Acetylcholinesterase Levels in Brains of Fishes from Polluted Waters

by A. K. WILLIAMS and CARL R. SOVA
Southeast Water Laboratory
Department of the Interior, Athens, Georgia

One of the acute toxic effects of organophosphorous compounds is due to their ability to severely inhibit the enzyme, acetylcholinesterase. This inhibition has been demonstrated many times both in vitro and in vivo. Studies by Weiss (1,2,3) have shown that fish exposed to various sublethal and lethal concentrations of several organophosphorous compounds exhibited a reduced level of AChE activity in the excised brain tissue. Weiss has also shown that death of several species of freshwater fishes occurs when brain AChE activity is 40 - 70% inhibited when compared to that of nonexposed control fish of the same species (1). This author has also suggested that brain AChE levels of fish could be utilized as a means of detecting the presence of organophosphorous pollutants in natural waters (1,2,3).

The Ashley River, Charleston, South Carolina, was chosen as a study site because sporadic fish kills had been occurring

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since 1961 in an area which receives wastes from a plant which manufactures a variety of organophosphorous compounds, including pesticides and defoliants.

Experimental

Moribund and apparently healthy (live) Atlantic menhaden, Brevoortia tyrannus (Latrobe), and apparently healthy (live) Atlantic croakers, Micropogen undulatus (Linnaeus), were collected from the same general area of the Ashley River. Menhaden, for control purposes, were collected by net from the offshore waters in the vicinity of Charleston, South Carolina. Similarly, control croakers were taken by net from the Ashapoo River in South Carolina. Upon collection, all fish were frozen on dry ice, promptly shipped to the laboratory, and stored at -18 C prior to analysis. The collections were made in late June and early July, 1965.

The intact brain was removed from the frozen fish and weighed on tared aluminum foil. The entire brain was then homogenized in 2.0 ml of 0.2 M phosphate buffer, pH 8.2 containing 0.2 M NaCl and 0.002 M $MgCl_2$ using a glass tissue homogenizer. The resulting homogenate was diluted with buffer to a final concentration of 10 mg brain/ml. This stock brei was then diluted with buffer to obtain the desired concentration for assay. Reactions were carried out using 5.0 mg brain tissue (0.5 ml of stock brei), 0.5 ml of buffer, and 4.0 μM of acetylcholine chloride in a total volume of 2.0 ml. Reactions were

allowed to incubate 20 minutes at 25 C. Residual acetylcholine was determined by the alkaline hydroxylamine - $FeCl_3$ method of Hestrin (4). The reactions were clarified by brief centrifugation immediately after color development. This entire procedure is essentially that of Weiss (1) with minor modifications. The results of these experiments are shown in Table 1.

TABLE 1

Acetylcholinesterase Activity of Fish Brain Homogenates

Source	Number Assayed	Specific Activity* Extremes	Mean	Percent Inhibition
Control Menhaden	18	0.71-1.49	1.09	----
Moribund Menhaden Ashley River	8	0.48-0.67	0.58	46.8
Live Menhaden Ashley River	8	0.72-1.14	0.91	16.5
Control Croakers Ashapoo River	8	1.02-2.34	1.48	----
Live Croakers Ashley River	8	0.72-1.13	0.95	35.8

*Specific Activity = μm acetylcholine hydrolyzed/mg. brain tissue/hr. Reactions were incubated at 25 C for 20 min.

Menhaden taken from the Ashley River in a distressed condition showed a 46.8% inhibition of brain AChE when compared to

the control group; while menhaden collected from the same source, showing no obvious sign of distress, were found to be 16.5% inhibited. Although moribund croakers were not observed, croakers taken from the Ashley River were found to have 35.8% less AChE activity than the controls.

Although the specific activity values of some of the Ashley River fishes fall within the range of values of the control fishes, statistical analysis has established that the two groups are, in all cases, significantly different.

The statistical significance of the mean AChE activity values shown in Table 1 was determined with a "t-test" (1). For menhaden, the null hypothesis that the difference between two population means was zero was tested for each of the following cases: control minus live; control minus moribund; and live minus moribund. Similarly, for croakers, the following was tested: control minus live.

All "t" values were greater than the 0.05 probability of a larger value. Thus, the hypothesis was rejected and the following conclusions were drawn about specific AChE activity in brain tissue: The activity of menhaden collected from off-shore areas was greater than both live and moribund menhaden from the Ashley River; in the Ashley River, the activity of live menhaden was greater than that of moribund menhaden; and the activity of croakers from the Ashapoo River was greater than that of croakers from the Ashley River.

In an effort to locate possible sources of organophosphorous pollution, water samples were taken from several industrial outfalls along the Ashley River, including those of the pesticide and defoliant manufacturing plant. These water samples were extracted with chloroform, and the chloroform fraction was adjusted to a volume such that 2.5 ml chloroform was equivalent to 1.0 ml of water. The chloroform fractions were tested for the presence of AChE inhibiting materials by placing a known quantity of chloroform extract in a test tube and evaporating in air stream. Approximately 3 units of purified bovine erythrocyte AChE was then added to the tubes and allowed to incubate for 30 minutes at 35 C. Acetylcholine was added and the enzyme activity determined as previously described. From a total of 12 samples assayed, only 3 exhibited anti-AChE activity when compared to control reactions (Table 2). It is significant that samples one and two were taken from effluents of the pesticide plant and sample three was obtained from a fertilizer plant.

Analysis of the water samples by gas chromatographic techniques indicated the presence of at least two anti-AChE compounds. Subsequent analysis of these materials by infra-red, nuclear magnetic resonance, and mass spectroscopy confirmed the identity of these compounds as 0,0 diethyl-0(2,4-dichlorophenyl) phosphorothioate and S,S,S-tributyl phosphoro-trithioate (5).

TABLE 2

In Vitro Inhibition of AChE in Extracts of Waste Water Being Discharged into the Ashley River

Sample Number	ml of Chloroform Extract Added	Percent Inhibition
1 1/	1.25	95
1 1/	0.10	30
2 1/	1.25	42
3 2/	1.25	29
4-12 3/	1.25	none

1/ Collected from the waste effluent of the pesticide plant.

2/ Collected from the waste effluent of the fertilizer plant.

3/ Collected from other waste sources.

Discussion

The moribund menhaden collected from this area exhibited a degree of AChE inhibition which has been shown to result in the death of certain species of freshwater fishes (Weiss 1,2,3). Furthermore, apparently healthy croakers collected from the Ashley River exhibited AChE inhibition which approached that thought to be critical for the more sensitive species of freshwater fishes (Weiss 1,2,3). While we have not shown that the extensive fish kills which have occurred in the Ashley River over

a period of years are due solely to the inhibition of AChE, it is our opinion that these fish kills are due, at least in part, to the inhibition of this enzyme. The data also indicate, as Weiss (1,2,3) suggested, that AChE inhibition in fish-brain tissue in conjunction with chromatographic or chemical analysis has considerable potential as a means of monitoring waters for the presence of organophosphorous compounds.

Summary

Distressed menhaden collected from the Ashley River, South Carolina, were found to have 46.8% less acetylcholinesterase (AChE) activity in brain homogenates as compared to menhaden collected from offshore waters. Menhaden and croakers also taken from the Ashley River, but not in a distressed condition, were found to be 16.5 and 35.8% inhibited, respectively. AChE inhibiting materials were found in three of twelve waste water samples collected from the vicinity of the Ashley River.

References and Notes

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