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Different Sensitivities to Paraoxon of Brain and Serum Cholinesterases from Pacu, an Indigenous Brazilian Fish

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The importance of fish-farming using economic indigenous fish in agricultural areas has been steadily increasing in Brazil. Despite the usage of organophosphate pesticides (OP) to protect crops in these lands, there is a lack of information concerning the biochemistry of OP intoxication of freshwater Brazilian fish.

Inhibition of brain acetylcholinesterase (AChE) activity has been considered the main event responsible for the toxicity of organophosphorus compounds to vertebrates (Saunders and Harper 1994). Among some non-Brazilian fish species, the correlation between reduced activity levels of brain ACE and mortality have been established as being variable (Gibson et al. 1969; Coppage 1972; Kozlovskaya and Mayer 1984; Chambers and Carr 1995). As a result, the use of brain AChE inhibition to evaluate fish intoxication by anticholinesterase compounds is controversial. Such variability may be due to assays of brain AChE performed without proper kinetic adaptations. Aside from that, acetylcholine may not be the only substrate hydrolyzed in the brain of a fish; propionylcholine and butyrylcholine might also have their ester bonds split. It is also reasonable to suppose that before reaching the brain, a considerable amount of pesticide should be found in serum of fish, producing inhibition of serum propionyl, butyryl and acetylcholinesterases, as well. Consequently, sensitivity of brain and serum cholinesterases to inhibition by OP should be biochemically characterized in a given fish species, in order to make it easier to evaluate how much intoxicated the fish is after OP absorption.

The present investigation was designed to learn about the kinetic properties of cholinesterases from pacu, *Piaractus mesopotamicus*, an economically important Brazilian fish species. Our main point was to look for the response of serum and brain cholinesterase activities of pacu to paraoxon, a potent anticholinesterase organophosphate pesticide which results from parathion bioactivation (Neal 1967; Cunha Bastos et al. 1992). Also, this work was intended to study whether serum and brain cholinesterases activities could be equally used for biodetecting sublethal exposure of pacu specimens to parathion.

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MATERIALS AND METHODS

Pacu specimens (*Piaractus mesopotamicus* Holmberg 1887, Characidae) were kindly donated by the Research and Training Center for Aquaculture (CEPTA), which is supported by the Brazilian Environment and Renewable Natural Resources Institute (IBAMA). Pacus measured roughly 25 cm and weighed approximately 160 g. Animals were kept in 500 L aerated tanks. Fish were acclimated for at least 10 days before being used.

Blood was collected by puncture of the dorsal aorta and transferred to glass centrifuge tubes. After being refrigerated for 30 min the tubes were centrifuged at 3,000 rpm and the serum taken. The sera used for the cholinesterase assays were up to 5 days old (kept at -20 °C). The brains were removed and homogenized using a Potter-Elvehjem type apparatus in 5.6 volumes of a 0.1 M sodium phosphate buffer, pH 7.2, containing 0.25 M sucrose at 4-6 °C. The homogenate was used on the same day for enzyme determinations. Protein concentration was determined as described by Peterson (1977).

The iodides of acetylthiocholine (ACh), propionylthiocholine (PCh) and butyrylthiocholine (BCh), 5, 5-dithiobis (2-nitrobenzoic acid) (DTNB) and paraoxon (O,O-diethyl O-p-nitrophenyl phosphate) were purchased from Sigma Chemical Company, St. Louis, MO, USA. Hexane specific for pesticide analysis was purchased from Grupo Química Industrial Ltda., Rio de Janeiro, Brazil. Contaminating p-nitrophenol was extracted by mixing the commercial paraoxon with 8 volumes of sodium phosphate buffer 0.1 M, pH 7.7, and 8 volumes of hexane in an assay tube. This content was vigorously shaken to ensure paraoxon solubilization, changing the hexane as repeatedly as needed. Each time, after phase separation, the superior layer was transferred to a brownish-glass flask and the hexane was evaporated under a stream of nitrogen. Cholinesterase activities were determined based on the method of Ellman et al. (1961) at 25 °C. The final incubation volume was 2.0 mL, reached with an adequate volume of buffer. Detection of the thiocholine was done by adding 64 µL of 0.01 M DTNB and following the absorbance at 412 nm. The optimum pH was determined using adequate volumes of a 0.1 M phosphate buffer (pH 5.7; 6.7; 7.2; 7.7 and 8.0) or a 0.1 M Glycine-NaOH buffer (pH 8.6 and 9.4). The substrate and protein concentration curves were performed by pipetting appropriate volumes of two substrate solutions (0.075 M or 0.0075 M in 0.1 M phosphate buffer, pH 7.7), and brain homogenates or serum, as required. Once the ideal assay conditions were established, the ordinary determination media of BChE, PChE and AChE activities contained every reagent dissolved in a 0.1 M phosphate buffer, pH 7.7. In these solutions, for brain cholinesterases determinations, a volume of brain homogenate containing 50 µg of proteins was mixed for AChE and PChE, and a volume containing 100 µg was used for BChE. Serum activities were assayed using 50 µL of serum. Reactions were started by adding 50 µL of 0.075 M of substrate (ACh, PCh or BCh). The paraoxon inhibition curves were obtained by adding different amounts of paraoxon to the reaction mixture (from a 1 ppm

solution, made using 1.0 mg of the purified paraoxon dissolved in 0.01 ml, benzene plus a volume of 0.1 M phosphate buffer, pH 7.7, up to 1.0 L).

RESULTS AND DISCUSSION

As a step to perform precise evaluation of pacu exposure to anticholinesterase pesticides, we needed to learn about the biochemistry of its serum and brain cholinesterases inhibition by paraoxon. In order to know if the higher activity could also be the one with the higher sensitivity to OP, we looked for which choline ester was best hydrolyzed by serum and brain of pacu.

The velocity versus pH curves shown in Figure 1-A (bottom) and 1-B (bottom) clearly show that the best pH to assay pacu brain or serum cholinesterase activities is 7.7. Figure 1-A also shows that AChE is the higher activity present in brain of pacu, although lower levels of PChE and BChE activities are also present. Even with the higher affinity (lower K_M) for BCh as it can be seen in Figure 2-A, brain hydrolysis of BCh occurred at a rate 10 times lower than the rate of ACh and PCh (Figure l-A, top). Comparing with the results in brain (Figure l-A), the results presented in Figure 1-B for AChE, PChE, and BChE activity levels in serum show that BChE are not so low in serum of pacu. In fact, BChE showed the higher maximum activity measured in serum. On the other hand, AChE presented the lower activity. Even showing the lowest K_M for its substrate (Figure 2-B), serum PChE activity proved to be steadily intermediate between serum BChE and serum AChE (Figure 1-B). On the basis of the calculated ratio V/K_M, Table I clearly shows that BChE is the "best" activity in serum and that AChE is the "best" activity in the brain of pacu.

Table 1. Calculated V/K_M ratio of Brain and Serum Cholinesterases of Pacu. The data were obtained from Figure 2.

	Ratio V/K_M	
Enzyme activity	Brain ($mg^{-1} \times min^{-1} \times L$)	Serum (min^{-1})
AChE	$2{,}80 \times 10^{-3}$	$2{,}95 \times 10^{-3}$
PChE	$6{,}49 \times 10^{-4}$	$7{,}64 \times 10^{-3}$
BChE	$2{,}10 \times 10^{-4}$	$8{,}39 \times 10^{-3}$

Figures 3-A and 3-B indicate that paraoxon concentrations 5 times lower than those used to produce inhibition of brain cholinesterase activities were able to produce nearly total inhibition of the serum activities. Calculations of paraoxon concentrations able to inhibit 50% (I_{50}) of brain AChE or serum BChE of pacu (Dixon 1953) demonstrated average values of 254 nM and 1.8 nM, respectively. It is reasonable to suppose that in intoxicated pacu a circulating paraoxon amount corresponding to 25 nM— a tenth of its I_{50} brain AChE— will be the lowest concentration capable of affecting its cerebral acetylcholine dependent functions. Such a concentration would be exceedingly inhibitory to serum BChE, as shown in Figure 3-B. So, it is possible to conclude that serum BChE determinations can be much more effective in showing if a pacu specimen had contact with sublethal

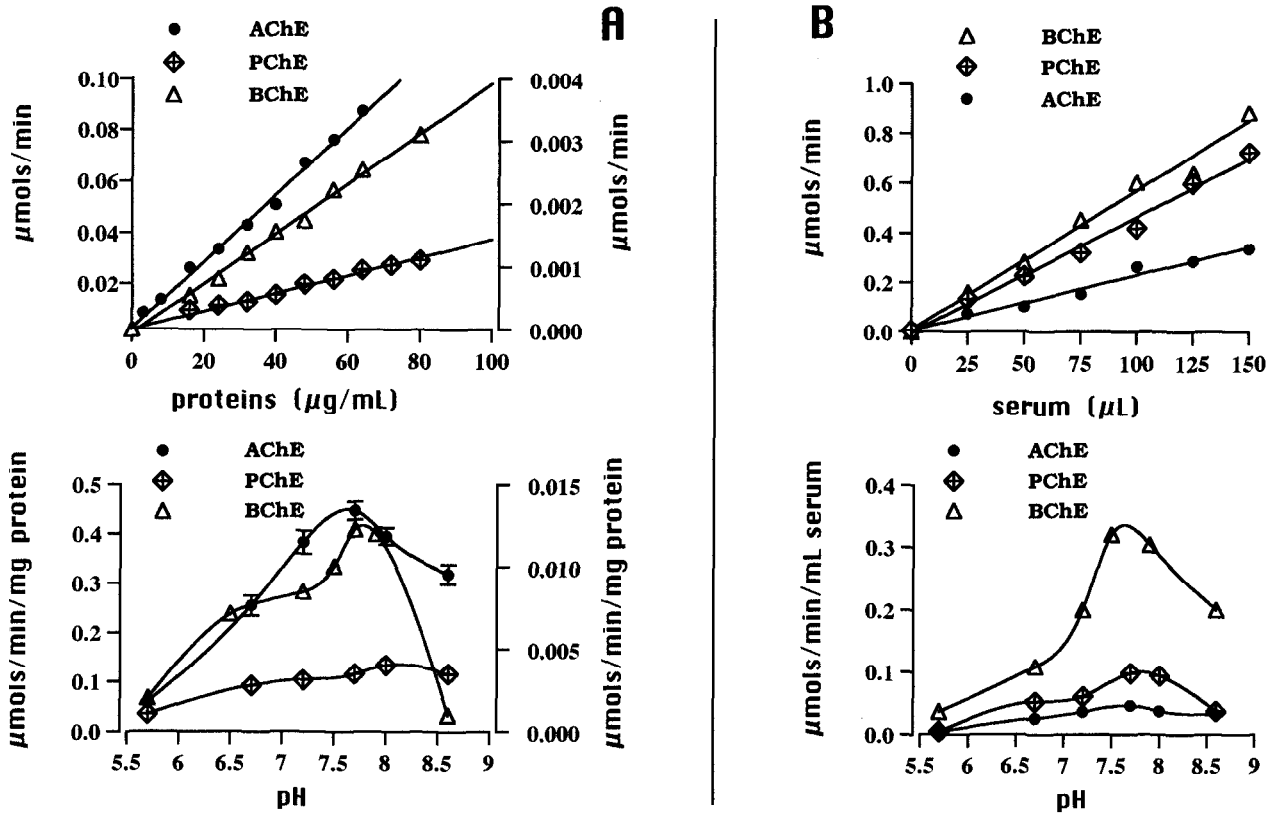

Figure 1. Velocity of brain (A] and serum (B) cholinesterases of pacu as a function of protein concentration or µL of serum and of pH. The ordinate at the right in (A) shows activities corresponding to BChE. Every value is the mean of triplicates assays from each of 7 fish specimens. Some standard errors of media (S.E.M.) were minor than 9%, too small to be drawn to scale and are not shown.

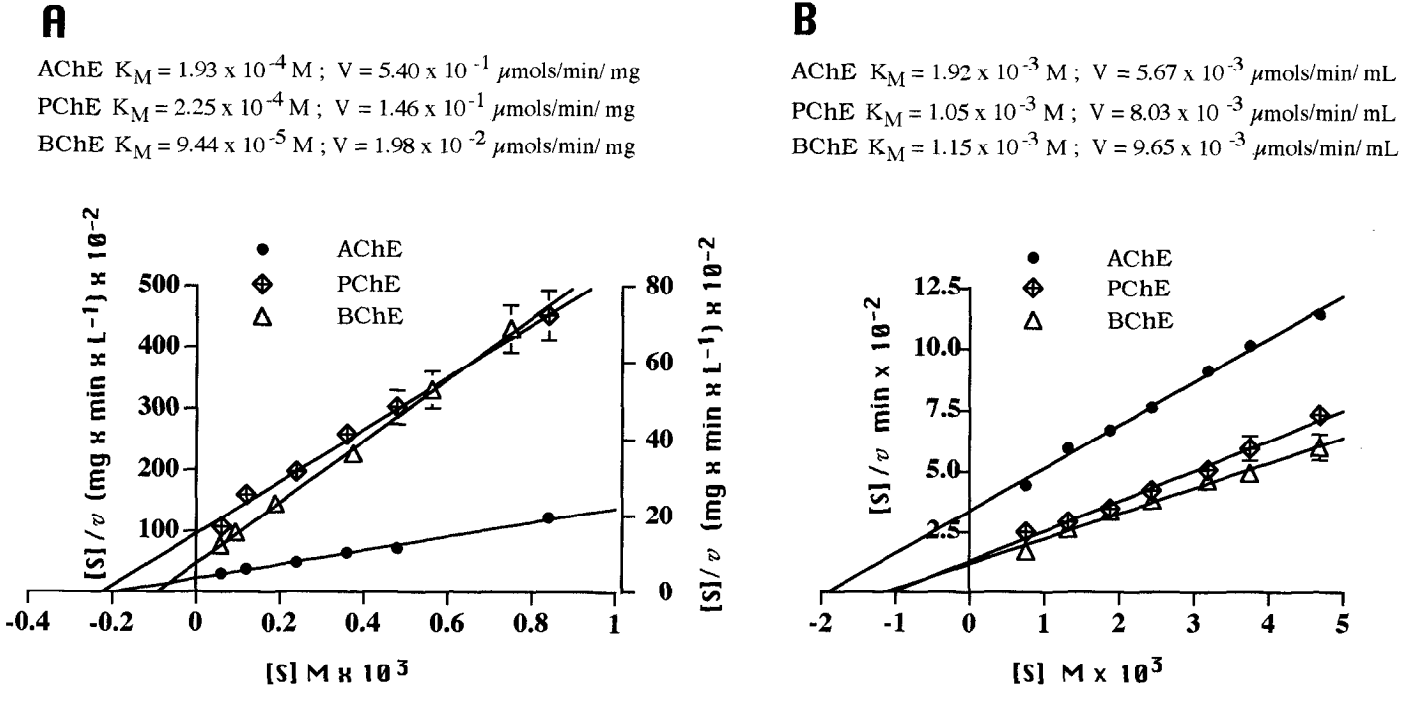

Figure 2. Hanes-Woolf plot of AChE, BChE and PChE activities from pacu brain homogenate (**A**) and serum (**B**). The ordinate at the left in (A) shows [S]/v ratios corresponding to BChE. Every point is the mean of triplicate assays from each of 7 fish specimens ± S.E.M. Some S.E.M. are too small to be drawn to scale and are not shown.

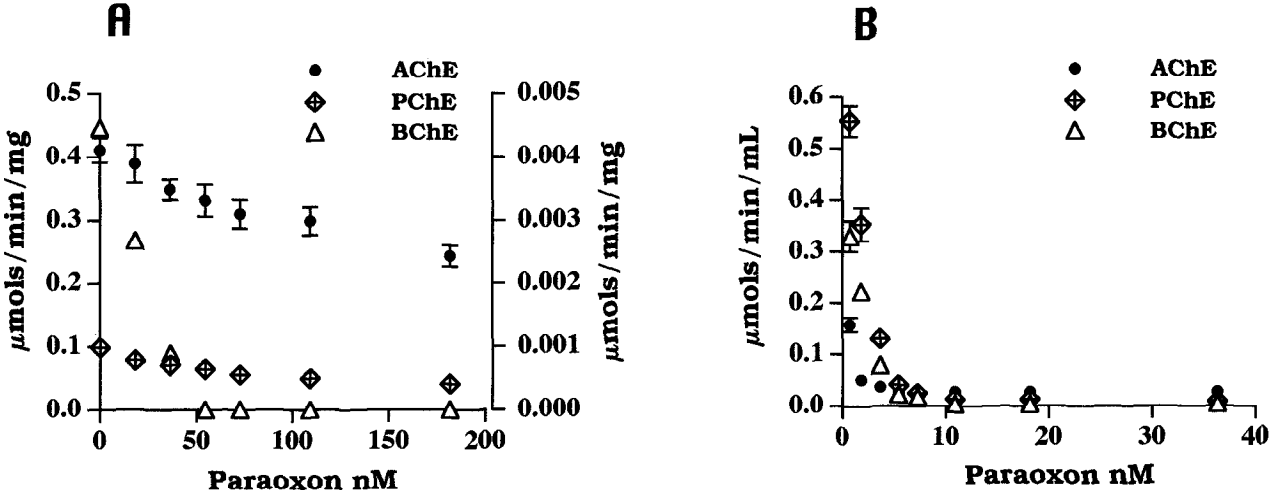

Figure 3. Paraoxon inhibition curves of AChE, PChE and BChE from brain (A) and serum (B) of pacu. The ordinate on the right in (A) shows *v* corresponding to BChE. Every point is the mean of triplicate assays from each of 7 fish specimens± S.E.M. Some S.E.M. are too small to be drawn to scale and are not shown.

organophosphate concentrations than assays of brain AChE, for it would be possible to detect the organophosphate in the fish bloodstream before it could hinder cholinesterases nervous mechanisms in the fish.

The inhibitory interaction of paraoxon molecules with esterases result into their stoichiometric, non-catalytic, hydrolysis (Chambers et al. 1990). Moreover, data from our laboratory showed that the hydrolysis of paraoxon occurs in serum of pacu *in vitro* with half of its maximum rate at 3.3 mM paraoxon (unpublished). As a consequence, chances are that paraoxon serum concentrations capable of nearly 100% inhibition of serum cholinesterases of pacu—values above 14 nM, as shown in Figure 3-B—might circulate almost intact in pacu serum. Taking these facts into account an important fact arises: only when nearly 100% of serum BChE inhibition is found in pacu it will be possible to argue that some paraoxon coming from serum might be affecting its brain.

The *in vitro* inhibition curves we performed pointed out that either of BChE determinations, using brain or serum of pacu, accurately detected paraoxon concentrations around 10 ng/mL in brain homogenates and 3 ng/mL in serum (Figures 3-A and 3-B), which are amounts under the detection limit of gas chromatography employed for paraoxon determinations in biological samples (Abbas and Hayton 1996). Hence, interpolating the data of paraoxon BChE inhibition curves performed *in vitro* with the activity levels of BChE found in serum and brain of pacu, would be a highly sensitive method for the detection of sublethal levels of paraoxon in those specimens suspected of being intoxicated.

The results reported in this paper indicate that determining the organophosphate inhibition of BChE from serum or brain homogenates can be more sensitive to establish if pacu specimens are intoxicated, than assaying only their brain AChE. Sensitivities of both pacu serum and brain BChE activities to paraoxon indicate that using BChE determinations is more accurate to detect sublethal intoxicating levels of this organophosphate than using gas chromatography. Our findings suggest that seeking for which concentrations of anticholinesterase pesticide will cause quantitative inhibition of hydrolysis of different choline esters in serum and brain of fish should be part of any precise approach to evaluate if a given species is being exposed to important subintoxicating levels of organophosphate pesticides.

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