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Storage and Handling As Sources of Error in Measuring Fish Acetylcholinesterase Activity

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Organochlorine chemicals have been replaced by less persistent organophosphate and carbamate chemicals in the agriculture industry. However, the acute toxicity of organophosphate and carbamate chemicals may be the same as or greater than that of organochlorine chemicals, and thus, fish kills and other adverse effects to aquatic biota continue. Generally, investigation of causative agents in fish kills suspected of involving organochlorine chemicals was straightforward and based on the presence or absence of organochlorine residues in water and fish. However, the absence in water and fish samples of short-lived organophosphate and carbamate residues may not be conclusive evidence that they were not involved with a fish kill.

Inhibition of acetylcholinesterase (AChE) activity in cholinergic parts of nervous tissue has been used as evidence of organophosphate poisoning (Gibson and Ludke 1971; Benke and Murphy 1974; Coppage et al. 1975) and carbamate poisoning (Haines 1981) in fish and invertebrates. It is generally accepted that a 60 to 80% inhibition of AChE activity is required before death occurs in fish. Death is believed to be caused by blocking neurotransmission in the respiratory center of the brain. Although organophosphate residues in water and fish usually disappear within several days, brain AChE activity remains inhibited in fish for several weeks (Coppage and Duke 1971).

Inhibition of AChE activity in combination with chemical residues in fish and water have been used as a tool in diagnosing orgnaophosphate and carbamate poisoning. Few researchers have, however, examined the potential sources of error in measuring fish AChE activity that may result from different storage and handling techniques. In this study, we investigated changes in channel catfish (Ictalurus punctatus) brain AChE activity caused by different storage and handling techniques. We compared measurements derived from controlled laboratory populations to those seen in wild populations of fish.

MATERIALS AND METHODS

The laboratory population of channel catfish was obtained from a commercial fish breeder. The fish were allowed to acclimate to laboratory conditions for one month prior to testing. Quality of the laboratory water in the 1,000-L circular flow-through fish acclimation tank was 10° C temperature, 10.6 ppm dissolved oxygen, 7.1 pH, and 18 mg/L $CaCO_3$ hardness. Fish were fed a commercial trout pellet food during acclimation and were 105 ± 14 mm (mean ± SD) fork length and weighed 15 ± 5 g at the time of testing.

The method used to measure AChE activity in channel catfish brains was developed by Ellman et al. (1961) as modified by Hill (1979). The Hill (1979) modification, developed for avians, has been successfully used for fish by Haines (1981). The chromagen was prepared monthly in pH 7.4 0.05 M tris buffer. The 0.156 M ASChI substrate was prepared daily.

The brain of catfish was removed by cutting the head open with a scalpel. The entire brain was excised and weighed to the nearest milligram on clean aluminum foil. The brain was homogenized with pH 8 0.05 M tris buffer at a ratio of 100 mg (wet weight) brain tissue to 1 mL buffer in a Pyrex 7-mL glass tissue grinder.

The brain homogenate, reagents, and optically matched spectrophotometer photocells were warmed to 25° C in a waterbath. The analysis was carried out in duplicate with a Bausch and Lomb Spec 20® spectrophotometer at 405 nm wavelength. The spectrophotometer and Omni Scribe D500® script chart recorder were adjusted to read zero absorption with a reagent blank. The reaction was allowed to stabilize for 30 seconds before the photocell was inserted back into the spectrophotometer. The reaction was monitored on script chart paper for 3 minutes. At 3 minutes, the absorption curve began to plateau and was no longer useful.

The slope of the absorption curve was calculated by regression analysis. The slope was multiplied by 130 to yield µmole acetylcholine hydrolyzed/g tissue·min (Hill 1979). The analytical precision (Shewhart 1931) of the method was ± 2.6%. Significant ($p \leq 0.05$) differences between treatment groups were detected by analysis of variance and least significant difference tests (Sokal and Rohlf 1969).

Three methods of storing fish were investigated for introducing error in measuring fish brain AChE activity: (1) refrigeration of brain tissue homogenate; (2) refrigeration of whole fish; and (3) freezing of whole fish.

A total of 50 fish were killed by MS-222 intoxication and arranged in 10 groups of 5 each using a random number table. The 10 groups were: one control group for refrigerated and frozen stored fish; three groups of fish stored refrigerated for 1, 2, and 5 days;

five groups of fish stored frozen for 2, 7, 14, 30, and 70 days; and one group of fish brain homogenate in buffer.

To determine effects of refrigerating and freezing whole fish on brain AChE activity, the control group (no freezing or refrigerating) was assayed the same day that they were killed. Treatment groups of whole fish were double wrapped in aluminum foil and then refrigerated at 7° C or frozen at -5° C for the above time intervals. The frozen fish were thawed for two hours prior to excising brain.

To determine the effect of refrigerating brain homogenate in buffer on brain AChE activity, the homogenate was reassayed after refrigeration at 1, 2, and 5 days.

To determine the effect that freezing whole fish would have on detecting inhibition of AChE activity following exposure to a known AChE inhibitor, two experiments were conducted where fish were exposed for 6 hours to several concentrations of methyl parathion. In one test the fish were assayed for AChE immediately after exposure while in the other test the fish were assayed after being forzen for 12 days. The test solutions were prepared using a commercial methyl parathion formulation. Concentrations of methyl parathion were estimated by dilution. The tests were conducted in 20-L glass aquaria with water volume adjusted to 10 L.

RESULTS AND DISCUSSION

Fish brain AChE activity was not significantly ($p \leq 0.05$) affected by storing the homogenate in buffer at 7° C for up to 5 days (Table 1). However, storing the whole fish at 7° C for 1 to 5 days significantly inhibited fish brain AChE activity, and the degree of inhibition increased with increased storage time: 15% inhibition after 1 day; 25% inhibition after 2 days; and 31% inhibition after 5 days. Thus, homogenizing the brain in tris buffer (pH 8.0) immediately after death is necessary for preserving AChE activity. Refrigerating the fish does not stop loss of AChE activity; proteins continue to decay.

Brain AChE activity was significantly inhibited (17 to 24%) by storing the whole fish frozen at -5° C for 2 to 14 days. The degree of inhibition did not appear to be dependent on storage time. Fish brain AChE activity returned to control value after 30 days freezing and was significantly increased (17%) after 70 days storage. Thus, freezing the whole fish caused an initial inhibition of brain AChE activity but increased after 30 days storage. The initial AChE inhibition was probably due to protein destruction as the fish became frozen. The subsequent increase of brain AChE activity could have been due to indeterminant moisture loss from fish tissue during storage at -5° C thereby, concentrating AChE (μmole/g wet weight).

Channel catfish exposed for 6 hours to 10 and 30 mg/L methyl parathion exhibited loss of equilibrium and were near death.

Regardless of whether the fish were fresh of frozen, AChE activity was significantly inhibited 58 to 61% and 65%, respectively (Table 2). Channel catfish exposed to 5 mg/L did not exhibit loss of equilibrium, although AChE activity was signifi- cantly inhibited (47%). Coppage et al. (1975) indicated that 60 to 80% inhibition of brain AChE activity was near the lethal threshold for fishes, and our data (58 to 65% depression) confirm these findings.

Table 1. Acetylcholinesterase activity (μmole/g·min) of channel catfish brain tissue following different storage techniques.

Storage Time (days)	Treatment		
	Refrigerate Homogenate	Refrigerate Fish	Freeze Fish
0 (Control)	41.2±1.2[a/]	42.3±1.6	42.3±1.6
1	39.4±1.8(4)	35.8±2.0(15)*	-
2	42.8±1.0(-4)	31.6±1.1(25)*	35.2±1.9(17)*
5	42.2±1.7(-2)	29.2±1.2(31)*	-
7			32.2±0.6(24)*
14			35.2±1.8(17)*
30			42.3±1.5(0)
70			49.5±3.7(-17)*

[a/] Mean ± S.E. for groups of 5 fish; percent inhibition from controls in paretheses.
* Significantly different than controls ($p \leq 0.05$).

Freezing fish for 12 days did not appear to alter the relationship in brain AChE activity between methyl parathion exposed and control fish even though there was 18% inhibition of AChE activity due to freezing for 12 days. Thus, little relative error in measuring AChE activity will occur providing that control and treatment groups are handled in the same manner. Hill and Fleming (1982) concluded that for avians concurrent controls must be handled in the same manner as specimens in question if diagnosis of AChE activity is to be optimized.

Brain AChE activity measured for channel catfish in the laboratory (29.1 to 42.3 μmole/g·min) which were frozen for 10 to 30 days prior to analysis were slightly higher than those measured for fish collected from an agricultural drain (24.4 μmole/g·min)

which were handled in a similar manner (Table 3). The agricultural drain contains return water from rice fields and up to 3 to 4 ug/L methyl parathion (Finlayson and Lew 1983). Methyl parathion concentrations may be responsible for the slightly lower brain AChE activity in drain fish. The difference may also be due to differences within and among populations (Gibson and Ludke 1971).

Table 2. Acetylcholinesterase activity (μmole/g·min) of channel catfish after exposure to parathion.

Methyl Parathion Concentration	Treatment	
	Fresh	12-Day Freezing
Control	35.6±5.2[a]	29.1±3.5
5 mg/L	-	15.3±1.4(47)*
10 mg/L	13.9±0.9(61)*	12.2±1.2(58)*
30 mg/L	12.6±1.2(65)*	-

[a] Mean ± S.E. for groups of 4 fish; percent inhibition from controls in paretheses.
* Significantly different than controls ($p \leq 0.05$).

Table 3. Acetylcholinesterase activity (μmole/g·min) of fish from an agricultural drain (data from Finlayson and Lew 1983).

Species	Collection Times	
	May 1983	June 1983
Ictalurus punctatus	-	24.4±3.8(4)
Ictalurus catus	19.1±1.8(13)[a]	17.6±1.0(15)
Ictalurus nebulosus	19.4±0.9(12)	-
Cyrpinus carpio	14.4±0.5(11)	18.5±1.0(4)

[a] Mean ± S.E.; number of fish in parentheses.

Our study indicates that: (1) freezing or refrigerating whole fish significantly alters fish brain AChE activity; (2) homogenizing fish brain in tris buffer shortly after death preserves AChE activity for up to 5 days when refrigerated; and (3) little relative error in measuring AChE activity will occur provided that control and treatment groups are handled in the same manner.

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