Phenol-Sulfate Conjugating Activity in Liver-Soluble Fraction of Fish

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The phenol-sulfate conjugation by the liver subcellular fractions from goldfish Carassius auratus, carp Cyprinus carpio, rainbow trout Salmo gairdneri, and Tilapia nilotica has been studied using [35S] K2S4.

The cell fractionation of goldfish liver was examined by the determination of respective markers for nuclear, mitochondrial, lysosomal, microsomal, and soluble fractions. Among those subcellular fractions, only the soluble fraction displayed the conjugating activity. The maximum activity of the enzyme in the soluble fraction was obtained in the presence of 20 μmol of both ATP and Mg2+ in 1.5 ml of medium, although the activity decreased with an excess of ATP and Mg2+. This conjugation was inhibited by more than 0.1 μmol of Ca2+ in 1.5 ml of medium. The enzyme exhibited the conjugating activity in the pH range 7.5-9.5; the optimum value was at 8.

In the temperature range 5-40°C, the maximum conjugation in goldfish, Tilapia, and carp occurred at 35°C, in rainbow trout it occurred at 30°C. The phenol-sulfate conjugating activities in goldfish, carp, rainbow trout, Tilapia and albino rat were approximately 340, 82, 66, 200 and 720 nmol/g-liver per h, respectively.

In our previous papers,1-6 it has been demonstrated that goldfish Carassius auratus, is able to detoxicate phenol and pentachlorophenol by both mechanisms of the sulfate and glucuronide conjugations. Their sulfate conjugates are excreted in surrounding water1,2 and also in the urine of the fish,3,4 while the glucuronide conjugates are excreted in the bile.4,5

In the both detoxication mechanisms, however, the sulfate conjugation is more important in the fish, especially from a viewpoint of the elimination of phenols from the fish body, because the phenols released from their respective glucuronides after secretion into the intestine with bile must be reabsorbed there, as shown in our paper.7

Although the sulfate conjugation of phenol by liver slices from several fishes has been demonstrated,8 the present study was undertaken to confirm the presence of the enzyme responsible for the sulfate conjugation of phenol in various subcellular fractions of the liver from goldfish, and also to demonstrate some properties of the enzyme.

The enzyme activities of the liver-soluble fractions from carp Cyprinus carpio, rainbow trout Salmo gairdneri, Tilapia nilotica and albino rat were also determined.

Materials and Methods

Test Animals

The approximate body weights of fishes and rats used in the experiment were as follows: goldfish, 60 g; carp, 60 g; rainbow trout, 250 g; Tilapia, 70 g; albino rat, 100 g.

Preparation of Liver Subcellular Fractions

After two days starvation, fishes were dissected after blood-letting by decapitation. The livers obtained from the fishes were cut into slices and washed with ice-cold 0.9% NaCl solution, removing the connective and adipose tissues. Then the liver slices were put into 9 volumes of ice-cold 0.25 M sucrose-10 mm tris (hydroxymethyl) aminomethane (Tris)-HCl buffer solution (pH 7.5) and homogenized by a Potter type glass-teflon homogenizer in a cold room at 5°C. The homogenate was fractionated by a procedure8 shown in Fig. 1, using an ultracentrifuge (Hitachi 55P-2; rotor RP-65).

Analysis of Each Subcellular Fraction

The cell fractionation was confirmed by the


*2 Mostly according to S. Mayemura's thesis for Master Degree of Science, Kyushu University, Fukuoka, Japan (1975).
Fish liver homogenate with 0.25 M sucrose-10 mm Tris-HCl buffer solution (pH 7.5) was centrifuged at 900 × g for 10 min. The supernatant (Nuclear fr.) was centrifuged at 5,000 × g for 10 min. The supernatant (Mitochondrial fr.) was centrifuged at 10,000 × g for 15 min. The supernatant (Lysosomal fr.) was centrifuged at 100,000 × g for 60 min. The precipitate (Soluble fr.) was centrifuged at 100,000 × g for 60 min. Determination of Phenol-Sulfate Conjugating Activity

The phenol-sulfate conjugating activity of each subcellular fraction of fish liver was determined by a procedure shown in Fig. 2, using [35S]-K2SO4.

Measurement of Radioactivity

The precipitate of BaSO4 containing 35SO4 was filtered off on the Millipore filter (pore size, 0.45 μ; diam., 25 mm) as a uniform thin layer of 18 mm in diameter. The radioactivity of the precipitate was measured by a G-M counter (Aloka TDC-1) with a mica window (1.5 mg/cm²).

Effects of Some Factors on Phenol-Sulfate Conjugation

To demonstrate some properties of the enzyme responsible for the phenol-sulfate conjugation, the effects of ATP, Mg2+ and Ca2+ concentrations on the activity of the enzyme in the soluble fraction of goldfish liver were examined.

0.5 ml aliquots of subcellular fractions of fish liver

Add 0.5 ml of 0.5 M Tris-HCl buffer (pH 8) and each 0.1 ml of 0.2 M KCl, 0.2 M MgCl2, 0.2 M ATP-NA, 0.01 M [35S] K2SO4 and 0.05 M phenol.

Incubate for 2 h at 30 or 35°C.

Add 1 ml of 25% TCA and centrifuge at 1,600 × g for 5 min.

Wash with 2 ml of 5% TCA.

Centrifuge at 1,600 × g for 5 min.

Add 0.2 ml of 0.3 M K2SO4 and 0.5 ml of 6% BaCl2.

Filter with Millipore filter.

Repeat 3 times this precipitation to remove free 35SO4.

BaSO4 (free 35SO4)

Add 2 ml of 2 N HCl and heat for 20 min at 100°C.

Add 0.2 ml of 0.3 M K2SO4 and 0.5 ml of 6% BaCl2.

Filter with Millipore filter.

BaSO4 (conjugated 35SO4)

Subject to measurement of radioactivity.

Fig. 2. Determination of phenol-sulfate conjugating activity in each subcellular fraction of fish liver.
The effects of pH and temperature on the enzyme activity were also examined, using 0.5 M sodium acetate-HCl buffer for pH 3-6 and 0.5 M Tris-HCl buffer for pH 6.5-9.5.

Results and Discussion

Analysis of Each Subcellular Fraction

Fig. 3 shows the protein distribution and the DNA content in each subcellular fraction of goldfish liver. Approximately the half amount of protein was found in the soluble fraction.

As shown by the DNA content in Fig. 3 and the enzyme activities in Fig. 4, the cell fractionation of goldfish liver was fairly successful. For example, the activity of succinate-cytochrome c reductase which is the marker enzyme of mitochondria shows low contamination of the microsomal fraction with mitochondria. Among the marker enzymes, however, β-glycerophosphate phosphatase (acid) was not satisfactory as a marker of the lysosomal fraction.

Phenol-Sulfate Conjugating Activity

Table 1 shows the phenol-sulfate conjugating activities in the nuclear, mitochondrial, lysosomal, microsomal and soluble fractions prepared from goldfish liver. Among those subcellular fractions, only the soluble fraction displayed the conjugating activity.

Table 1. Phenol-sulfate conjugating activities in various subcellular fractions of goldfish liver

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Activity (nmol/g-liver per h)</th>
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</thead>
<tbody>
<tr>
<td>Nuclear</td>
<td>ND</td>
</tr>
<tr>
<td>Mitochondrial</td>
<td>ND</td>
</tr>
<tr>
<td>Lysosomal</td>
<td>ND</td>
</tr>
<tr>
<td>Microsomal</td>
<td>ND</td>
</tr>
<tr>
<td>Soluble</td>
<td>338</td>
</tr>
</tbody>
</table>

ND: Not detected.

The activity of sulfatase which might hydrolyze the phenyl sulfate produced in the reaction mixtures with respective subcellular fractions was also determined under the same condition as in the assay of the phenol-sulfate conjugation, using p-nitrophenyl sulfate as the substrate. All the fractions examined, however, did not exhibit any sulfatase activity under this condition.

As shown in Fig. 5, the amount of the [35S] sulfate conjugated with phenol by the soluble fraction increased almost linearly with time at least up to a 2-h incubation.

Fig. 5. Phenol-sulfate conjugation by the liver-soluble fraction of goldfish.

Effects of Some Factors on Phenol-Sulfate Conjugation

1. ATP, Mg²⁺ and Ca²⁺: Fig. 6 shows the effects of ATP, Mg²⁺ and Ca²⁺ concentrations on
Fig. 6. Effects of ATP, Mg\(^{2+}\) and Ca\(^{2+}\) on phenol-sulfate conjugation by the liver-soluble fraction of goldfish.

The values in the figure express the respective amounts (\(\mu\text{mol}\)) of ATP, Mg\(^{2+}\) or Ca\(^{2+}\) added into 1.5 ml of each medium.

The phenol-sulfate conjugating activity of the enzyme in the soluble fraction of goldfish liver. The total volume of each reaction mixture was 1.5 ml. The maximum activity was obtained in the presence of 20 \(\mu\text{mol}\) of both ATP and Mg\(^{2+}\). By adding excess ATP, however, the enzyme activity decreased, as reported by SEGAL\(^{14}\) on the 18,000xg supernatant of rat liver. The enzyme activity also decreased in the presence of an excess of Mg\(^{2+}\). This conjugation was inhibited approximately by 10, 25 and 85\% in the presence of 0.1, 1 and \(10\ \mu\text{mol}\) of Ca\(^{2+}\) in 1.5 ml of media, respectively.

2. pH: Fig. 7 shows the effect of pH on the enzyme activity. The enzyme exhibited the conjugating activity in the pH range 7.5-9.5, optimally at pH 8.

3. Temperature: Fig. 8 shows the effect of temperature on the activity of the phenol-sulfate conjugation in the soluble fractions prepared from the livers of goldfish, carp, rainbow trout and Tilapia. The maximum conjugation in goldfish, carp and Tilapia occurred at 35\(^\circ\text{C}\), whereas that in rainbow trout at 30\(^\circ\text{C}\). However, the conjugating activities in all the test fishes declined markedly at 40\(^\circ\text{C}\).

ADAMSON has mentioned in his review paper\(^{13}\) on the drug metabolism in marine vertebrates that the reaction of some drug metabolizing enzymes in the subcellular fractions of rainbow trout liver occurred optimally at 20-26\(^\circ\text{C}\). The difference in temperature effect on the phenol-sulfate conjugating activity among the test fishes must be attributed to the reflection of their optimum water temperature.

Table 2. Phenol-sulfate conjugation by liver-soluble fractions of several fishes and albino rat

<table>
<thead>
<tr>
<th>Species</th>
<th>Number of repeated experiments</th>
<th>Conjugated (^{35}\text{S-}\text{SO}_3) (nmol/g-liver per h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goldfish</td>
<td>20</td>
<td>340 (\pm) 64(^{4,2})</td>
</tr>
<tr>
<td>Carp</td>
<td>2</td>
<td>82 (\pm) 1(^{2})</td>
</tr>
<tr>
<td>Rainbow trout</td>
<td>3</td>
<td>66 (\pm) 7(^{1})</td>
</tr>
<tr>
<td>Tilapia</td>
<td>2</td>
<td>200 (\pm) 48(^{2})</td>
</tr>
<tr>
<td>Albino rat</td>
<td>5</td>
<td>720 (\pm) 36(^{3})</td>
</tr>
</tbody>
</table>

\(^{1}\) Assayed at 30\(^\circ\text{C}\).

\(^{2}\) at 35\(^\circ\text{C}\).

\(^{3}\) at 37\(^\circ\text{C}\).

Table 2 shows the phenol-sulfate conjugating activities in the liver-soluble fractions from the test fishes and albino rat. Although the fairly large individual variation was observed, goldfish
showed the highest activity among the test fishes, corresponding to approximately half of that in albino rat. The individual variation in the activity might be due to the previous exposure to drugs. The induction of the enzyme activity by pre-exposure to phenol will be shown in subsequent papers.

Acknowledgements

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References