Characterization of Fish Brain Acetylcholinesterase with an Automated pH Stat for Inhibition Studies[1]

by DAVID L. COPPAGE
Gulf Breeze Laboratory
Pesticides Office
Environmental Protection Agency
Sabine Island, Gulf Breeze, Fla.

Fish brain acetylcholinesterase has been inhibited by organophosphate pesticides under controlled conditions in the laboratory (1, 2). Also, water pollution by these pesticides in the environment has been monitored by measuring the activity of the enzyme in brains of fish (3, 4). However, Gibson and co-workers (5) exposed fish to organophosphate pesticides, found great inhibition of acetylcholinesterase without death and death with little inhibition, and therefore questioned the usefulness of acetylcholinesterase activity in fish brains for monitoring. Before regarding inhibition measurement as an undependable monitoring tool, a better understanding of the properties of the enzyme and improved assay methods should be studied to provide activity measurements that correlate well with exposure and observed toxicity.

Several conditions should be studied to improve our present methods of assay of acetylcholinesterase. (a) Animal tissue may contain two general types of cholinesterase, acetylcholinesterase (AChE, EC 3.1.1.7 acetylcholine acetyl-hydrolase) and cholinesterase (ChE, EC 3.1.1.8 acylcholine acyl-hydrolase) and these types must be differentiated. Both are assayed by measuring the rate at which they hydrolyze suitable esters and they may be differentiated by the rate at which each hydrolyzes specific choline esters. In general, AChE requires a lower concentration of acetylcholine for maximal hydrolysis than ChE, and is inhibited by excess acetylcholine. Acetyl-β-methylcholine is specific for AChE and butyrylcholine is specific for ChE. (b) Assay conditions should be such that the rate of substrate (acetylcholine) hydrolysis is linear and proportional to the tissue homogenate (enzyme) concentration. (c) Temperature and pH should be such that the enzyme and substrate are stable and it may be desirable to have temperature and pH at values where maximal substrate hydrolysis by AChE occurs.

[1]Contribution No. 125, Gulf Breeze Laboratory

Bulletin of Environmental Contamination & Toxicology,
Vol. 6, No. 4, 1971, published by Springer-Verlag New York Inc.

This report concerns characterization of brain AChE of sheepshead minnows, Cyprinodon variegatus, by means of an automated pH stat for use in inhibition studies. The function of enzyme and choline ester concentration, the action of enzyme on specific choline esters, and the effects of pH and temperature on AChE activity were studied to determine suitable assay conditions for brain AChE. Also, the effects of several inhibitors were studied in vitro and compared with toxicity. A method for in vivo inhibition studies is proposed.

Materials and Methods

Enzyme Preparation: Adult fish (40 to 70 mm total length) were seined from marsh ditches on Santa Rosa Island, Escambia County, Florida. Fish were killed by placing them in 100 percent acetone for 3 to 5 minutes. The scales were removed from the heads and the fish were placed in acetone for another 3 to 5 minutes. After drying, the top of the skull was clipped away and the brain removed. For each assay, 5 to 10 brains were pooled, weighed wet, homogenized in distilled water, and diluted with distilled water to the desired tissue concentration.

Ester Substrates: Acetylcholine iodide (ACh), acetyl-β-methylcholine iodide (MeCh), and butyrylcholine iodide.

In Vitro Inhibitors: Guthion®, phorate, parathion, Diazinon®, and eserine sulfate.

Enzyme Assay Instrument: A Sargent® recording pH stat was employed in the AChE assay. It titrates the acid liberated by enzymatic hydrolysis of a substrate. The function of the pH stat is to record the delivery rate of titrant (NaOH) by controlling voltage at electrodes in the enzyme-substrate mixture. The instrument combines the functions of pH control, temperature control, reagent delivery and mixing, and titrant volume recording.

Enzyme Assay Method: Selected concentrations of ester substrates were prepared in distilled water and 2 ml were mixed with 2 ml of brain homogenate. Acid liberated during hydrolysis of the ester substrate was titrated with carbonate-free 0.01 N NaOH. The pH was held constant by addition of NaOH and the amount added was continuously recorded (titragraphed). Nitrogen was passed over the liquid surface to prevent adsorption of atmospheric carbon dioxide. Esterase activity is reported as micromoles of substrate hydrolyzed per mg wet weight of brain tissue per hour. All assays were conducted at pH 7 and 22°C unless otherwise indicated. All data are the means of at least two determinations.

®Trademark: Guthion, Bayer Co., Germany; Diazinon, Geigy Co., N.Y.; Sargent, E.H. Sargent & Co., Illinois. Mention of commercial products does not constitute endorsement by the Environmental Protection Agency.

Enzyme Properties

The rate of hydrolysis of acetylcholine varied linearly with increasing amounts of enzyme (brain homogenate) (Figure 1). Therefore, subsequent analyses were conducted with 10 mg of brain in the reaction vessel.

Hydrolysis by brain homogenate was inhibited by excess ACh and MeCh, yielding bell-shaped curves typical of AChE when hydrolytic activity is plotted against the negative logarithm of molar concentrations (pS) (Figure 2). Hydrolysis of butyrylcholine was not detected in a similar concentration range, indicating lack of hydrolysis by ChE. The optimum ACh concentration was near 10 mM; therefore, a 15 mM concentration was used in subsequent assays to insure enzyme saturation. Titragraphs at greater than optimum concentrations of ACh were straight lines for at least 30 minutes of reaction time, but titragraphs at less than optimum concentrations were not linear.

Maximal ACh hydrolysis took place between pH 7.0 and 7.5 (Figure 3). Destruction of the enzyme in relation to pH was studied by exposing the enzyme to each pH for 15 minutes, then readjusting the pH to 7.0. No destruction took place, but considerable nonenzymatic hydrolysis of ACh took place above pH 8.0 and increased with increasing pH.

AChE activity was relatively constant between 20° C and 30° C but when temperature reached 40° C activity declined (Figure 4). AChE activity was irreversibly destroyed at 50° C, as demonstrated by complete loss of activity when temperature was returned to 20° C. Considerable nonenzymatic hydrolysis of ACh took place above 35° C and increased with increasing temperature.

In Vitro Inhibition and Toxicity

Eserine, an inhibitor specific for AChE, and four organophosphate pesticides were incubated separately with brain homogenate for 30 minutes before addition of ACh, and the inhibition values for the organophosphate pesticides were compared to their 48-hour LD 50 values for sheepshead minnows (Table 1). Eserine completely inhibited hydrolysis of ACh at 1×10^{-4} M concentration, and inhibited hydrolysis by 81.5 percent at 1×10^{-6} M concentration, indicating hydrolysis is primarily by AChE.

The inhibition values indicate that the presence of organophosphate pesticides can be detected by the pH stat brain AChE assay, but it is obvious that *in vitro* inhibition is not closely related to the toxicity of the compounds. For example, Diazinon, the greatest inhibitor of the four compounds, is approximately one-

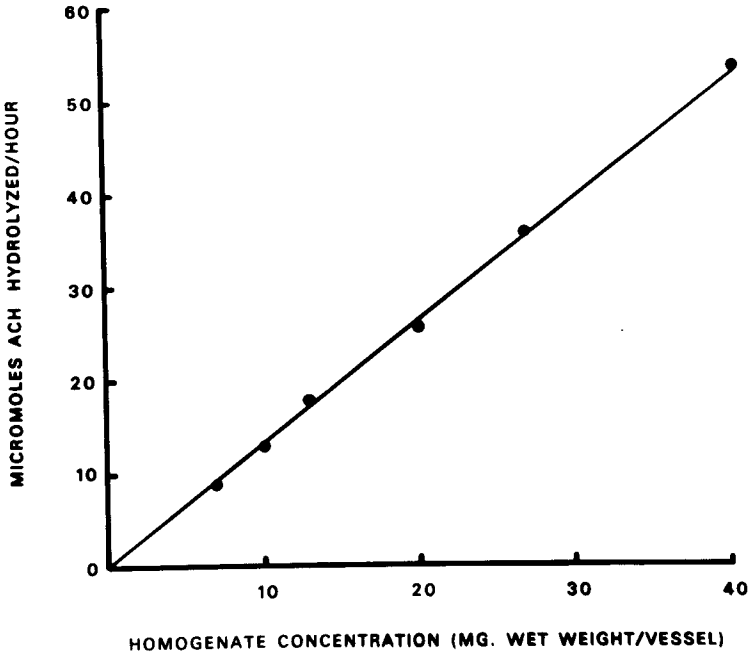

Figure 1. Hydrolysis of acetylcholine (15 mM) by sheepshead minnow brain homogenate as a function of homogenate concentration.

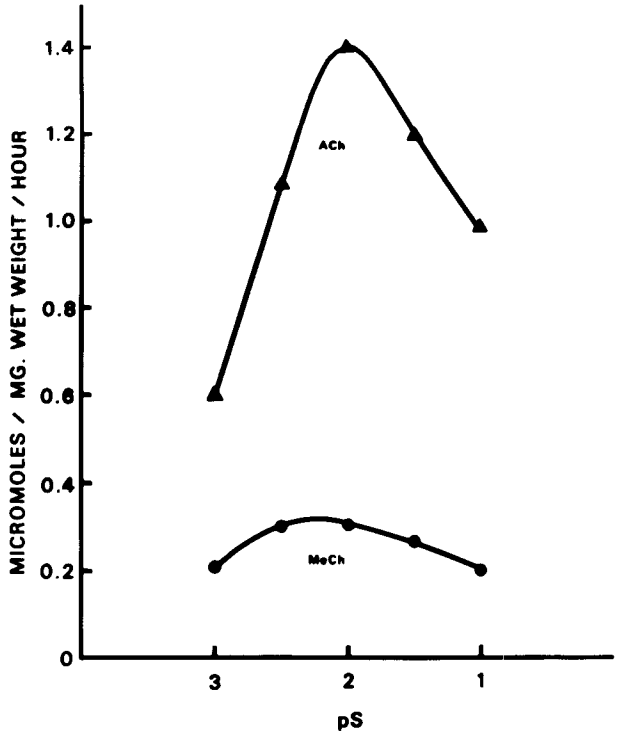

Figure 2. Effect of substrate concentration on hydrolytic activity of sheepshead minnow brain homogenate. pS = negative logarithm of molar concentration. ACh = acetylcholine. MeCh = acetylβ methylcholine.

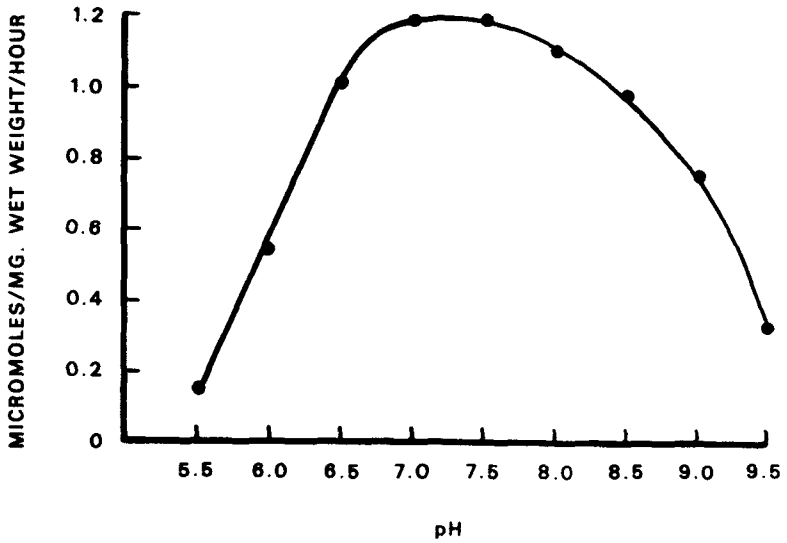

Figure 3. Effect of pH on the rate of hydrolysis of acetylcholine (15 mM) by sheepshead minnow brain homogenate.

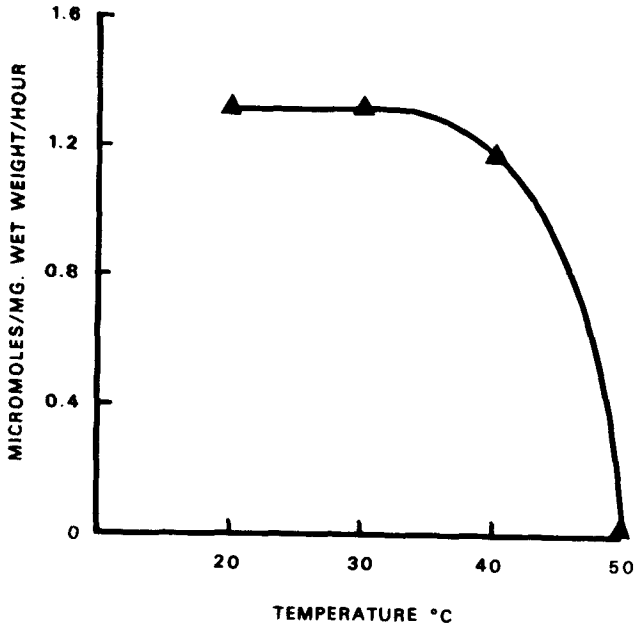

Figure 4. Effect of temperature on the rate of hydrolysis of acetylcholine (15 mM) by sheepshead minnow brain homogenate.

TABLE 1

In vitro organophosphate pesticide inhibition of sheepshead minnow brain AChE compared to toxicity.

Pesticide	Percent inhibition at 1×10^{-4} M concentration	48-hour LD 50 (µg per liter of aquarium water)
Guthion	59.3	3.5
Phorate	31.5	9.0
Parathion	27.8	100.0
Diazinon	100.0	100.0

thirtieth as toxic as Guthion, one-tenth as toxic as phorate, and as toxic as parathion. Although inhibitions by Guthion, phorate, and parathion are in the order of toxicity, the inhibitions are not related to the magnitude of their toxicity. Guthion is approximately thirty times as toxic as parathion, but causes only about twice the inhibition. Phorate is about ten times as toxic as parathion, but causes about the same inhibition. The poor correlation between *in vitro* inhibition and *in vivo* toxicity can be explained by the fact that toxicity depends on *in vivo* AChE inhibition which, in turn, depends on the rate at which the pesticide penetrates certain membranes and the rate at which the pesticide is converted metabolically to more or less toxic compounds. Therefore, only *in vivo* inhibition could be a meaningful indicator of toxicity.

Proposed Techniques

Data from this study indicate the following procedure is suitable for measuring normal and *in vivo* inhibited brain AChE with the automated pH stat: pool 5 to 10 brains from fish of similar size, weigh wet, homogenize in distilled water, and dilute with distilled water until tissue concentration is 5 mg per ml; mix 2 ml of diluted brain homogenate with 2 ml of 0.03 M acetylcholine iodide in distilled water; titrate the liberated acetic acid with carbonate-free 0.01 N NaOH; carry out the reaction at pH 7 and 22° C while passing nitrogen over the liquid to prevent adsorption of atmospheric carbon dioxide. Calculate the micromoles of substrate hydrolyzed per unit of time from the number of micromoles of NaOH required to neutralize the liberated acetic acid per unit of time, and express AChE activity as micromoles of ACh hydrolyzed per hour per mg brain tissue.

To establish normal AChE activity, a sufficient number of fish brain samples should be taken for a period of 12 months to determine mean normal brain AChE activity and the standard deviation. For interpretation of in vivo inhibition, bioassay tests of fish in the laboratory should be made to determine the relationship of AChE inhibition to pesticide concentration, length of exposure, and death. The assay method derived from studies in this report, when applied in tests comparing in vivo brain AChE inhibition and toxicity in sheepshead minnows, yields AChE activity measurements that correlate well with exposure and observed toxicity (6). It is likely that a similar characterization and assay method would lead to improved correlation between brain AChE inhibition and observed toxicity in other fish. The confusing relationship between mortality and degree of in vivo AChE inhibition reported by Gibson et. al. (5) is not evident in my work with the pH stat. The difference could be in methodology, since the spectrophotometric method used in their work is subject to several limitations and possible sources of error (7).

The method utilizing the pH stat overcomes many of the limitations and sources of error of other AChE assay methods. It does not utilize buffers, and is rapid and simple to operate. Rate curves are obtained by continuous recording of hydrolysis. Also, pH, temperature, and enzyme and substrate concentrations can be adjusted and maintained to permit studies of kinetics and optimum conditions. It is not subject to errors from color interference inherent in spectrophotometric methods. It is not necessary to use substrates foreign to the enzyme, and small errors in substrate concentration would not significantly alter results as would be the case where residual ACh is measured.

Acknowledgment

I thank Mr. Edward Matthews for his assistance in the assays.

References

1. WEISS, C. M., Ecology 39, 194 (1958)
2. WEISS, C. M., Trans. Amer. Fish. Soc. 90, 143 (1961)
3. HOLLAND, H. T., COPPAGE, D. L., and BUTLER, P. A., Bull. Environ. Contam. Toxicol. 2, 56 (1967)
4. WILLIAMS, A. K., and SOVA, R. C., Bull. Environ. Contam. Toxicol. 1, 198 (1966)
5. GIBSON, J. R., LUDKE, J. L., and FERGUSON, D. E., Bull. Environ. Contam. Toxicol. 4, 17 (1969)
6. COPPAGE, D. L., Unpublished data
7. WITTER, R. W., Arch. Environ. Health 6, 537 (1963)