

PHYSIOLOGY AND TOXICITY STUDIES

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Introduction

Fish acetylcholinesterase (AChE) activity is often referred as one of the most successful example of clinical test in aquatic toxicology. As it is well known the AChE is extremely sensitive to organophosphate (OP) and carbamate (CP) pesticides. This property makes the determination of the enzyme activity very useful in diagnosis of OP and CP poisoning. However, this toxicity index has several serious defects limiting its practical use: a) Sharp decrease in AChE activity may be induced by non-cholinergic pollutants and non-toxic stressors (Lukyanenko, 1983); b) Based upon AChE activity determination only it is impossible to distinguish the OP from the CP poisoning; c) Considerable seasonal changes of the AChE activity plus its individual, populational and species specific variability could mask the toxicant-induced enzyme inhibition (Pavlov, 1992; 1994a) or, at least demand expensive and time-consuming studies on background (normal) enzyme activity dynamics in variety of fish species; d) Despite very intensive studies the functional role (biological importance) of cholinergic system and of changes in the AChE in fish is far from complete clarification. Consequently, AChE does not meet the criteria of "perfect" clinical test (Mehrle & Mayer, 1980).

The aim of the present paper is to study process of induced reactivation of inhibited AChE in fish. The study is directed towards the development of a methodological approach allowing to overcome above limitations. The approach based upon the use of specific AChE reactivators should help to certainly attribute the enzyme activity decrease to the OP intoxication and should be useful for diagnostic purposes and for elucidation of the functional role of cholinergic system.

Materials and methods

Farm raised rainbow trout (Oncorhynchus mikyss) and common carp (Cyprinus carpio) were used in the experiments. In the in vitro study the brains were removed from sacrificed intact rainbow trout and homogenated in phosphate buffer. The AChE activity was measured in the homogenates according to

Ellman's technique (Ellman et al., 1961) and referred further as control. The homogenates were incubated (at 30°C) with tested toxicants and the extent of the enzyme inhibition was determined. Then, the reactivator was added into test tube and after known period of incubation the AChE activity was measured to give an estimate of the reagent reactivation potential. In the control respective volumes of buffer and reactivators were added into reagent/homogenate mixtures. In the second series of experiments carps were exposed flow-through to tested toxicants. Exposure protocol followed recommendations in (Methods for Acute Toxicity Tests, 1975). Reactivation dynamics were measured in the brain homogenates of exposed fish in vitro as described above. The AChE reactivators tested, 2-PAM (used in the in vitro study), TMB-4 and LuH-6 (in vivo study) are well known antidotes of OP poisoning in human beings (Golikov & Zaugolnikov, 1970). Their reactivation potential have been estimated against OP diclorvos (DDVP), malathion and paraoxon, carbamate compound eserine and heavy metal mercury. The toxicants tested belong to different chemical classes and are known to inhibit fish brain AChE in vitro or in vivo. All in vitro assays have been performed in 10 and in vivo in 4 replicates. Mann-Witney u-test was used to evaluate the differences between assays.

Results and discussion

Incubation of the trout brain homogenates with paraoxon, DDVP, mercury and eserine resulted in fast and strong AChE activity decrease. That is, these toxicants do really act as strong AChE inhibitors. Not surprisingly, malathion (demanding metabolic activation to become effective AChE inhibitor) exhibited much less pronounced effect in vitro: no one concentration tested caused 100% inhibition. 2-PAM has no effect on the AChE activity decreased by mercury and eserine while the AChE inhibited by OP recovered fast.

The example of 2-PAM-induced recovery of the enzyme fully inhibited (up to "0" activity) by DDVP is given in Table 1. As it is shown the effect of reactivator was concentration-dependent. When 0.01 M solution was used complete AChE recovery was achieved after 10 min of incubation with the reactivator. Lower 2-PAM concentrations resulted only in about 10 % recovery even after 2 h of incubation. The in vitro experiments have shown that 2-PAM is really highly specific against OP-inhibited AChE and has no effect on other enzyme inhibited by other toxicants. High specificity of this compound as well as of other dipiroximes is also known for mammals (Golikov & Zaugolnikov, 1970).

Table 1. 2-PAM-induced reactivation of trout brain AChE fully inhibited by DDVP

2-PAM concentr.	Incubation time, min	AChE activity uM/g tissue/h
0.3 uM	30	192± 14.3*
0.3 uM	120	198± 7.3
3.0 uM	30	193± 21.0
3.0 uM	120	190± 12.2
0.01 M	10	1298± 31.9
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Control		1300±47.1

* - average±S.E; N=10

For the in vivo study DDVP was chosen as a model OP. This compound acts against AChE directly, i.e without metabolic activation and as it was revealed in the in vitro study is quite potent enzyme inhibitor. Exposure of carps to 15 mg.l^{-1} DDVP for 96 h resulted in about 10% fish mortality. In the survived fish the 75-79% decrease in the brain AChE activity was recorded. In the moribund specimens and in the fish died 20 to 40 min before the analyses AChE activity dropped down to 8-15% of control level. Incubation of the brain homogenates of exposed fish with TMB - 4 and LuH - 6 resulted in the recovery of the enzyme activity (Fig. 1 and 2). This recovery was concentration dependent. It was revealed that LuH-6 is more powerful reactivator than TMB-4. The LuH-6 effect became evident in the concentrations exceeding 0.0002 M while TMB-4 reactivated DDVP-inhibited AChE only starting from the concentration one order higher. It was revealed that tested reactivators are equally effective in the cases when survived, moribund and dead fish were analyzed. That is, the full enzyme recovery was induced by reactivators if the AChE was 75-92% inhibited by DDVP.

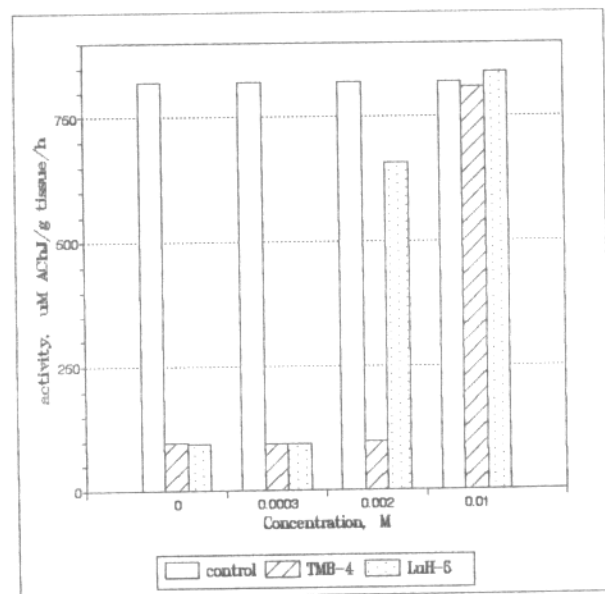


Figure 1. Induced reactivation of carp brain AChE inhibited by DDVP: reactivation rate vs. reactivator concentration.

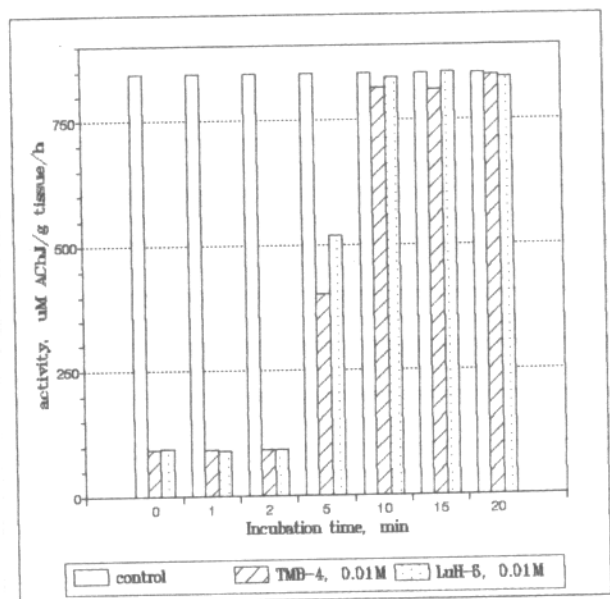


Figure 2. Induced reactivation of carp brain AChE inhibited by DDVP: reactivation rate vs. time.

The results of the experiments given above have shown that tested drugs, 2-PAM, TMB-4 and LuH-6 are powerful and specific reactivators of fish brain AChE inhibited by OP. This suggests the use of these compounds to be promising for fish toxicity and physiology studies. Previously, we have shown that the use of the AChE reactivator TMB-4 helped to elucidate the role of the enzyme inhibition in the impaired efficiency of feeding behavior in bream (Abramis brama) exposed to DDVP (Pavlov et al., 1992). In that study the injection of TMB-4 resulted both in fast reactivation of fish brain AChE and

in recovery of the feeding behavior in the OP-exposed fish. Similar procedure, exploited the use of cholinergic drugs, the n- and m-choline receptor blockers, pediphen and atropine, contributed to understanding of the functional role of cholinergic system in the control of respiration in perch (Perca fluviatilis) (Pavlov, 1994b).

The data given here allow to propose a procedure for diagnosis of the OP poisoning in fish. In case when either visible signs of intoxication or fish mortality appear and suspected reason for that is the poisoning, the routine AChE activity determination should be performed. If the activity be found decreased than the in vitro procedure described here should be carried out. If the incubation of the fish tissue homogenates with AChE reactivator will result in the enzyme recovery it will certainly indicate the OP intoxication. The procedure is applicable both in the cases when live or dead fish are analysed. As the further development of this approach (although demanding further detailed studies) we could propose the following. In the case of suspected OP or CP poisoning or for elucidation of the functional role of cholinergic system the fish should be injected with the cholinergic drugs, such as choline receptor blockers, as it is described elsewhere (Pavlov, 1994). Disappearing signs of intoxication will indicate the cholinergic system to be involved. Then it is possible to determine whether the fish were intoxicated by OP or by CP. To do this, AChE reactivators-based in vitro protocol should be followed as described above. Again, in this case recovery of the AChE activity will allow to certainly attribute fish poisoning to an OP particularly. If the reactivator will have no effect it will indicate CP poisoning. Combined application of the OP treatment, cholinergic drugs and reactivators could become a powerful tool for the studies on the functional role of fish cholinergic system.

It must be noted that the methodological approach proposed here needs further investigations and must be treated now as only a general scheme demanding detailisation. Obviously, these is question worthy of further research.

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