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Concentration Effects of Selected Insecticides on Brain Acetylcholinesterase in the Common Carp *(Cyprinvs carpio* L)

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Abstract : The differential inhibition of acetylcholinesterase (AChE) by organophosphate (OP) and carbamate (C) is followed by the distinct duration of exposure effect on common carp AChE. Hence, in the present study *in vivo* exposure period effect and *in vitro* concentration-response of chlorfenvinphos, chlorpyrifos diazinon, and carbofuran were investigated on *Cyprinus carpio* L. AChE. Individuals of 1-year-old carp were exposed for 96 h to different concentrations of insecticides- after which AChE activity was measured in the brain. The highest concentrations of carbofuran (2.44 mg • L^{-1})-chlorfenvinphos (2.9 mgL^{-1}), and diazinon (2.5 $mg.L^{-1}$) killed at] the test animals after only 4 h, although there was no statistically significant difference from the control group's brain AChE activity. The lowest concentration significantly inhibited brain AChE after 96 h. Chlorfenvinphos was the most potent inhibitor *in vivo* and chlorpyrifos the least active inhibitor after 96 h of exposure time. *In vitro* experimentation with the same pesticide indicated that several concentrations inhibited 50% of the AChE activity (I_{50}) ranging from 4.1 x 10^{-7} to 8.12X 10^{-4} M in both single inhibitory action and joint inhibitory effect. The results suggest that in biomonitoring programs carp brain AChE can be a good diagnostic tool for chronic OP nd C pollution.

Key Words: organophospbate; carbamate; synergy; AChE; common carp.

Introduction

The recent development of a biomarker based on the study of the biological response of organisms to pollutants has provided essential tools for the implementation of programs for contamination monitoring (Peakall and Shugart, 1991).
Organophosphate (OP) and carbamate (C) insecticides are known to disrupt transmission in the central and peripheral cholinergic nervous systems in vertebrates by inhibiting acetylcholinesterase activity (Sahib and Rao, 1980; Sharma *et al.,* 1993). These pesticides are produced and used in large amounts, and they enter the environment in greater quantities than chlorinated hydrocarbon insecticides (Coppage and Mathews, 1974), The possible hazard of AChE-inhibiting pesticides in the aquatic environment should not be ignored, since these pesticides act as a nerve poison by inhibiting the respiration center of the brain and neuromuscular junctions of the respiratory apparatus (Coppage and Braidech, 1976).
Aquatic organisms exhibit a broad range of inhibitory response to OP and C pesticides, depending on the compound, exposure time, water conditions, and species (Copp-age and Mathews, 1974). Considering the number of OP and C compounds and the difficulty in detecting their highly toxic oxygen analogue, measuring AChE is probably the best general indicator of serious OP and C pesticide pollution (Cool *et al,* 1976). Determination of brain AChE activity is widely used to diagnose OP and C poisoning (Hart, 1993). Taking into consideration that the activity of AChE is reduced to less than 50% of the normal level by OP and C, this degree has been regarded as a good indicator of poisoning (Coppage and Mathews, 1974; Westlake *et al.,* 1981a, b).
This article reports an investigation carried out to evaluate the concentration-response to some widely used ag-rochemical insecticides, and the length of time necessary for an *in vivo* inhibitory effect on carp brain AChE to occur. For testing this chlorfenvinphos, chlorpyrifos, diazinon, and car-bofuran were chosen, because these compounds are widely used in Belgium. A high density agricultural area and a river with cyprinidea coincide in Wallonia. In this region, data for 1996 indicate that the cholinesterase inhibitor concentration is estimated to be 0.17 $\mu g\text{-}L^{-1}$, a yield higher than the WHO limit of 0.1 $\mu g \bullet L^{-1}$. The present study evaluates the effects of agricultural pesticides on the teleosts of the river Meuse.

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Materials and methods

Chemicals

Commercial preparations of Birlan (244 g • L^{-1} chlorfen-vinphos (2-chloro-l-(2,4-dichlorophenyl)-vinyl diethyl phosphate)), Pychlorex (480 g.L^{-1}, chlorpyrifos (0,0-di-ethyl-0-(3,5,6-trichloro-2-pyridyl)-phosphorothioate)), Dis-onex (162 g • L^{-1} diazinon (0-0-diethyl-0-(-2-isopropyl-4-methyl-6-pyrimidyl)phosphorothionate)), and Curater (200 g L^{-1} carbofuran (2,3-dihydro~2,2-dimethyl-7 benzo-furanol methylcarbamate)) were purchased from Shell Chemical Co, and were used for the *in vivo* study. Toxicity assays were performed with stock solution prepared by dissolving 10 ml of Birlan, Disonex, and Pychlorex in 100 ml of ethanol, and 10 ml of Curater in 100 ml of distilled water. These solutions were further diluted to obtain the experimental concentrations in aquariums.
Active preparations of chlorfenvinphos (97%), chlor-pyrifos (99%), diazinon (98%) and carbofuran (99%) from Sigma Chemical Co. were used for the *in vitro* study. *In vitro* test solutions were initially prepared at 10^{-1} M concentration in absolute ethanol. These initial solutions were further diluted to obtain final concentrations ranging from $10"^{2}$ to 10^{-8}M.
The dye 5-5'-dithiobis-(2-nitrobenzoic acid) (DTNB) and the substrate acetylthiocholine chloride (AcSCh) were used to determine acetylcholinesterase activity (Ellman *et* a/., 1961). The chromogen was prepared as follows: 186 mg of DTNB was mixed with 75 mg of calcium bicarbonate and dissolved in 50 ml of distilled water. AcSCh was diluted to 10^{-1}M in distilled water. The chemicals were obtained from Sigma Chemical Co. Bovine serum albumin (BSA) was used for determination of protein quantity and was obtained from Bio-Rad.

Biological Reagent

Test animals were 1-year-old carp belonging to the "mirror variety." Separate groups of 400 fish, 6-10 cm long, were obtained from an industrial fish farm (Piscimeuse, Thiange: Belgium). Acclimatization took place in a 6 x 3 x 0.75-m aquarium in the experimental piscicultural installation of the Gembloux Agricultural University (Belgium). During acclimatization the fishes were fed every 72 h for 12 days with Trouvit compound (Trouw, Fontaine les Vervins, France). Temperature was kept at 8°C ± 2°C and aeration at 0.66 ± 0.1 g .L^{-1} of oxygen.
The experimentation was conducted in two ways. The first was performed *in vitro* and the effect of chlorfenvin-phos, chlorpyrifos, diazinon, and carbofuran on AChE activity was checked, as well as the effect of the mixtures carbofuran-chlorfenvinphos, carbofuran-chlorpyrifos, car-bofuran-diazinon, and chlorfenvinphos-chlorpyrifos. Second, *in vivo* effects were determined by exposing groups of 10 individuals to four concentrations of each pesticide in separate 100-L acrylic plastic aquariums. For all experiments, the static test was performed as described by the European Organization for Cooperation and Development (OCDE, 1981), modified for measuring brain AChE activity.

Acetylcholinesterase Assay

After decapitation, the fish brain was quickly removed and homogenized in a low salinity solution (LSS) containing 50 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.0, and 1 mg/ml bacitracin. The crude homogenate was centrifuged at 18,000g for 15 min at 4°C. The supernatant was collected for protein and AChE assays.
The protein determination was performed using the original Lowry method {Lowry *et al.* 1951). Twenty-five microliters of supernatant was incubated with 1125 µl of folin reagent (125 µl of A and 1000 µl of B). The content was then mixed and after 15 min of reaction the absorbance was read at 750 nm. Brain AChE activity determination was carried out using the method of Ellman *et al.* (1961). The supernatant (100 µl) was added to a test tube (1.5 ml) containing 880 µl of 0.1 M phosphate buffer (pH 7.5), 10 µl of 100 mM DTNB, and 10 ul of 0.1 M acetylthiocholine chloride at 412 nm. The contents was then mixed and the absorbance read continuously for 1 min using a Shimadzu 160A UV spectrophotometer. At least three samples were assayed for each brain. Enzyme activity was reported as millimoles of product formed per milligram protein per minute. The percentage of AChE inhibition was derived by expressing the activity levels of exposed animals as a percentage of the activity in controls. For the determination of the *in vitro* inhibitory effect, 10 µl of chlorfenvinphos (97%), chlor-pyrifos (99%), diazinon (98%), and carbofuran (99%) in concentrations ranging from 10^{-2} to 10^{-8} M (concerning the final reaction mixture) was added to the curve for 2 min. The absorbance was then

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determined at 412 nm. The combined effect of insecticides on AChE activity was determined as follows: the two insecticides being tested were added in the curve at the same quantity and concentration; the preincubation took 2 min. AChE activity was determined according to the method of Ellrnan *et ai* (1961). The I_{50} value was then determined by probit regression (Finney, 1971).

In Vivo Study

The experiments were conducted for 2 weeks after acclimatization. Chlorfenvinphos, chlorpyrifos, and diazinon were dissolved in ethanol and carbofuran in water. Four trial dosages were selected according to the toxicity of the pesticides on other animals described in the literature, and a preliminary experiment was conducted on carp. Every week two different concentrations of each of the four pesticides were chosen randomly with a control group. Fish were exposed to those concentrations for 96 h. Before the end of the previous experiment (96 h), the dead fish were decapitated and AChE activity was determined.
Pesticide concentration in the water was expressed in theoretical mg • L^{-1}. The analysis of residues was not performed because criteria were aimed at the duration and concentration effect on AChE inhibition. Control animals were maintained in a pesticide-free medium (100 L) containing 25 ml of ethanol, because three of the four pesticides were dissolved in ethanol. After the stipulated periods the surviving fish were decapitated and the brains were removed and kept at 0°C for determination of AChE activity and protein quantity. AChE was measured even If fish died after a few hours in order to demonstrate the longer period of time necessary to exhibit significant inhibition of brain AChE, as it is generally stipulated that OP and C inhibit the respiratory center in the brain.

Statistical Analysis

The general statistical analysis was analysis of variance.
(1) Brain AChE activity of the control groups for the first 2 weeks was compared by general linear model (GLM) hierarchical method at 0.05 level of significance in order to eliminate the interference of the experimental period duration.
(b) One-tailed Dunnett's test for significant differences ($\alpha = 0.05$) between treated animals and controls (Dagnelie, 1975) was also used.
Statistical analyses were performed using Minitab statistical software.

Results

Exposure of fish to different levels of OPs chlorfenvin-phos, chlorpyrifos, and diazinon and carbofuran (C) caused severe abnormalities in their behavior, such as disequilibrium in swimming and retardation in opercula movement The test animals exhibited abnormal behavior earlier at higher than lower concentrations. With all insecticides tested, test animals died rapidly when exposed to the highest concentration.
Data on the inhibition of AChE incubated with OPs and C are provided in Figs. 1 and 2, which depict logarithmic concentration response plots for the insecticides used. Figure 1 indicate that C causes a more potent inhibition than OPs. This is indicated by the broad range of I_{50} from 4.1×10^{-7} (carbofuran) to 8.12×10^{-4}M (chlorpyrifos), both in a single inhibitory action (Fig. 1). In Fig. 2, the presence of carbofuran induces a more rapid inhibition than that produced by OPs. Table 1 provides the I_{50} values for the concentration - related inhibition of AChE.

In vitro the highest $/_{so}$ values were detected with chlor-pyrifos ($I_{50} = 8.12 \times 10^{-4}$M) and the lowest were found with carbofuran ($I_{50} = 4.1 \times 10^{-7}$ M). The highest inhibitory effect was obtained with the mixture of carbofuran and chlorpyrifos ($I_{50} = 3.68 \times 10^{-7}$ M) and the lowest inhibitory effect was with the mixture of two organophosphates ($I_{50} = 9.8 \times 10^{-5}$M) (Table 1).
The experiment to determine *in vivo* inhibition of brain AChE had a 2-week design. There were two control groups: controls- for the first week and contro!s_2 for the second week. No mortality was observed in controls. The comparison of AChE activity of the controls indicates no variability between control groups for the same week and between the 2 weeks (GLM;$df=1, P < 0.05, F = 1.38$ and 2.02, respectively) (Table 2).
In the 96-h static test, the LC_{50} value for chlorfenvinphos was 0.74×10^{-4}mg.L^{-1} determined by probit

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analysis with CL (confident limit) = 95% (Raymond, 1985).

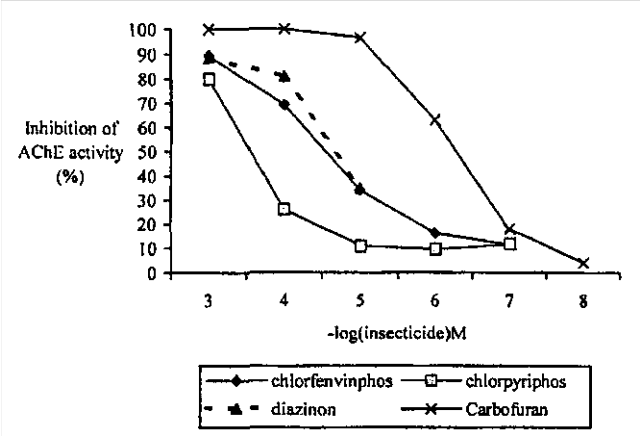

FIG. 1. Concentration - response of the common carp brain acetylcholinesterase (AChE) exposed to organophosphorous and carbamate. The result is expressed as a percentage of a control group. Each value is the mean of two replicated measurements. Point-to-point composite curves are provided to help visualization. At all concentrations tested the carbamate (carbofuran) caused more rapid inhibition than organosphophates (chlor-fenvinphos, chlorpyrifos, and diazinon).

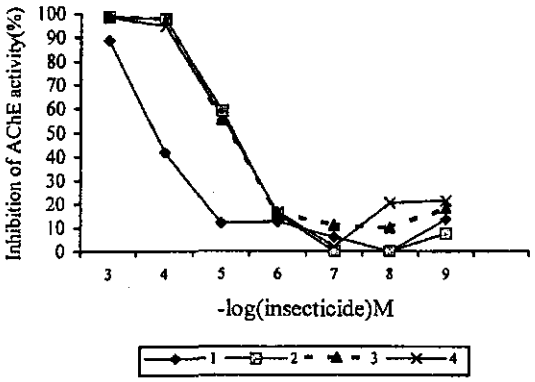

FIG. 2. Concentration-response of common carp brain acetylcholinesterase (AChE) exposed to a mixture of organophosphates and carbamate. The result is expressed on a percentage of a control group. Each value is the mean of two replicated measurements. Point-to-point composite curves are provided to help visualization. The presence of car-bofuran in the mixture caused a more potent inhibition than that of organophosphates. 1, chlorfenvinphos-chlorpyrifos; 2, carbofuran-dia-zinon; 3, carbofuran-chlorpyrifos; 4, carbofuran-chlorfenvinphos.

TABLE 1 I_{50} Values of Organophosphate and Carbamate Insecticides, and I_{50}Values of Mixtures of Organophosphates and Carbamate on Cyprinus carpio Brain AChE

Insecticides	I_{50} (M)	Insecticides mixtures	I_{50}(M)
Chlorfenvinphos	1.9×10^{-5}	Carbofuran-chlorfenvinphos	6.9×10^{-7}
Chlorpyrifos	8.1×10^{-4}	Carbofuran-chlorpyrifos	$3.7 + 10^{-7}$
Diazinon	1.9×10^{-5}	Carbofuran-diazinon	5.6×10^{-7}
Carbofuran	4.1×10^{-7}	Chlorfenvinphos-chlorpyrifos	9.8×10^{-3}

Note: Acetylcholinesterase (AChE) inhibition was determined following 2 min of incubation at 25°C with a range from 10^{-2} to 10^{-8}M. That includes concentrations giving between 10 and 90% of inhibition. Concentrations inhibiting AChE activity by 50% at that time and temperature (I_{50}) were obtained by regression method (Finney, 1971).

All four groups tested with chlorfenvinphos exhibited significant inhibition of brain AChE activity. The observed inhibition ratio observed was higher than 80%. The highest concentration induced 80% mortality after only 4 h (Table 3). The medium concentration (4.9 mg. L^{-1}) induced 100% mortality within 24 h. With higher concentrations (19 $mg.L^{-1}$) death occurred earlier in the experimental period. For chlorpyrifos, an LC_{50} of $0.49 \times 10^{-4} mg.L^{-1}$ was determined by log-probit analysis (Raymond, 1985). Three of the four concentrations (14; 7.2; 3.6 $mg\text{-}L^{-1}$) tested significantly inhibited brain AChE

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activity. A concentration three times the LC_{50} decreased brain AChE activity of the control group by 11.6%. This concentration killed all fish after 4 h (Table 3). The lowest concentration (3.6×10^{-4} mg . L^{-1}) killed only 30% of the test animals; the other 70% spent 96 h in contact with the insecticide and AChE activity decreased by about 85% of the control activity.

In the groups treated with two times the LC_{50} and LC_{50}, at least seven fish spent 72 h in contact with insecticides and AChE activity decreased by about 75%. The concentration two times LC_{50} showed 79% inhibition of the control group brain AChE activity, and a similar range was observed for the concentration LC_{50}.

The concentration one-half the LC_{50} exhibited 86.4% inhibition of the control group AChE activity (Table 4).

For diazinon, LC_{50} determined by probit analysis method was estimated to be 0.72×10^{-4} mg·L^{-1}. The concentration three times LC_{50} induced no inhibition. All 10 fish died after 4 h. The three other concentrations revealed different inhibitory levels. The two times LC_{50}, LC_{50}, and one-half LC_{50} indicated 18.3, 58, and 49% inhibition, respectively, compared to control group brain AChE (Table 4). The concentration two times LC_{50}, killed 70% of the fish before 72 h. In the two other concentration groups about 80% of the fish spent 96 h in contact with the insecticide.

The first concentration of carbofuran (10^{-3} mg.L^{-1}) exhibited no significant difference in activity with that of the control group. All 10 fish died 4 h after exposure. Fish exposed to 0.22×10^{-3} mg.L^{-1} die after 24 h; in this group the decrease in AChE activity was 75%. The two other concentration groups, 0.1×10^{-3} mg.L^{-1} and 0.05×10^{-3}mg.L^{-1}, induced 27.8 and 64.5% inhibition, respectively, compared to control group brain AChE activity (Table 4).

TABLE 2 Control Group Brain Acetylcholinesterase Activity (mM/min/mg Protein)

	AChE
$Control_1$	2.20×10^{-3}
$Control_2$	1.64×10^{-3}
$Control_3$	2.40×10^{-3}

Note. Carp in control group were exposed to water without insecticide for 96 h at 8 + 2°C. After the exposure period the 10 fish were sacrificed and brain AChE was extracted. Three measurements per brain were performed. Contrail represented the controls of the first week and control -represented those of the second week.

TABLE 3 Mortality (%) related to Exposure Period (96 h)

Pesticide	Concentration x 10^{-4} (mg.L^{-1})	24 h	48 h	72 h	96 h
$Carbofuran_1$[a]	10	100	0	0	0
Carbofuran2	2.2	100	0	0	0
Carbofuran $_2$	1	0	0	0	0
Carbofuran1	0.5	20	0	0	0
$Diazinon_1$[a]	15	100	0	0	0
Diazinon $_2$	7.9	20	10	40	0
$Diazinon_2$	3.9	10	10	0	0
$Diazinon_1$	1.9	0	10	0	0
$Chlorfenvinphos_1$	19	80	10	10	0
$Chlorfenvinphos_1$	9.8	70	0	20	0
$Chlorfenvinphos_2$	4.9	100	0	0	0
$Chlorfenvinphos_2$	2.4	20	0	0	0
Chorpiryfos $_2$[a]	29	100	0	0	0
$Chorpiryfos_1$	14	20	20	10	20
Chorpiryfos $_2$	7.2	40	10	0	0

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Chorpiryfos 1	3.6	0	30	0	0

Note. The highest concentration killed all the fish and the lowest killed at least 30%. Carp were exposed to four different concentrations of car-bofuran, chlorfenvinphos, chlorpyrifos, and diazinon. Exposure was performed in concentrations able to kill fish after 4 h or up to 4 days exposure period. The activity of brain AChE was measured and compared to controls.

[a]Concentration that induced 100% mortality after 4 h.

TABLE 4 Dunnett Test Result

Pesticide	Concentration x 10^{-4} ($mg.L^{-1}$)	Mean of AChE activity x(10^{-4})±SD (mmol mg^{-1} min^{-1})	% Activity/control	Z
Carbofuran 1	10	24.8 ± 0.2	116.7 B	3.5
Carbofuran 2	2.2	5.5 ± 0.2	25.8 C	15.8*
Carbofuran 2	1	15.4 ±0.2	72.6 D	5.8
Carbofuran 1	0.5	7.9 ± 0.2	37.0 C	13.4*
Diazinon 1	1.5	25.8 + 0.2	121-4 B	4.5
Diazinon 2	7.9	17.3 ± 0.2	81.4 C	3.9
Diazinon 2	3.9	8.8 ± 0.2	41.3 C	12-5*
Diazinon 1	1.9	12.0 ± 0.3	56.3 C	***9.2***
Chlorfenvinphos 1	19	1.1 ±0.3	5.2 C	20.1*
Chlorfenvinphos 1	9.8	2.1 ± 0.2	9.8 C	19.2*
Chlorfenvinphos 2	4.9	1.3 ±0.2	6.3 C	19.9*
Chlorfenvinphos 2	2.4	1.7 ± 0.2	8.0 C	19.5*
Chorpiryfos 2	29	18.6 ± 0.2	87.6 B	2.6
Chorpiryfos 1	14	5.1 ± 0.2	24.2 C	16.1*
Chorpiryfos 2	7.2	4.7 ± 0.2	22.2 C	16.5*
Chorptryfos 1	3.6	2.9 ± 0.2	13.8 C	18.3*

Note. A, AChE activity means that presented significant difference from controls, B, concentration that killed all the test animals within 4 h; C, concentration that killed all the test animals between Day 1 and Day 4; D, concentration for which all the tested animals were still alive after 4 days of exposure; 1, concentration tested the first week; 2, concentration tested the second week. Carp were exposed to four different concentrations of carbofuran, chlorfenvinphos, chlorpyrifos, and diazinon. Exposure was performed in concentrations able to kill fish after 4 h or up to 4 days exposure period. The activity of brain AChE was measured and compared to the control. Each value represents the mean of measurements on 10 fish. Three measurements of AChE activity were perfonned'for each fish. I= d_1 = 11,13, Dunnett test value; Z is the difference between the mean of control and the activity of the treated fish brain AChE activity; When Z < I there is a significant difference between the group and the control.

DISCUSSION

It is worth noting that, in in *vitro* inhibitory tests, all of the pesticides inhibited the enzyme by at least 20% at a concentration of 10^{-3} M. Similar results were observed by Bastos *et al.* (1991) for other organophosphates and carbamates.

The observed values of I_{so} are in agreement with those observed with carbofuran and carbaryl on common prawn (Bocquene *et at,* 1995).

When two insecticides were mixed it was noted that the effect of the lowest inhibitor of the two was enhanced. It was also observed that when chlorfenvinphos was combined with carbofuran, the I_{50} represented 3.6 % of its value compared to chlorfenvinphos alone. In the case of chlor-pyrifos, the I_{50}

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was reduced to 12% of its value. Diazinon and chlorpyrifos exhibited a synergistic effect when combined with carbofuran. The percentage of cabofuran used was 90% (of the I_{50} when used alone) and only 0.04% of the I_{50} of chlorpyrifos. It qualified as a synergistic interaction. When diazinon was associated with carbofuran, the I_{so} (concentration that inhibited 50% of AChE activity) was in the same range of that of carbofuran used alone. Although 2.9% of the I_{50} of diazinon was added, this association seemed to have no interaction. Carbofuran appeared to be the most efficient inhibitor (I_{50} = 4.1 x 10^{-7} M) compared to the other pesticides tested (I_{50} > 4.1 x 10^{-7} M). A similar result was reported by Bocquene *et al* (1995). Chlorfenvinphos and diazinon are moderate inhibitors, whereas chlor-pyrifos is a weak inhibitor This observation on chlorpyrifos is in agreement with the work of Bocquene *et al.* (1995) and Olson and Christensen, (1980), who reported that many organophosphate pesticides are less efficient inhibitors before becoming metabolically transformed from thio to oxy analogs.

The abnormalities in fish behavior observed in this study could be related to failure of energy production or the release of stored metabolic energy, which may cause severe stress, leading to the death of the fish (Chakraborty *et al.,* 1989).

Treating carp with chlorfenvinphos, chlorpyrifos, carbo-furan, and diazinon caused modification of acetylcholinesterase activity. Some marked differences in efficiency are indicated between the insecticides examined when tested with the Dunnett test pagnelie, 1975). The fish that died within 4 h after exposure of OP and C generally revealed no statistical difference with the control group. These results can be explained by death of the fish by asphyxia or irritation of the gill (Pesson, 1976). With chlorfenvinphos, the inhibitory action was proportional to the concentration and the time of exposure. The quicker the fish died, the lower the inhibition rate was. The same observation was found for chlopyriphos and diazinon. Those differences can be explained by the fact that chlorpyrifos and diazinon are the two sulfite forms and the duration in the water induced their oxidation to the more inhibitory oxon form.

In vivo the activation of the thiono-type OPs is mainly due to photolysis. Phosphorothionate (P=S) pesticides such as chlorpyrifos are converted into their oxygen analog, oxon (P=0), by different degradation pathways, including proteolysis, before becoming strongly irreversible inhibitors (Bocqueneet *al,* 1995).

CONCLUSION

In conclusion, although the highest concentrations of cabofuran and diazinon killed all the fish, this occurred without a significant decrease in brain AChE activity. It is well established by this study that the chronic exposure to all tested insecticides in aquatic medium can significantly depress the AChE level in the brain. This level of depression induced by chronic exposure can be used as a biomarker to predict agrochemical pollution. Further work is needed to determine the specific effect of high concentrations of insecticides that kill fish without inhibiting brain AChE, and the reaction of AChE in field conditions.

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