Effects of pH and Temperature on Mixed-Function Oxidases in the Liver of Cultured Fish*1

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Effects of pH and temperature on two mixed-function oxidases, aniline-p-hydroxylase and aminopyrine-N-demethylase, in the liver of cultured carp Cyprinus carpio, eel Anguilla japonica, yellowtail Seriola quinquergadiata, and red sea bream Pagrus major were studied.

The pH optima for aniline-p-hydroxylase were 7.5, 7.8, 7.5, and 7.3 in carp, eel, yellowtail, and red sea bream, respectively; and those for aminopyrine-N-demethylase were 7.3 in carp and 7.5 in other fishes. After 30 min incubation, aniline-p-hydroxylases of carp and yellowtail showed the highest activity at 33°C and those of other fishes at 27°C. After 15 min incubation, aminopyrine-N-demethylase showed the highest activity at 35°C, 37°C, and 40°C in red sea bream, carp, and two other fishes, respectively.

Liver mixed-function oxidases in a variety of organisms play an important role in the metabolism of many compounds and xenobiotics, and it is well known that environmental pollution and physiological conditions of organisms alter their activities in the liver.1-4) Thus, it is possible that these enzyme activities can serve as good indices to environmental pollution and physiological conditions of cultured fish. However, the mixed-function oxidase activities are much lower than in mammals and cannot be detected when assayed under the conditions normally used for mammalian enzymes. Because of this low activity, it was even believed until early 1960's5) that fish cannot metabolize drugs. It is therefore of value to establish the method by which the mixed-function oxidase activities of fish can be measured accurately.

Considering the importance of the mixed-function oxidases in fish, especially their relations to environmental pollution and fish pathology, we have attempted to establish optimal assay methods for their activities. From many chemical compounds that are used as the substrate for the enzyme, aniline and aminopyrine were chosen, because (1) the enzymes which metabolize these chemicals, i.e., aniline-p-hydroxylase and aminopyrine-N-demethylase, have been well examined in various species of animal and (2) special apparatus is not necessary to measure their activities. This paper deals with the effects of temperature and pH on these two enzymes in the liver of four cultured fishes.

Materials and Methods

Materials
Carp Cyprinus carpio, eel Anguilla japonica, yellowtail Seriola quinquergadiata, and red sea bream Pagrus major were obtained from fish farms in Miyazaki Prefecture. Aminopyrine was purchased from Aldrich, and NADP*3 and glucose 6-phosphate were obtained from Sigma. Other reagents were of analytical grade.

Enzyme Solutions
Fishes were anesthetized with MS-222 or ethyl carbamate. The liver was immediately removed and placed on crushed ice. Twenty percent (w/v) homogenate in 50 mM Tris-HCl buffer (pH 7.4) containing 0.25 M sucrose and EDTA*4 was prepared at 0°C with a Potter homogenizer equipped with Teflon pestle. The homogenate was then centrifuged at 9,000×g for 20 min at 4°C. The supernatant was used for the enzyme assay. It is well known that mixed-function oxidases are localized in the microsomal fraction of the liver. In order to use these enzymes as indices to environ-
mental pollution and physiological conditions of fish, their activities in many individuals must be analyzed. Moreover, it is difficult to obtain the amount of microsome required for the enzyme assay from the liver of small fish. Therefore, we used the supernatant as the enzyme solution. The mixed-function oxidases require NADPH for their activities. Glucose-6-phosphate dehydrogenase is localized in the cytosol fraction and is thus present in 9,000 × g supernatant. Therefore, we have chosen glucose-6-phosphate dehydrogenase as a NADPH generator. In mammals, 9,000 × g supernatant is widely used in the studies of the metabolism of drugs.6)

Enzyme Assays

Aniline-p-hydroxylase and aminopyrine-N-demethylase were assayed by the method of Kamataki.6) For aniline-p-hydroxylase, the reaction mixture contained, in a final volume of 3.0 ml, 0.5 ml of enzyme solution, 22 mM aniline, 0.8 mM MgCl₂, 8 μM MnCl₂, 130 μM NADP, 5 mM glucose-6-phosphate, and either of the following buffers; 50 mM sodium phosphate buffer (pH 6.0-6.7), Tris-HCl buffer (pH 7.0-8.0) and glycine NaOH buffer (pH 8.3-9.3). The mixture was incubated for 30 min at 15-50°C. After incubation, reaction was stopped by cooling in crushed ice. Two ml of the mixture was withdrawn and mixed with 2.0 ml of saturated NaCl and 25 ml of peroxide-free ether. This mixture was shaken for 10 min and then centrifuged at 2,000 rpm for 5 min. Twenty ml of upper ether layer was withdrawn and 3 ml of 1% phenol in 0.1 N NaOH was added to it. The mixture was shaken for 5 min and centrifuged at 2,000 rpm for 5 min. The upper ether layer was discarded. After 30 min, the absorbance of the aqueous layer at 620 nm was measured.

For aminopyrine-N-demethylase activity, the reaction mixture contained, in a final volume of 3.0 ml, 0.5 ml of the enzyme solution, 16.7 mM aminopyrine, 0.8 mM MgCl₂, 8 μM MnCl₂, 5 mM glucose-6-phosphate, 130 μM NADP, 5 mM neutralized semicarbazide hydrochloride, and either of the following buffers; 50 mM sodium phosphate buffer (pH 6.3-6.7), Tris-HCl buffer (pH 7.0-8.0), and glycine NaOH buffer (pH 8.3-9.3). After incubation for 15 min at 15-50°C, the reaction was stopped by the addition of 2 ml of 15% ZnSO₄. The mixture was allowed to stand for 5 min at room temperature, and 2 ml of saturated Ba(OH)₂ was added to it. It was then vigorously shaken, kept for 5 min at room temperature, and centrifuged at 3,000 rpm for 5 min. The amount of formaldehyde formed in the supernatant was determined according to the methods of Nash7) modified by Cochran and Arendro.8)

The reaction mixture without substrate was routinely used as a blank. All enzyme assays were carried out in triplicate and product formation was calculated from calibration curves. The enzyme activities are expressed in n mole of the product formed/g tissue/hr.

Results and Discussion

Effects of pH on Aniline-p-hydroxylase and Aminopyrine-N-demethylase

Fig. 1 shows the effects of pH on aniline-p-
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hydroxylase activity in the liver of four fishes. The pH optima appeared at 7.5 in carp and yellow-tail, at 7.3 in red sea bream and at 7.8 in eel. Fig. 2 shows the effects of pH on aminopyrine-N-demethylase activity in the liver of four fishes. The pH optima appeared at 7.3 in carp and at 7.5 in other fishes. Buhler and Rasmussen9) have reported that the optimum pH of aniline-p-hydroxylase in rainbow trout is approximately 7.4. Their result is similar to the results presently obtained in carp, yellowtail and red sea bream. Dewaide and Henderson10) have reported that the optimum pH of aminopyrine-N-demethylase of rainbow trout is about 8.0. Their result is different from the results obtained in four fishes used in the present study.

Effects of Incubation Temperature on Aniline-p-Hydroxylase and Aminopyrine-N-demethylase

Fig. 3 shows the effects of incubation temperature on aniline-p-hydroxylase activity of four fishes. After 30 min incubation, aniline-p-hydroxylase of carp and yellowtail showed the highest activity at 33°C, and those of eel and red sea bream showed at 27°C. Fig. 4 shows the effects of incubation temperature on aminopyrine-N-demethylase activity of four fishes. After 15 min incubation, the highest activity was observed at 37°C, 40°C, 40°C, and 35°C in carp, eel, yellowtail, and red sea bream, respectively. In contradiction to these results, Buhler and Rasmussen9) have reported that aminopyrine-N-demethylase of rainbow trout shows the highest

Fig. 3. Temperature dependence of aniline-p-hydroxylase activity in the liver of four cultured fishes.

Fig. 4. Temperature dependence of aminopyrine-N-demethylase activity in the liver of four cultured fishes.
Fig. 4. Temperature dependence of aminopyrine-N-demethylase activity in the liver of four cultured fishes.

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