KOREAN JOURNAL OF ZOOLOGY
Vol. 16, No. 2, 79~96 (1973)

Studies on the Effects of Copper on the Lactate Dehydrogenase and Esterase Isozymes in Various Tissues of *Carassius carassius*

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붕어(*Carassius carassius*)의 조직내 젖산수소이탈효소와 에스테라아제 아이소자임에 미치는 동의 영향에 관한 연구

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(Received March 2, 1973)

적 요

붕어(*Carassius carassius*)에 미치는 동의 영향을 밝히기 위하여 다음과 같은 연구를 하였다. 1) 셀루로오스 아세테이트 전기영동법에 의한 젖산수소이탈효소 아이소자임 상, 2) 분광비색법에 의한 LDH활성과 LDH효소계에 대한 동의 영향, 3) 한천 박층 전기영동법에 의한 에스테라아제 아이소자임 상, 4) 전분 겔 전기영동법에 의한 혈색소 상, 및 5) 조직학적 연구.

1. LDH 아이소자임 영동대는 정상 붕어의 아가미에 2개 (LDH-3 및 LDH-5), 간에 3개 (LDH-2, LDH-4, 및 LDH-5), 그리고 근육에서 2개 (LDH-3 및 LDH-4) 나타났다. LDH-1은 이상의 세가지 조직에서 나타나지 않았다. 동을 처리한 붕어의 간에서는 LDH-3이 나타났고 근육에서는 LDH-5가 나타났다. 그러나 아가미에서는 새로운 영동대가 나타나지 않았다.

2. LDH활성은 정상 붕어의 아가미, 간, 및 근육 중 아가미에서 가장 낮았고 근육에서 가장 높았으며, 아가미를 제외한 간과 근육에서는 1일부터 10일간의 동처리에 따라 점차적으로 감소되었다.

3. 동을 처리한 붕어의 간과 근육의 LDH활성 감소는 주로 체내 M-LDH에 대한 동의 억제에 기인된다.

4. 정상 붕어의 아가미, 간, 근육, 혈액, 뇌, 및 신장의 에스테라아제 아이소자임 영동대 수는 각 각 3, 6, 2, 2, 2, 및 2개였고 이들은 동을 처리한 경우에서도 같았다. 동처리군의 아가미, 간, 혈액, 그리고 신장의 에스테라아제 영동대의 상대이동도는 대조군의 그것들

과 상이하였다.

5. 정상 붕어의 혈색소 영동대는 양극에 1개 있었다. 동을 처리한 붕어의 혈색소 영동대의 이동도는 정상 붕어의 그 것 보다 약간 빠른 것으로 보였다.

6. 동(20 ppm)에 5일간 처리한 붕어의 조직학적 연구는 다음과 같았다.

1) 아가미에서는 처음에 표피층이 분리되었다가 분해된 후 완전히 파괴되었다.

2) 간에서는 많은 지방립이 나타났다.

3) 정상 붕어와 동에 처리된 붕어의 근육 사이에서는 조직학적 차이가 없었다.

4) 신장조직은 기부요세관에 손상을 받았다. 요세관세포의 높이가 작아진 후 요세관의 내강이 확대되었다. 많은 기부요세관이 분홍빛의 과립성 형태와 여러 단계의 변성을 나타내었다.

INTRODUCTION

Copper is one of the chemicals in algicides and wastes from industrial plants. The frequent introduction of copper into the aquatic environment and the sensitivity of aquatic life to the pollutant may be serious biological problems of fish toxicology.

When fishes were exposed to copper, the copper content level in the body was increased (Kariya *et al.*, 1967) and the survival, growth, and reproduction were decreased (Mount and Stephan, 1969; McKim and Benoit, 1971). Jackim *et al.*(1970) proposed that the changes in liver enzyme activity might be useful as a kind of biochemical autopsy tool for diagnosing sublethal metal poisoning in fish.

The enzyme lactate dehydrogenase (LDH) occurs in the tissues of many organisms in multiple molecular forms (termed isozymes by Markert and Møller, 1959). In mammalian tissues, five principal LDH isozymes usually are found. The five forms are all tetramer combinations of two kinds of subunits, known as H and M. Adult heart muscle sinthesizes predominantly the H subunit and contains an abundance of LDH-1, while most skeletal muscles produce primarily the M subunit resulting in a preponderance of LDH-5. In a study of LDH isozymes in 30 species of fish, Markert and Faulhaber (1965) found one major isozyme system in all fish and two minor systems restricted to eyes and gonads in many fish. Fishes resemble birds and mammals containing two basic functional types of LDH, heart and muscle. The LDH in the heart and muscle groups of trout appear to be homologous with the H_4 and M_4 enzymes of higher vertebrates (Bailey and Wilson, 1968). But the electrophoretic patterns found in many fishes are not the same as the five-isozyme patterns of higher vertebrates. Physico-chemical characterizations (Bailey and Wilson, 1968; Markert and Holmes, 1969; Whitt and Booth, 1970; Wunsch and Goldberg, 1970) and interspecies comparisons and genetic variations (Markert and Faulhaber, 1965; Clayton and Gee, 1969; Lush *et al.*, 1969; Utter

and Hodgins, 1969; Chen and Tsuyuki, 1970; Clayton and Franzin, 1970; Lush, 1970; Whitt, 1970; Williscroft and Tsuyuki, 1970; Massaro, 1972; Whitt *et al.*, 1972) of the fish LDH have also been studied.

There are distinct migratory patterns of the esterase isozymes in three tissues of carp, funa, and their hybrids (Takayama *et al.*, 1966). In recent years, increased attentions have been attributed to esterase isozyme properties (Holmes *et al.*, 1968; Tsuyuki, 1970; Hogan, 1971) and esterase polymorphisms of fishes (De Ligny, 1968; Nyman, 1969).

The electrophoretic patterns of hemoglobins of various fishes have been reported (Yamanaka *et al.*, 1965, 1967; Ohno and Morrison, 1966; Tsuyuki *et al.*, 1966, 1969; Chen and Tsuyuki, 1970; Tsuyuki and Ronald, 1970; Westrheim and Tsuyuki, 1971).

Pathological changes attributable to copper poisoning (Baker, 1969) and cadmium (Gardner and Yevich, 1970) were observed in the liver, kidney, gills, and intestinal tract of fishes.

Copper, one of the chemical pollutants in water, may affect some of the enzymatic and histological activities of *C. carassius*, because this species is known to drink water. Although the study on the changes in enzyme activity and histology is one of the important fields in fish toxicology, little work has been done on this subject and little is known about the physiological responses of fish to copper.

The purpose of the present study is to investigate the effects of copper on the LDH isozyme patterns, activities, and *in vitro* experiments; esterase isozyme patterns; hemoglobin patterns; and histological structures in various tissues of *C. carassius*.

MATERIALS AND METHODS

Medium sized specimens, 140—170mm in total length, of *Carassius carassius* were used in the study. These fish were obtained from a live material supplier in Seoul.

Exposure System

The test tanks were polyethylene-lined wooden boxes of 150 liters in capacity and provided with aquarium filters. All tanks were aerated steadily, filtered, and covered with 5×5mm mesh plastic screens during the experimental period. Twenty fish were placed in each tank containing 100 liters of dechlorinated tap water. Copper was supplied in the form of copper sulfate solution; various concentrations were maintained by dropping the solution into the tanks at predetermined rates. The fish were exposed to 0.2 and 1.0 ppm of copper at a water temperature of about 17.5°C for 10 days for the LDH examination, and to 20 ppm of copper at

about 14°C for 5 days for the esterase, hemoglobin, and histological studies. One of the tanks, kept free of exogenous copper, acted as a control.

Water temperature was checked with an alcohol thermometer at 10 : 00 a.m. and the chemical oxygen demand (COD) was determined by the methods described by the American Public Health Association *et al.* (1965).

Homogenate Preparation

Tissues were immediately removed from the fish after treatment and frozen in a refrigerator. The weighed tissues were homogenized in distilled water at a half weight of the material and centrifuged at 20,000 g at 2°C for 20 minutes. The supernatants were then used for lactate dehydrogenases and esterases.

LDH Isozyme Patterns

Electrophoretic separation of the isozymes of lactate dehydrogenase was performed on the cellulose acetate strip for 90 minutes with a current of 1mA per strip or a constant voltage of 200 according to the method of Preston *et al.* (1965). A sample of homogenate was applied by serum applicator, 1.5 to 2.0 cm on the cathode side of the center of the cellulose acetate strip. After electrophoresis, the acetate strip was stained, and the LDH was visualized with nitroblue tetrazolium and phenazine methosulfate at 37°C. The isozymes of lactate dehydrogenase appeared as discrete blue bands. The strip was fixed in methanolacetic acid solution. The percentage of each zymogram was determined by densitometry. The bands of fish were compared with the standard of zymograms obtained from a mouse, and then numbered.

LDH Activity and Protein Assays

LDH activity was assayed spectrophotometrically at pH 7.4, 25°C, by measuring the changes in absorbance due to changes in the NADH concentration coupled with the reduction of pyruvate or the oxidation of lactate. Protein concentration was determined spectrophotometrically.

***In vitro* Experiment**

Purified M-LDH(rabbit muscle, Sigma Chemical Co.), NAD, and substrate(lactate) were put in three test tubes separately. In one of the three tubes, the copper sulfate solution of 1 ppm was added. Then a little amount of each sample from the above three tubes was taken and put into another test tube. In this way, the M-LDH, NAD, and substrate were treated by both 1 and 10 ppm of copper sulfate solutions respectively. Absorbance changes were measured by the method of LDH activity determination.

Esterase Isozymes

Electrophoretic separation of the isozymes of esterase was principally carried out with agar thin layer electrophoresis according to the method of Takayama *et al.* (1966). The supernatant from each sample was applied on the agar gel by

means of a capillary applicator. The agar plate was connected to the buffer solution (ionic strength, 0.05 μ) in electrode vessels with Whatman filter paper. Electrophoresis was carried out at a constant current of 1.5 mA/cm of the agar plate for 70 minutes at 4°C. After electrophoresis, the agar gel was covered with an alpha— and beta-naphthyl acetate solution and incubated at 37°C for 60 minutes. The esterae isozymes were developed by spreading a 2% aqueous solution of naphthanil diazo blue B. After staining, the agar plate was washed with water and dried in an incubator.

Hemoglobins

Blood sample was obtained by cutting the tail of live fish, and put into heparinized bottles. In preparing the hemoglobin solution, erythrocytes were washed two times with isotonic salt solution and hemolysed in a mixture of distilled water and toluene. After centrifugation at 16,000 rpm, 4°C for 30 minutes, clear red supernatant was used for electrophoresis. The gel buffer prepared for this system was Tris-EDTA-borate buffer adjusted to pH 8.9, and the cell buffer used was sodium-borate buffer solution. The starch gel was prepared by using potato starch in the usual way. Blood hemoglobins were separated by vertical starch gel electrophoresis at 100 v or 4-7 mA (3 v/cm) for 16 hours. After electrophoresis, the gels were sliced horizontally and developed with amido black 10B stain.

Histological Observations

Tissues taken for histological examination were gill, liver, muscle, and kidney. All the tissues were routinely fixed in Bouin's fluid, embedded in 56°C wax, sectioned at 6-8μ, stained with hematoxylin and eosin, and mounted in Canada balsam.

RESULTS AND DISCUSSION

Water Characteristics

Three groups of fish for LDH assays were kept in tanks at the copper concentrations of 0.0, 0.2, and 1.0 ppm for 10 days. During the 10-day exposure, the water temperatures in the tanks were checked on the lst, 5th, and 10th days

Table 1. Temperatures (°C) of the test water containing different concentration of copper

Period (day)	Concentration of copper (ppm)		
	0.0	0.2	1.0
1	17.5	17.5	17.5
5	17.3	17.3	17.3
10	17.5	17.5	17.5

(Table 1).

Water temperatures were relatively constant, ranging 17.3 to 17.5°C, throughout the experimental period.

Chemical oxygen demand determinations were made at the same time during the period. The values of COD were between 64.0 ppm and 84.0 ppm throughout the period (Table 2). The water temperatures and chemical oxygen demands did not vary greatly during the experimental period.

Table 2. Chemical oxygen demands (ppm) of water from the test tanks containing different concentration of copper

Period (day)	Concentration of copper (ppm)		
	0.0	0.2	1.0
1	74.0	64.0	80.0
5	80.0	84.0	84.0
10	80.0	84.0	80.0

General Behavior of Fish

When the fish were exposed to copper of 1 ppm at about 17°C, they began to swim around. After a few days, the mucus secretion from the gills was increased. Ten of the 20 fish died within 2 days at a 14°C of water temperature after the exposure to 20 ppm of copper. Kariya *et al.* (1967) reported that the survival time of goldfish was about 4 hours in 10 ppm of copper, and about 24 hours in 1 ppm of copper at 8 to 11°C. It seems that the fish, *C. carassius*, has higher tolerance to copper pollutant than goldfish.

1. EFFECTS OF COPPER ON LDH ISOZYMES

LDH Isozyme Patterns

The isozyme patterns of lactate dehydrogenase in the tissues of gill, liver, and muscle were investigated. The isozyme patterns of lactate dehydrogenase in the tissues of controlled *C. carassius* were compared with the patterns of isozymes from the tissues of the fish exposed to 1 ppm of copper for 10 days (Fig. 1). In figure 1, the normal gill, liver, and muscle had 2, 3, and 2 LDH isozyme bands of different relative mobilities respectively. The diagram of these zymograms suggests that essentially every normal tissue exhibits

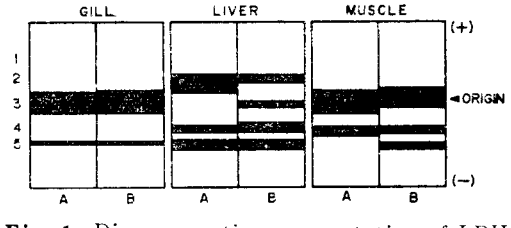


Fig. 1. Diagrammatic representation of LDH isozyme patterns in various tissues of *C. carassius* exposed to copper. A, control; B, exposure to copper (1ppm) for 10 days. Numbers indicate the LDH isozyme bands.

a specific pattern of LDH isozyme. The tissue specificity is based on the presence or absence of particular LDH isozymes among the normal gill, liver, and muscle of *C. carassius*. The LDH isozyme patterns of the liver and muscle tissues of the normal fish represented different patterns from those of the fish exposed to copper for 10 days. The fish subjected to copper revealed the presence of additional isozymes, LDH-3 in the liver and LDH-5 in the muscle, but no new LDH isozyme band appeared in the gill.

There was no LDH-1 band in all of tissues, gill, liver, and muscle, of *C. carassius* (Fig. 1 and Table 3). In a scope of evolution, the absence of the LDH-1 might be related to the level of lower vertebrates, like the brook lamprey, standing at the lowest systematic position in vertebrates, which has no sign of LDH-1 in heart muscle (Kusa, 1966).

The distribution of LDH isozymes in percentage was represented in table 3. The LDH-1, LDH-2, and LDH-4 were not found in the gills of the controlled and exposed groups of the fish. The percentage of LDH-3 was 88.6 in the gill of the control, and decrased to 82.4 when exposed to copper for 10 days. But the LDH-5 of the gill was increased from 11.4 to 17.6 per cent when exposed to copper.

Table 3. Distribution of lactate dehydrogenase isozymes in various tissues of *C. carassius* exposed to copper (1ppm) for 10 days

Tissue	Condition	LDH isozymes (%)				
		LDH-1	LDH-2	LDH-3	LDH-4	LDH-5
Gill	Control	0	0	88.6	0	11.4
	Exposure	0	0	82.4	0	17.6
Liver	Control	0	50.7	0	17.6	31.7
	Exposure	0	21.5	15.6	26.1	36.8
Muscle	Control	0	0	69.8	30.2	0
	Exposure	0	0	51.3	24.0	24.7

In the liver, the LDH-2 of the controlled fish predominated and decreased remarkably from 50.7 to 21.5 per cent, and LDH-4 and LDH-5 were increased from 17.6 and 31.7 per cent to 26.1 and 36.8 per cent respectively under the exposed condition. This increase of LDH-5 in the liver exposed to copper showed the same tendency of that in rats exposed to sulfur dioxide (Kwon, 1969) and in rabbits exposed to carbon monoxide (Rim, 1970). In addition, the LDH-3, absent in normal liver, appeared in the liver after bing treated to copper.

In the muscle, the percentages of the LDH-3 and LDH-4 were 69.8 and 30.2 in the control, and reduced to 51.3 and 24.0 respectively by the exposure to copper. However, 24.7 per cent of LDH-5, absent in normal muscle, appeared after the exposure.

The LDH-5 was increased in all tissues of the gill, liver, and muscle after the

exposure to copper. This phenomenon suggests that the M type anaerobic isozyme is stimulated by copper and affects the anaerobic glycolysis. It is well known that the synthesis of H-LDH is increased in the aerobic environment or aerobic tissues, and M-LDH predominates in the anaerobic environment or anaerobis tissues, because the H-LDH is related to the aerobic metabolism, and the M-LDH to anaerobic metabolism. In the anaerobic tissues, liver and muscle, the M type of LDH may be responsible for the adaptability to unfaverable conditions in *C. carassius*.

LDH Activities

The protein concentrations and the specific activities of LDH of the gill, liver, and muscle of *C. carassius* exposed to copper were compared with those of the control (Table 4 and Fig. 2). The concentration of the liver protein was gradually decreased during the 10 days, but there was no remarkable change in all tissues.

Table 4. Protein concentrations (mg/ml homogenate) in various tissues of *C. carassius* after the exposure to 10 ppm of copper

Tissue	Day after exposure			
	0	1	5	10
Gill	1.7	1.6	1.8	1.8
Liver	3.8	3.0	2.6	2.0
Muscle	2.8	2.5	2.7	2.6

The LDH activities in the normal gill, liver, and muscle were 32.4, 79.3, and 88.0 units (μM NADH/min/mg of protein) respectively (Fig. 2). The LDH activity of the gill was varied with a slight fluctuation under the treatment of copper during 10 days. After 10 days of the exposure to copper, this LDH activity revealed the tendency of recovery. It seems that the gill may not be affected by 1 ppm of copper. The LDH activity of the liver was dropped rapidly from 79.3 units (control) to 62.3 units after the 1-day exposure to copper and continued to fall gradually to 58.2 units in the 10-day exposure as shown in figeur 2. The LDH activity of the muscle was gradually decreased from 88.0 units in the control to 68.6 units after the exposure to copper. These data indicate that

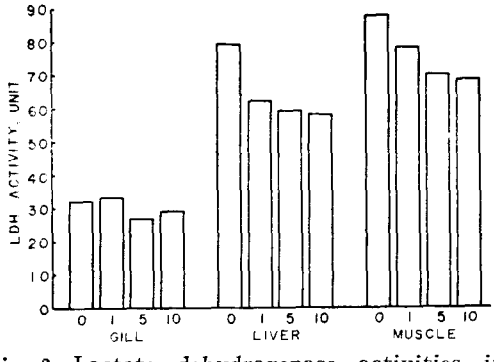


Fig. 2. Lactate dehydrogenase activities in various tissues of *C. carassius* exposed to copper. The unit of LDH activity is μM NADH /min/mg of protein. 0, control; 1, 5, 10, days after the exposure to copper.

the LDH activities in the liver and muscle were inhibited by copper poisoning in *C. carassius*.

***In vitro* Experiment**

In order to find out whether copper acts on the pyruvate⇄lactate reaction directly, the direct (*in vitro*) effect of copper on the LDH enzyme system was examined (Table 5). The absorbance in the control group of M-LDH, NAD, and lactate was 0.90. When each one of M-LDH, NAD, and lactate was treated by 1 ppm of copper sulfate solution, their absorbances were 0.86, 0.91, and 0.92 respectively. There was no significant differences among the above absorbances. When each one of them was treated by 10 ppm of copper sulfate solution, however, the

Table 5 Effects of copper on the lactate dehydrogenase enzyme system *in vitro*

Concentration of copper (ppm)	M-LHD	NAD	Lactate	Absorbance (△E340/min)
Control	−	−	−	0.90
1	+	−	−	0.86
	−	+	−	0.91
	−	−	+	0.92
10	+	−	−	0.32
	−	+	−	0.82
	−	−	+	0.70

+, added; −, none

absorbance was 0.32 in one case in which only M-LDH was treated by copper *in vitro*. This value was about one-third of that of the control group. Through an *in vitro* experiment, it is clear that the LDH enzyme system was greatly inhibited on the M-LDH by copper (10 ppm), but not on NAD or lactate in *C. carassius*. Therefore, it is concluded that the decrease of LDH activities in the liver and muscle of the fish exposed to copper was mainly caused by the inhibition on the M-LDH in the fish, *C. carassius*.

It was reported that both LDH and coenzyme (NAD) were not affected directly by sulfur dioxide in a rat (Chung, 1970) and NAD was affected directly by carbon monoxide in a rabbit (Rim, 1970). It seems that there are some differences between the higher and lower vertebrates, and between terrestrial and aquatic animals, in enzyme system characteristics.

2. EFFECTS OF COPPER ON ESTERASE ISOZYMES

Figure 3 shows the different esterase patterns obtained from the various tissues of *C. carassius* of normal and exposed to copper by agar thin layer electrophoresis. The numbers of esterase bands of the gill, liver, muscle, blood, brain, and kidney

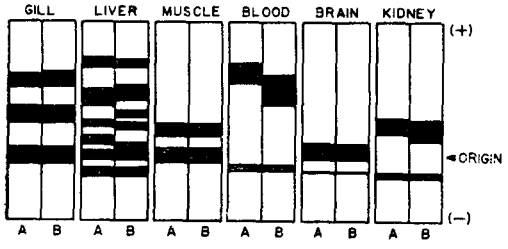


Fig. 3. Diagrammatic representation of esterase isozyme patterns in various tissues of *C. carassius* exposed to copper. A, control; B, exposure to copper.

of normal *C. carassius* were 3, 6, 2, 2, 2, and 2 respectively, and these numbers were the same as in the fish exposed to copper. In both of the normal and exposed groups, the gill, liver, muscle, and brain had the bands on the line of origin. Each of the liver, blood, and kidney had a band on the cathode side, and the brain had traces on the same cathode, while most tissues except brain had one to four esterase bands on the anodes. There were some differences in the intensity of staining and speed, and the direction of mobility between the normal and copper groups. In the blood and kidney, the normal tissues had faster and narrower bands than those of the treated group.

The esterase isozymes in the muscle and brain were not affected by copper. But in the liver the relative mobilities of most esterase isozyme bands were accelerated by copper treatment, except the band which migrated most to the anode, with a similarity in the mollusca (Wright and File, 1968).

Takayama *et al.* (1966) classified the esterase bands in three tissues of funa into four groups. In his work, there were four bands on the anode side in the liver, and two bands were on the anode and the other one on the cathode side in the kidney. But there was no band on the line of origin. His data differ a little from those in figure 3.

3. EFFECTS OF COPPER ON HEMOGLOBINS

The hemoglobin patterns of *C. carassius* in the three groups which were controlled and exposed to copper and to sodium chloride respectively are shown in figure 4. Each of these three groups had one hemoglobin band on the anode. In comparing their hemoglobin patterns, it seems that the two groups exposed to copper and sodium chloride had somewhat faster mobilities of the bands than that of the control.

For the comparative study, the number and the relative mobility of hemoglobin bands of *C. carassius* were compared with those of *Ophicephalus argus* and *Misgurnus anguillicaudatus* in different conditions (Fig. 5). The hemoglobin patterns of three normal species were different from each other. The number of the hemoglobin band of *C. carassius* was one on the anode, whereas the crucian carp, *C.*

auratus, had two bands according to the study of Yamanaka *et al.* (1965). The *O. argus* had two hemoglobin bands on the anode and the mobilities of them

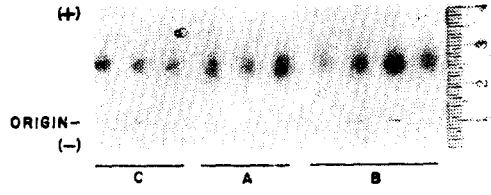


Fig. 4. Starch gel electropherogram of hemoglobins in *C. carassius* exposed to chemicals. A, control; B, exposure to copper; C, exposure to sodium chloride.

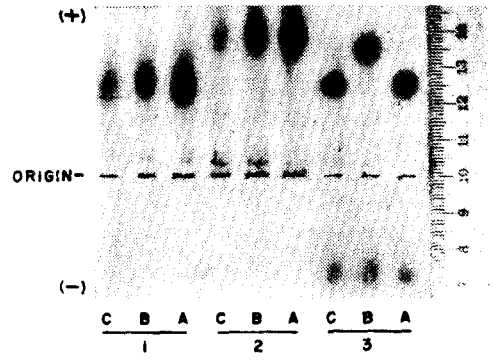


Fig. 5. Comparative starch gel electropherogram of hemoglobins in *C. carassius* (1), *O. argus* (2), and *M. anguillicaudatus* (3) exposed to chemicals. A, control; B, exposure to copper; C, exposure to sodium chloride.

were faster than those of *C. carassius* and *M. anguillicaudatus*. Normal *M. anguillicaudatus* had two hemoglobin bands with nearly identical mobilities; one band migrating anodically and the other cathodically. In the experiment on the effects of the chemicals, neither the number of the hemoglobin bands of both *C. carassius* and *O. argus* nor the mobility of them was affected by copper or sodium chloride. In *M. anguillicaudatus*, the control and the exposed group to sodium chloride had identical hemoglobin mobilities; however, when *M. anguillicaudatus* was exposed to copper, the mobility of the band on the anode was faster than those of the other groups of the control and the group exposed to sodium chloride, whereas the hemoglobin band on the cathode was not affected.

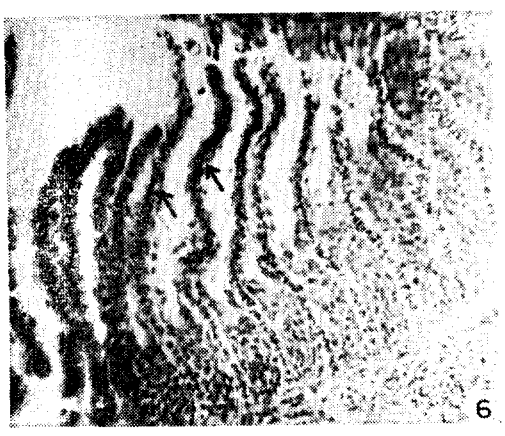
4. EFFECTS OF COPPER ON TISSUES

The gill, liver, muscle, and kidney of *C. carassius* were examined for histological study.

Gill

Each normal gill lamella was composed of dense central pillar cells having arms that stretched out to touch those of adjacent pillar cells. There were numerous mucus cells along the layer of epithelial cells (Fig. 6). When the fish was exposed to copper, the epithelial layer was divorced from the remaining parts of the lamella first and distintegrated (Fig. 7). Then they were completely destroyed with some fusion of adjacent lamellae. The remains of the epithelial layer became completely detached from the central portion of each lamella. The above results were similar to the effects of copper on the winter flounder gill (Baker, 1969). The respirarion

of the fish may be reduced by the damage on the epithelia of gill first. Munshi suggested that mucus cells can either secrete mucus or chloride ions (Baker, 1969).



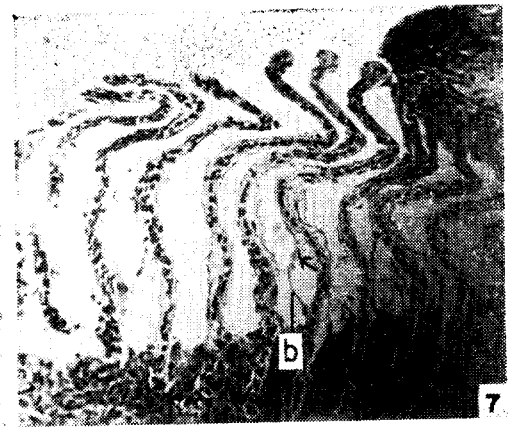
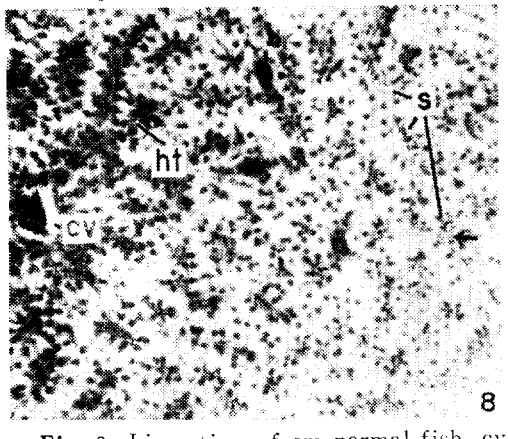


Fig. 6. Gill lamellae of normal fish consist of centrally located pillar cells and show a number of mucus cells (arrows). X150.

Fig. 7. Gill lamellae from fish exposed to copper. Epithelium, which has a balloon-like appearance (b), contains dense material (arrow) and does not adhere to the pillar cell substructure. X150.



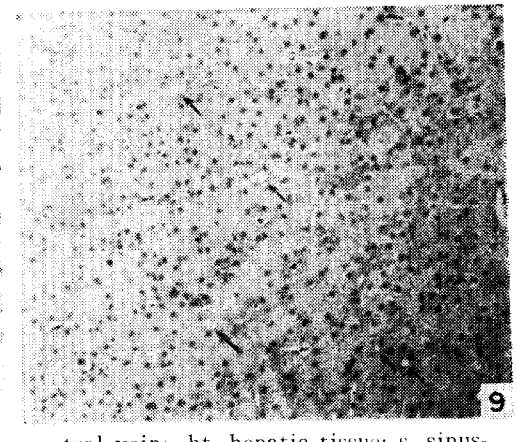


Fig. 8. Liver tissue from normal fish. cv, central vein; ht, hepatic tissue; s, sinusoids; →, erythrocytes. X150.

Fig. 9. Liver tissue from fish exposed to copper. Cells are characterized by the deposition of droplets of fat (clear spots). X150.

Liver

In the liver of normal fish, the central vein was prominent and had the cords of hepatic cells leading to it (Fig. 8). The sinusoids were separated from the columns of hepatic cells and were filled with erythrocytes. But many droplets of fat were found in the cells around the central vein of liver when the fish was subjected to 20 ppm of copper for 5 days (Fig. 9), with a similar pattern establi-

shed in the winter flounder (Baker, 1969). It may be assumed that the fatty droplets are accumulated by the lesion due to the fatty metamorphosis of the liver caused by copper. Deficiency of lipotropic factor cause the fatty liver. The lipotropic factor accelerates the fatty metabolism and prevents the accumulation of the fat in the liver.

Muscle

As shown in the figures 10 and 11, it is hard to find the histological difference between the muscle of the normal fish and that of the fish exposed to copper.

Kidney

Figures 12 and 13 show the kindey tissues of normal *C. carassius*. The tubules

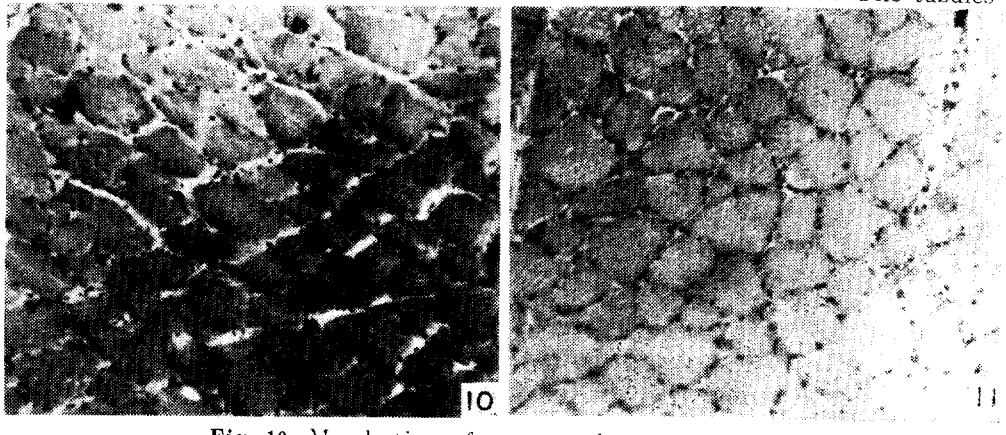


Fig. 10. Muscle tissue from normal specimen. X150.
Fig. 11. Muscle tissue from exposed to copper. X150.

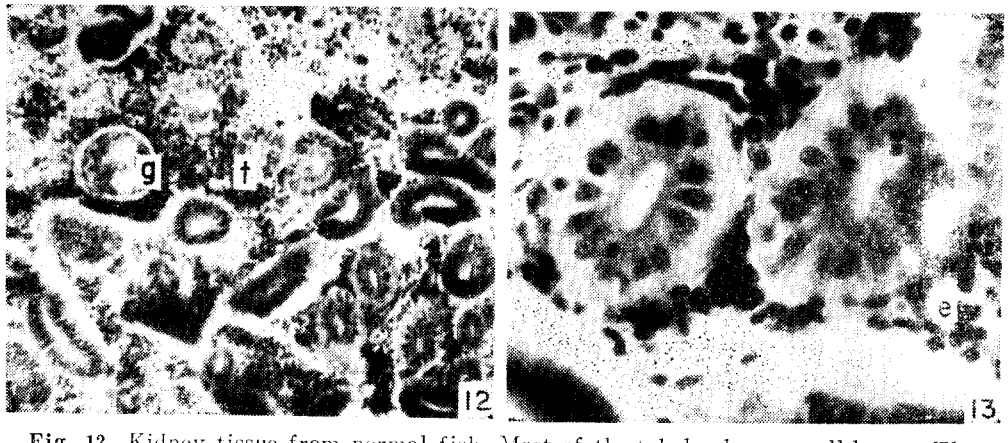


Fig. 12. Kidney tissue from normal fish. Most of the tubules have small lumen. The area between the tubules (t) and the glomerulus (g) contains hemopoetic tissue. X150.

Fig. 13. Kidney tissue from normal fish. The area outside of the tubules contains nucleated erythrocytes (e). Each tubule has asmall lumen, and tubule cells are thicker than those of exposed to copper. X600.

were surrounded by hemopoetic tissues and the tubular cells of normal fish were well formed with little cellular debris. There was no pink-stained granular cast in the tissue (Fig. 12). Immature red blood cells were found around the tubules (Fig. 13).

When the fish was exposed to 20 ppm of copper for 5 days, on the other hand, the tubule cells were shrunken and reduced in height, and the lumens of tubules were enlarged (Figs. 14 and 15). These structures are similar to those of the winter flounder studied by Baker (1969). The kidney tissue of the fish exposed to copper

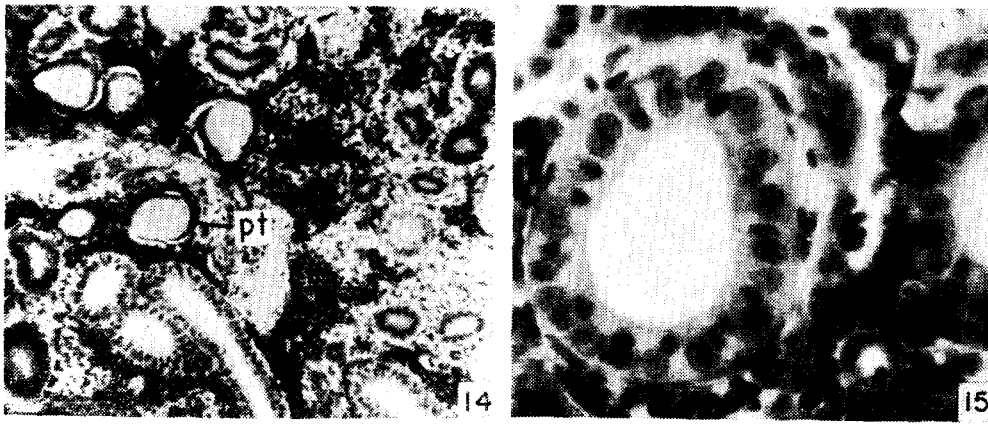


Fig. 14. Kidney tissue from fish exposed to copper. The height of tubule cells is decreased. Many of the proximal tubules (pt) are pinkish and others show various stages of degeneration. X150.

Fig. 15. Kidney tissue from fish exposed to copper. The tubule cells are decreased in height and the lumen of tubule is remarkably enlarged. X600.

also received damage on the proximal tubules. Consequently many of these tubules exhibited some pink-stained granular casts and various stages of degeneration (Fig. 14). Estuarine teleost, *Fundulus heteroclitus*, exposed to cadmium represented pink-staining granular casts in many proximal tubules and various stages of degeneration (Gardner and Yevich, 1970). The effect of copper on the proximal tubules of the freshwater teleost, *C. carassius*, was similar to that of the estuarine teleost.

SUMMARY

In order to elucidate the effects of copper on *Carassius carassius*, the following were studied: 1) lactate dehydrogenase isozyme patterns by cellulose acetate electrophoresis, 2) LDH activity and copper effect on LDH enzyme system by spectrophotometry, 3) esterase isozyme patterns by agar thin layer electrophoresis, 4) hemoglobin patterns by starch gel electrophoresis, and 5) histological study.

1. There were two bands of LDH isozymes (LDH-3 and LDH-5) in the gill, three bands (LDH-2, LDH-4, and LDH-5) in the liver, and two bands (LDH-3 and

LDH-4) in the muscle of the normal fish. The LDH-1 bond was not found in the above three tissues. When the fish were exposed to copper, LDH-3 appeared in the liver, LDH-5 in the muscle, but no new LDH band appeared in the gill.

2. The specific activities of the LDH were lowest in the gill and highest in the muscle of the normal fish, and they were gradually decreassed in the gill and highest in the muscle of the normal fish, and they were gradually decreased in the liver and mucle except in the gill from 1-day to 10-day exposure to copper. It indicates that LDH activities in the liver and muscle of the fish were inhibited by copper.

3. Through *in vitro* experiment, it is clear that the decrease of the LDH activities of the liver and muscle of the fish exposed to copper is mainly caused by the inhibition on the M-LDH in the fish.

4. The numbers of the esterase isozyme bands of the gill, liver, muscle, blood, brain, and kidney of the normal fish were 3, 6, 2, 2, 2, and 2 respectively, and these numbers were the same as those exposed to copper. The relative mobilities of the esterase bands in the gill, liver. blood, and kidney of the exposed group were different from those of the control.

5. There was one hemoglobin band on the anode in the normal fish. It seems that the mobility of hemoglobin band of the fish exposed to copper was slightly faster than that of the normal fish.

6. The normal gill lamellae of the fish consisted of centrally located pillar cells and a number of mucus cells. When the fish were exposed to copper, the epithelial layer was divorced first, disintegrated, and then destroyed completely.

7. The liver of the normal fish had prominent central veins, cords of hepatic cells, and sinusoids. When the fish were exposed to copper, numerous droplets of fat appeared in the cells around the central vein of the liver. It is assumed that the fatty droplets were accumulated by the lesion due to fatty metamorphosis of the liver caused by copper.

8. There was no histological difference between the muscle of the normal fish and that of the fish exposed to copper.

9. In the normal fish, the tubules of the kidney were surrounded by hemopoetic tissues. However, the kidney tissue of the fish exposed to copper received some damage on the proximal tubules. Since the tubule cells were reduced in height, the lumens of the tubules were enlarged. Consequently many proximal tubules exhibited some pink-stained granular casts and various stages of degeneration.

ACKNOWLEDGMENTS

We are grateful to Dr. Taek Jun Lee, Department of Biology, Chungang Uni-

versity, for his valuable advice. We also thank Dr. Ihl-Hyeob Ro of Sookmyung Women's University for his help in discussion.

REFERENCES

American Public Health Association, American Water Works Association, and Water Pollution Control Federation, 1965. Standard methods for the examination of water and waste water. 12th ed. New York, N.Y. 510-514.

Bailey, G.S. and A.C. Wilson, 1968. Homologies between isoenzymes of fishes and those of higher vertebrates. *J. Biol. Chem.* **243** : 5843-5853.

Baker, J.T.P., 1969. Histological and electron microscopical observations on copper poisoning in the winter flounder (*Pseudopleuronectes americanus*). *J. Fish. Res. Bd. Canada* **26** : 2785--2793.

Chen, F.Y. and H. Tsuyuki, 1970. Zone electrophoretic studies on the proteins of *Tilapia mossambica and T. hornorum* and their F_1 hybrids, *T.zillii*, and *T. melanopleura*. *J. Fish. Res. Bd. Canada* **27** : 2167-2177.

Chung, Y., 1970. Effects of sulfur dioxide on lactate dehydrogenase isozyme. *Kor. J. Prev. Med.* **3** : 111-119.

Clayton, J.W. and W.G. Franzin, 1970. Genetics of multiple lactate dehydrogenase isozymes in muscle tissue of lake whitefish (*Coregonus clupeaformis*). *J. Fish Res. Bd. Canada* **27** : 1115-1121.

Clayton, J.W. and J.H. Gee, 1969. Lactate dehydrogenase isozymes in longnose and blacknose dace (*Rhinichthys cataractae* and *R. atratulus*) and their hybrid. *J. Fish. Res. Bd. Canada* **26** : 3049—3053.

De Ligny, W., 1968. Polymorphism of plasma esterase in flounder and plaice. *Genet. Res. Camb.* **11** : 179-182.

Gardner G.R. and P.P. Yevich, 1970. Histological and hematological responses of an estuarine teleost to cadmium. *J. Fish. Res. Bd. Canada* **27** : 2185-2196.

Hogan, J.W. 1971. Some enzymatic properties of plasma esterases from channel catfish (*Ictalurus punctaus*). *J. Fish. Res. Bd. Canada* **28** : 613—616.

Homes, R. S., C. J. Masters and E.C. Webb, 1968. A comparative study of vertebrate esterase multiplicity. *Comp. Biochem. Physiol.* **26** : 837-852.

Jackim, E.,J.M. Hamlin and S. Sonis, 1970. Effects of metal poisoning on five liver enzymes in the killifish (*Fundulus heteroclitus*). *J. Fish. Res. Bd Canada* **27** : 383-390.

Kariya, T., Y. Haga, H. Haga and T. Tsuda, 1967. Studies on the post-mortem identification of the pollutant in the fish killed by water pollution-V. Detection of copper in the fish. *Bull. Jap. Sci Fish.* **33** : 818-824.

Kusa, M., 1966. Lactic dehydrogenase isozyme in amphibian heart muscle. *Zool.*

Mag. **76** : 77-79.

Kwon, S.P., 1969. A study on the isozyme alterations of lactic dehydrogenase in the tissues of albino rat by the exposure in sulfur dioxide. *J. Pharm. Soc. Kor.* **13** : 101-110.

Lush, I.E., 1970. Lactate dehydrogenase isozymes and their genetic variation in coalfish (*Gadus virens*) and cod (*Gadus morrhua*). *Comp. Biochem. Physiol.* **32** : 23—32.

Lush, I.E.,C.B. Cowey and D. Knox, 1969. The lactate dehydrogenase isozymes of twelve species of flatfish (Heterosomata) *J. Exp. Zool.* **171** : 105-118.

Markert, C.L. and I. Faulhaber, 1965. Lactate dehydrogenase isozyme patterns of fish. *J. Exp. Zool.* **159** : 319—332.

Markert, C.L. and R.S. Holmes, 1969. Lactate dehydtogenase isozymes of the flatfish, *Pleuronectiformes*: kinetic, molecular and immunochemical analysis. *J. Exp. Zool.* **171** : 85-104.

Markert, C.L. and F. Mϕller, 1959. Multiple forms of enzymes: tissue, ontogenetic, and species specific patterns. *Proc. Nat. Acad. Sci.* **45** : 753—763.

Massaro E.J., 1972. Isozyme patterns of coregonine fishes: evidence for multiple cistrons for lactate and malate dehyhyrgenases and achromatic bands in the tissues of *Prosobtum cylindraceum* (Pallas) and *P. coulteri* (Eigenmann and Eigenmann) *J. Exp. Zool.* **179** : 247—262.

McKim, J.M. and D.A. Benoit, 1971. Effects of long-term exposures to copper on survival. gowth, and reproduction of brook trout (*Salvelinus fontinalis*). *J. Fish. Res. Bd. Canada* **28** : 655—662.

Mount, D.I. and C.E. Stephan. 1969. Chronic toxicity of copper to the fathead minnow (*Pimephales promelas*) in soft water. *J. Fish. Res. Bd. Canada* **26** : 2449—2457.

Nyman, O.L., 1969. Polymorphic serum esterases in two species of freshwater fishes. *J. Fish. Res. Bd. Canada* **26** : 2532—2534.

Ohno, S. and M. Morrison, 1966. Multiple gene loci for the monomeric hemoglobin of the hagfish (*Eptaretus stoutii*). *Science* **154** : 1034—1035.

Preston, J.A., R.O. Briere and J.G. Batsakis, 1965. Rapid electrophoretic separation of lactate dehydrogenase isozymes on cellulose acetate. *Am. J. Clin. Path.* **43** : 256—260.

Rim, C.E., 1970. A study on the alteration of lactic dehydrogenase activity in tissue homogenate of the rabbit exposed to carbon monoxide. *Yonsei J. Med. Sci.* **3** : 160—173.

Takayama, S., Y. Ojima and A. Hamaguchi, 1966. Cytogenetic studies in lower vertebrates III. Some aspects of esterase pattern in the carp (*Cyprinus carpio*), Funa (*Carassius*) and their hybrids. *Annot. Zool. Japon* **39** : 211—221.

Tsuyuki, H., E. Roberts and E.A. Best, 1969. Serum transferrin systems and the hemoglobins of the Pacific halibut (*Hippoglossus stenolepis*). *J. Fish. Res. Bd. Canada* **26** : 2351—2362.

Tsuyuki, H. E. Roberts, R.H. Kerr and A.P. Ronald, 1966. Micro starch gel electrophoresis. *J. Fish. Res. Bd. Canada.* **23** : 929—933.

Tsuyuki, H. and A.P. Ronald, 1970. Existence in salmonid hemoglobins of molecular species with three and four different polypeptides. *J. Fish. Res. Bd. Canala* **27** : 1325—1328.

Utter, F.M. and H.O. Hodgins, 1969. Lactate dehydrogenase isozymes of Pacific hake (*Mesluccius productus*). *J. Exp. Zool.* **172** : 59—68.

Westrheim, S. J. and H. Tsuyuki Texonomy, distribution, and boiology of the northern rockfish. *Sebates polyspinis J. Fish Res. Canada* **28** : 1621—1627.

Whitt, G.S, 1970. Developmental genetics of the lactate dehydrogenase isozymes of fish. *J. Exp. Zool.* **175** : 1—36.

Whitt, G.S. and G.M. Booth, 1970. Localization of lactate dehydrogenase activity in the cells of the fish (*Xiphophorus helleri*) eye. *J. Exp. Zool.* **174** : 215—224.

Whitt, G.S.,P.L. Cho and W.F. Childers, 1972. Preferential inhibition of allelic isozyme synthesis in an interspecific sunfish hybrid. *J. Exp. Zool.* **179** : 271—282.

Williscoroft, S.N. and H. Tsuyuki, 1970. Lactate dehydrogenase systems of rainbow trout: evidence for polymorphism in liver and additional subunits in gills. *J. Fish. Res. Bd. Canada* **27** : 1563—1567.

Wright, C.A. and S.K. File, 1968. Digestive gland esterases in the genus *Bulinus* (Mollusca, Planorbidae). *Comp. Biochem. Physiol.* **27** : 871—874.

Wuntch, T. and E. Goldberg, 1970. A comparative physico-chemical characterization of lactate dehydrogenase: isozymes in brook trout, lake trout and their hybrid splake trout *J. Exp. Zool.* **174** : 233—252.

Yamanaka, H., K. Yamaguchi, K. Hashimoto and F. Matsuura, 1967. Starch gel electrophoresis of fish hemoglobins-III. Salmonoid fishes *Bull. Jap. Soc. Sci. Fish.* **33** : 195—203.

Yamanaka, H., K. Yamaguchi and F. Matsuura, 1965. Starch gel electrophoresis of fish hemoglobins-II. Electrophoretic patterns of hemoglobin of various fishes. *Bull. Jap. Soc. Sci. Fish.* **31** : 833—839.