PHENOL ACTION ON FISHES (CARASSIUS AURATUS GIBELIO).
A HISTOCHEMICAL AND BIOCHEMICAL STUDY.

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The investigation of toxical action of phenol and its compounds represents a complex problem owing to the variety of its effects on the animals. Phenols from polluted waters are absorbed by the gill and digestive epithelium of fishes, reach the blood and by this way are transported to all tissues.

Reported toxical effects of phenols on aquatic animals and especially on fishes concerned: morphological modifications of tissues (Puckov, 1962; Mitrovic et al., 1968; Meşter et al., 1982), morpho-functional alterations of blood cells (Waluega, 1966; Liu & Krueger, 1968) and of the nervous tissues (Reichenbach-Klinke, 1965; Swift, 1978; Meşter et al., 1982). Biochemical investigations established the interference of phenol on the activity of some enzymes (Bosstrom & Johansson, 1972; Ołowska et al., 1980) and in energy metabolism (Weinbach & Garbus, 1965; Wynn & Fore, 1965).

However, the literature data are controversial concerning the mechanisms of its action, that probably is dissimilarly expressed depending on species, tissue, physiological state of animals, a.s.o. It is evident that by accumulation of phenols in organism, there are produced morphological lesions and alterations of the cellular metabolism.

The aim of the present study is to follow the action of phenol and of mixture of phenolic compounds on the crucian carp, in order to specify the degree of accumulation of this contaminant from waters in different tissues. At the same time was investigated the action of phenol on the activity of
two enzymes involved in glycolysis (lactate dehydrogenase and cytochrome oxidase) from four tissues of fishes, in order to correlate the toxic effects with the metabolic modifications specific to every tissue.

**MATERIALS AND METHODS**

The experiments were conducted on two year crucian carps, obtained from the Pisciculture Station Nucet (Dimbovița). The fishes were kept in 10 l. aquaria and divided in control and exposure groups. The experiments were of two types: the first consisted in exposing fishes for 2 days in a mixture of phenol and hydroquinone (together 5 mg/l) and the second one consisted in longer duration exposing (10 days) with 1 mg/l and 5 mg/l respectively.

**Histological study.** Samples of gills, striated muscle, liver, spleen, ovary and kidney from control and intoxicated fish were prelevated. The tissues were fixed in Bouin-Holande's solution or in buffered formalin, dehydrated, cleared and embedded in paraffin. Tissue sections were stained in haemalum-eosin and P.A.S. (Li 11 i e, 1965).

**Histochemical study.** Phenol in tissues was evidenced by using the p-nitroanilin diazotized, that determined the formation of dark brown precipitates of azoic coloration (Li 11 i e, 1965). The trivalent iron was also histochemically identified, using Prussia's blue technique (Li 11 i e, 1965).

The tissues (liver, kidney, skeletal muscle and gill epithelium) were homogenized in a medium containing tris-HCl 0.05 M, pH 7.5, with a Potter homogenizer. The homogenates were centrifuged at 10.000 g. for 20 minutes and the supernatants obtained were used for enzyme assays.

Determination of lactate dehydrogenase activity was made by measuring the formation of NADH, at 340 nm, after the technique described by B e r g m e y e r (1965). The electrophoretic study of lactate dehydrogenase and cytochrome oxidase was done on polyacrylamide gel 7.5%, system disc-electrophoresis (D a v i s, 1964). The concentration of protein was assayed by the method of L o w r y et al. (1951) using bovine serum albumine as standard.

**RESULTS**

*Morphological modifications induced in some tissues of fishes under the phenols' action.* The fishes kept in waters containing phenol (5 mg/l) or in water with phenols mixture presented respiratory perturbations. In water with phenol mixture a 100% mortality after 32 hours and lesions at the level of gill and kidneys were noticed (Fig. 2A). At small concentrations of phenol the fishes behave normally, but in time they accumulate the toxicant in tissues.

After 10 days of keeping the fishes in water with phenol (1 mg/l) there were observed some morphological modifications in the analysed tissues.

The gills appear with altered respiratory epithelium and with phenol accumulations in chondrocytes and in supporting bony lamellae (Fig. 1A).

In the liver, the hepatocytes have a vacuolated cytoplasm and sometimes a granular chromatin. In some areas can be noticed the presence of granules of azoic coloration and some dilated sinusoids (Fig. 1C).
Fig. 1. Crucian carp kept 10 days in water with phenol (1 mg/l). A. Gills with precipitates of azoic coloration in hyperplasyed cartilaginous cells and in bone lamellae. 10×/16. B. Striated muscle with positive reaction for phenol. 10×/16. C. Liver parenchyma with phenol deposits in hepatocytes. 10×/40. D. Intestinal fold with brown granules in nuclei of enterocytes and blood cells. 10×/16. E. Brain with dilated blood vessels and positive reaction for phenol in blood cells. 10×/16. F. Spleen with dilated blood vessels and macrophages Fe+++ positive. 10×/40.
Fig. 2. A. Kidney of crucian carp, kept 32 jours in a mixture of phenol and hydroquinone (PAS reaction). 20×/40. B. Gill of crucian carp, kept 10 days in water with phenol (5 mg/l). There are granules of azoic coloration in blood cells nuclei and in bone supporting lamella. 10×/16. C. Ovary with positive reaction perinuclear and in the cytoplasm of oocytes. Crucian carp kept 10 days in water with phenol (1 mg/l). 10×/16. D. Muscle of crucian carp, kept 10 days in water with phenol (5 mg/l). Positive reaction for phenol at the level of sarcolemmatae and myofibrils. 10×/16. E. Congestion at the brain level and vacuolized aspect. Crucian carp, 10 days in water with phenol (5 mg/l.) 10×/16. F. Kidney with positive reaction in tubules. Crucian carp, 10 days in water with phenol (1 mg/l). 10×/40.
Fig. 3. — The electrophoretic pattern of lactate dehydrogenase from control and phenol treated fish.
The brain accumulates phenol, especially in the subcortical nuclei and in the blood cells of cerebral capillaries. Around some dilated capillaries appear empty spaces (Fig. 1E). The striated musculature presents a positive reaction for phenol in sarcoplemma and in cytoplasm among the myofibrils (Fig. 1B). In the intestine, the toxicant concentrates at the level of enterocytes brush border, in their nuclei and in the nuclei of infiltrated blood cells. There are noticed in submucoosa the presence of some dilated blood vessels (Fig. 1D). The spleen parenchyma appears altered by the dilatation of blood vessels. There are aggregates of macrophages, iron positive (Fig. 1F). In the kidney, accumulations appears at the apical poles of the cells belonging to proximal convoluted tubules of nephrons and the blood vessels are dilated. At the blood cells level was observed a positive reaction for phenol (Fig. 2F). The ovary presents phenol's accumulation in the cytoplasm of oocytes and perinuclear (Fig. 2G). The morphological alterations appear more obvious after 10 days of maintaining the fishes in water with higher concentrations of phenols (5mg/l). Gills epithelium appears congested, with accumulation of phenol in the musculature from the base of lamellae and in hypertrophied chondrocytes, where are formed brown nodules (Fig. 2B).

A high phenol accumulation was observed in the liver, especially in the hepatocytes from the blood vessels vicinity. The alteration of endothelial cells is marked by a reduced affinity of staining. The brain is characterized by congestion, vacuolized appearance of fundamental substance and neurons with lysated cytoplasm. Positive reaction for phenols appears at the blood cells level and in neuronal plasmatic membrane (Fig. 2E). Big precipitates of phenol appear in the striated musculature, in cells cytoplasm and in the connective tissue among the muscular fibers (Fig. 2D). The spleen presents numerous accumulations of lymphocytes, a congestion of blood vessels and the nuclei of blood cells with an intense brown reaction. Kidney shows evident morphological alterations, with the tubules epithelium oedematized and numerous dilated blood vessels. In the apical pole of the cells were identified phenol's accumulations.

**Enzymatic modifications. Electrophoretic study of lactate dehydrogenase.** Was analysed the isoenzymatic pattern of lactate dehydrogenase from the tissues of control fishes and of those maintained ten days in the presence of phenol, in 5 mg/l concentration. The comparative study of the enzymatic activity in the 4 tissues analysed from the crucian carp pointed out some peculiarities (Fig. 3). In normal conditions the electrophoretic patterns of lactate dehydrogenase appear characteristic tissues: three isoenzymes in the liver (LDH₂, LDH₃, and LDH₄), 2 in the kidney (LDH₂, LDH₃) and only one in gill (LDH₄) and muscles (LDH₃). In the tissues of intoxicated fish were identified isoenzymatic patterns similar to the control ones, but the staining intensity of some isoenzymes is modified, suggesting modifications in their activity. In this way, the staining affinity increased for all liver's isoenzymes, for muscle's isoenzyme and for one from kidney's isoenzymes. In exchange, the LDH₄ activity from the gills of controls appears more intense in comparison with that from intoxicated fish.

**The quantitative study of lactate dehydrogenase.** Quantitative determinations of lactate dehydrogenase activity from the tissues (liver, muscle,
Fig. 4.—Lactate dehydrogenase activity in the tissues of control (open bars) and phenol treated fish (hatched bars). The enzymatic activity was expressed in specific activity (µmol/mg protein/min).
Fig. 5.—The electrophoretic pattern of cytochrome oxidase from control and phenol treated fish.
kidney and gill) in control and phenol treated fishes are presented in the
fig. 4. The quantitative data appear in agreement with electrophoretical
results. Under the action of phenol is increased the activity of lactate dehy-
drogenase in the liver (+230% relative to control), in the kidney (+276%
relative to control) and in muscle (+44% relative to control). In the gills of
fishes intoxicated with phenol, activity of lactate dehydrogenase is smaller
with about 38% in comparison with enzymatic activity from controls.

The electrophoretic study of cytochrome oxidase. The isoenzymatic pattern
of cytochrome oxidase from the tissues of control fishes appears characte-
ristic for each one: 4 isoenzymes in the liver, 3 in striated muscle, 3 in kidney
and 2 in gill. The staining intensities for each molecular form are specific
for tissue. In the fishes maintained in water with phenol, the comparative
study of the enzyme shows some significant modifications, that appear tissue
specific. Thus, under the influence of phenol was identified only one iso-
enzyme in the liver, two in skeletal muscle, four in kidney, three in gill. Compa-
rative electrophoretic data show that by the influence of phenol is produced
the diminution of cytochrome oxidase activity in the liver and muscle and
the increase of enzymatic activity in kidney and gill (Fig. 5).

DISCUSSION

It is well known that a great amount of the phenol penetrated in the
organism is conjugated in the liver (50—70%) and in this form is eliminated.
The free form remnants of phenol can be partially oxidated, eliminated or
accumulated in the cells, producing variable morpho-functional alterations.
The fate of phenol absorbed by fishes is little known. Kobayashi & Nakamura (1979) indicated that a part of pentaclorophenol is excreted
through the gills of goldfish in a free form (30%), whereas all the quantity
excreted by urine is in a conjugated form. The detoxification mechanisms
in fishes have only a low efficiency in comparison with those from higher
vertebrates, so that a considerable amount of phenol is accumulated in the
tissues. Our experimental observations clearly show that a part of phenol
present in water is accumulated in the majority of tissues of fishes. In this
way, specific azoic granules were pointed out in the gill epithelium, skeletal
muscle, liver, ovary, kidney, intestine, brain and in blood cells nuclei. The
induced morphological alterations appear variable depending on tissue and
the experimental condition: the oedematisation of the proximal convoluted
tubule cells, necrosis in the kidney distal convoluted tubule cells, necrosis in
liver parenchyma, congestions in the brain accompanied by the cytoplasmic
lysis of some neurons and accumulation of specific granules in the perika-
ryons. In all tissues was noticed an alteration of blood capillaries. that reduced
the ability of crucian carp to physiological adaptation. Our morphological
and histochemical data agreed with the findings of literature, obtained on
another species of fishes or mammals (Veselov, 1957; Mackiewicz et al

Parallel to morphological study was followed the influence of phenol
on the activity of two glycolytic enzymes. The isoenzymatic pattern of lactate
dehydrogenase was well specified both at the lower vertebrates (Slika,
1976; Pl ace & P owers, 1984) and at the higher ones (K a p l a n, 1968; M a r k e r t & W h i t t, 1968; H o l b r o o k et al., 1957). In exchange the influence of phenol on the lactate dehydrogenase isoenzymes is little known. In our experimental conditions, the lactate dehydrogenase isoenzymatic pattern is not altered under the influence of phenol. In all the analysed tissues appeared qualitative modifications, characterized by the increase of staining intensity of the bands in the liver, muscle and kidney and the diminution of stain intensity in gill epithelium. Quantitative study of lactate dehydrogenase activity rendered evident modifications similar to electrophoretic ones: a significant increase of enzymatic activity in the liver, muscle and kidney and diminution of enzymatic activity in gill epithelium. Cytochrome oxidase, better known in mammals, appears heterogenous, depending on the tissue and alters under the influence of pollutants (C a l d w e l l, 1969; P e t r u n, 1973; M a r k a s s i a n et al., 1981). The electrophoretic data presented here show the existence of isoenzymatic polymorphism of cytochrome oxidase depending on the tissue. The accumulation of phenol in the tissue deeply affects the isoenzymatic pattern, both quantitative and qualitative. Under the phenol's influence was observed a diminution of the number of bands with enzymatic activity in liver and muscle and an increase of isoenzymes in kidney and gill. The mechanism of phenol action at the cellular level is not well known and available data referring to the interference of toxicant with the enzymatic activity are controversial (L i u & K r u e g e r, 1968; P e t r u n, 1973; O l o w s k a et al., 1980). H ence, B o s t r o m & J o h a n s s o n (1972) following the pentaclorophenol effects on some liver enzymes from cell show that this pesticide inhibits the activity of pyruvate kinase and lactate dehydrogenase, but activates the cytochrome oxidase and glucose-6-phosphate dehydrogenase. On the other hand, L i u & K r u e g e r (1968) recorded an increased activity for the glycolytic enzymes in fishes after the intoxication with pentaclorophenol.

The present study pointed out that the influence of phenol on glycolytic enzymes manifested differently depending on tissue, suggesting the interference of other cellular specific mechanisms in the control of metabolic functions, produced in the pesticide presence.

ACȚIUNEA FENOLULUI ASUPRA PESTILOR (CARASSIUS AURATUS GIBELIO). STUDIU HISTOCHIMIC ȘI BIOCHIMIC

REZUMAT

Fenolul în concentrație de 1 mg/l și 5 mg/l se acumulează în toate țesuturile analizate (granule de colorație azoică). Acumularea fenolului determină modificări morfologice variabile în țesuturi: ficat, mușchi, rinichi, epiteliul branbial, creer, intestin ovar, splină. Studiind efectul fenolului asupra lactat dehidrogenazei din ficat, mușchi, rinichi și epiteliul branbial s-a constatat că pesticidul nu afectează modelul izoenzimatic, dar modifică intensitatea de colorare a izoenzimelor: crește intensitatea de colorare în ficat, rinichi și
mușchi și scade intensitatea de colorare în epiteliul branbial. Datele cantitative ale activității enzimatice apar similare cu cele electroforetice. La animalele intoxicate cu fenol activitatea lactat dehidrogenazei crește în ficat (230%), în mușchi (44%) și rinichi (276%) și scade în branbie (38%), comparativ cu valorile din țesuturile normale. Sub influența fenolului se modifică, cantitativ și calitativ, modelele izoenzimatice ale citocrom oxidazei: numărul izoenzimelor scade în ficat și mușchi și crește în rinichi și epiteliul branbial.

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