined by the 4-aminoantipyrine method.

2. Extraction with xylene from steam-distillates: The acidic solutions (0.6 N HCl) of the conjugate, PCP–Na and PCP-SO_3K were subjected to a steam-distillation. The amount of PCP in the xylene extract from each distillate was determined by the method described above.

3. Thin layer chromatography: Thin layer chromatography was carried out on the conjugate, PCP-Na and PCP-SO_3K, using Wakogel B–0 as the adsorbent and acetone—n-hexane mixtures with ratios of 1:0.7 and 1:1 as the developing solvent. The spots were detected by spraying an aqueous solution of 1% $KMnO_4$.

4. UV-absorption spectra: The ultraviolet absorption spectra of aqueous solutions of the conjugate, PCP–Na and PCP–SO_3K were measured with HITACHI EPS-3T spectrophotometer.

5. Molar ratios of PCP to SO_4 in the conjugate and in PCP–SO_3K: PCP determinations were carried out on both steam-distillates of the conjugate and PCP–SO_3K by the 4-aminoantipyrine method. The amounts of SO_4 were determined by the St. LORANT method.[12]

6. Determination of glucuronic acid in the conjugate: The presence of glucuronide, which is another typical conjugate of phenols in mammals, was determined by measuring the amount of glucuronic acid in the conjugate and other fractions from the Sephadex G–10 gel filtration, by the carbazole method.[13,14]

Results and Discussion

Excretion of a bound-PCP by goldfish After a 24 hr exposure of 100 goldfish (93 g total body weight) to 20 *l* of 0.25 ppm PCP, the concentration of free-PCP in the medium decreased to 32% of its initial value. Of the PCP initially present in the medium 46% (2.3 mg) was found in the fish. The remainder of the PCP was not decomposed, but was in a bound-form in the medium, resulting, when added to the 2.3 mg found in the fish, in a recovery of almost 100% of the total quantity of PCP contained in the original 20 *l* of culture medium.

Fig. 1 shows the change with culture time in the amount of PCP retained by fish which had accumulated 24.8 μg-PCP/g-body weight and also the amounts of free- and bound-PCP excreted in the medium, when the fish were transferred to PCP-free water. Values given are expressed as percent of the initial amount of PCP in the fish of each group. The amount of PCP retained in the fish rapidly decreased to half the initial value after 17 hr, as was reported previously.[15] The PCP excretion increased with culture time with a corresponding decrease in the fish. Most of the PCP excreted was in a bound-form and only 10% was found as free-form PCP. The free-PCP was excreted shortly after the fish had been transferred to PCP-free water and its subsequent decrease (Fig. 1) in the medium was probably due to reabsorption by the fish. The bound-PCP excreted in the medium was a conjugated PCP biosynthesized by the goldfish.

Isolation of conjugated PCP excreted by goldfish 240 goldfish (each 30–40 g in body weight) which had accumulated a total of 168 mg PCP, excreted a total of 96.6 mg of conjugated-PCP and 8.4 mg of free-PCP, during the 24 hr culture period.

In the procedure shown in Scheme 1, PCP and its conjugate in the medium were almost

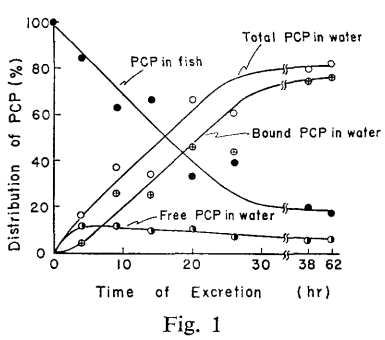

Fig. 1

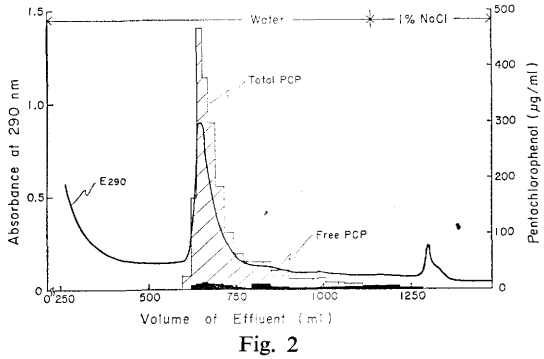

Fig. 2

Fig. 1. Change in amounts of PCP in goldfish and in free- and bound-PCP excreted in PCP-free water.
Amounts are expressed as percent of the initial amount of PCP (24.8 μg/g-body weight) in the fish.

Fig. 2. Elution pattern of the conjugated PCP excreted by goldfish on Sephadex G-10 column.
Sephadex G-10, 100 g; column size, 2.5 $cm^2 \times 70$ cm; flow rate, 1.2–5.0 ml/hr; temperature, 5°C.

completely adsorbed on activated charcoal columns. With the treatment of acetone-ammonia mixture 76% of the adsorbed PCP and its conjugate was eluted. The eluate was concentrated under reduced pressure and passed through a Sephadex G-10 column, resulting in the elution patterns shown in Fig. 2. The acetone remaining in the concentrated eluate, which interferes with the determination of glucuronic acid by the carbazole method, was completely eliminated from the conjugate fraction by the gel filtration. Although a small amount of free-PCP was detected in the conjugate fraction, it did not affect the elution pattern of the conjugate based on the absorbance at 290 nm. This agreed with another elution pattern based on the amount of PCP released from the conjugate by steam-distillation. A conjugate containing 44 mg PCP was finally obtained from the peak fraction.

Identification of the conjugated PCP excreted by goldfish

1. Extraction with xylene from acidic solutions: The amounts of PCP directly extracted with xylene from the acidic solutions of the conjugate, PCP-Na and PCP-SO_3K are shown in Table 1.

Table 1. Change in values for xylene extractable PCP before and after steam-distillation of aqueous solutions of PCP-Na, conjugated PCP and synthesized PCP-SO_3K. Values given are expressed as μg-PCP/m*l*-solution.

	PCP-Na	Conjugated PCP	Synthesized PCP-SO_3K
Xylene extract from acidified (0.02 N HCl) aqueous solution	312	10.7	0.8
Xylene extract from steam-distillate of acidified (0.6 N HCl) aqueous solution	304	467.9	383.1

2. Extraction with xylene from steam-distillates: The amounts of PCP extracted with xylene from the steam-distillates of the conjugate, PCP-Na and PCP-SO_3K are shown in Table 1.

As shown in Table 1, PCP-Na is directly extracted with xylene from an acidic (0.02 N HCl) aqueous solution, but the conjugate and PCP-SO_3K is almost unextracted. Both the conjugate and PCP-SO_3K, however, are easily hydrolyzed in acidic (0.6 N HCl) aqueous solution on heating, yielding free-PCP which is then released by steam-distillation.

3. Thin layer chromatography: Thin layer chromotograms of the conjugate, PCP-Na and PCP-SO_3K are shown in Fig. 3. The Rf value of the conjugate agreed with that of PCP-SO_3K, using two developing solvents with different mixing ratios of acetone to n-hexane. The mixture of the conjugate and PCP-SO_3K gave only one spot on the chromatogram.

4. UV-absorption spectra: Fig. 4 shows the UV-absorption spectra of aqueous solutions of the conjugate, PCP-Na and PCP-SO_3K. Absorption maxima of PCP-Na

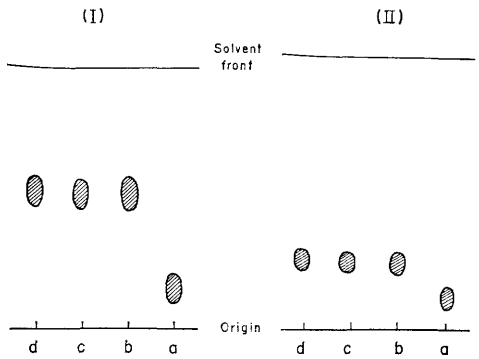

Fig. 3

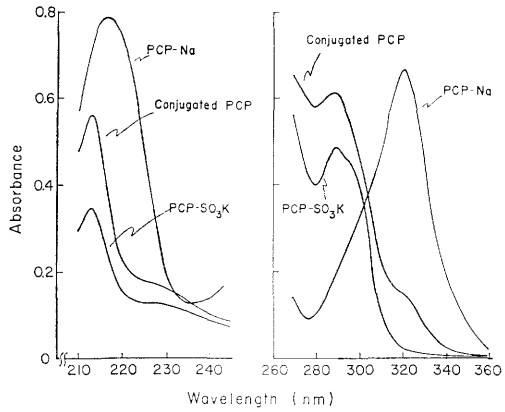

Fig. 4

Fig. 3. Thin layer chromatograms of PCP-Na, conjugated PCP and synthesized PCP-SO_3K.
Sample: a, PCP-Na; b, conjugated PCP; c, synthesized PCP-SO_3K; d, b+c.
Adsorbent: Wakogel B-0.
Solvent system: (I) acetone: n-hexane (1: 0.7); (II) acetone: n-hexane (1: 1).
Color developer: 1% $KMnO_4$ aqueous solution.

Fig. 4. Ultraviolet absorption spectra of PCP-Na, conjugated PCP and synthesized PCP-SO_3K in water.

Table 2. Molar ratios of PCP to SO_4 in conjugated PCP and in synthesized PCP-SO_3K

	Conjugated PCP	Synthesized PCP-SO_3K
PCP found (μ mole)	1.495	1.438
SO_4 found (μ mole)	1.610	1.560
PCP/SO_4	0.929	0.922

in the ultraviolet region were found at 217 and 320 nm, but those of the conjugate and PCP-SO_3K were found at 213 and 288 nm.

5. Molar ratios of PCP to SO_4 in the conjugate and in PCP-SO_3K: The analytical values obtained are shown in Table 2. The molar ratio of PCP to SO_4 in the conjugate was 0.929 which was in good agreement with that found in PCP-SO_3K (0.922).

6. Determination of glucuronic acid in the conjugate: The glucuronic acid was not detected in the conjugate fraction or in any other fractions of the effluent from the gel filtration. The glucuronic acid added to an aqueous solution of the conjugate was completely recovered, indicating no interfering substances in the conjugate solution.

From these results, the conjugate excreted by goldfish was identified as pentachlorophenylsulfate which is the same as that found in the short-necked clam. No other conjugates of PCP were detected. Even if other conjugates are excreted by goldfish, they would be present in trace amounts only when compared to the abundance of sulfate conjugated PCP.

It is very interesting from the comparative biochemical view point that goldfish and

the short-necked clam do conjugate PCP with sulfate unlike the PCP-glucuronide excreted in the urine of rabbits after oral administration of PCP–Na, TASHIRO *et al.*[2]

It will be shown in subsequent papers that goldfish also conjugate phenol with sulfate. This suggests that fish possess a detoxication mechanism for various phenolic compounds by sulfate conjugation, contrary to the conclusion of BRODIE *et al.*[5]

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