PARAOXONASE ACTIVITY IN SERA FROM *PIARACTUS MESOPOTAMICUS* HOLMBERG (CHARACIDAE) AND *HYPOSTOMUS PUNCTATUS* VALENCIENNES (SILURIDAE)

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ABSTRACT. A paraoxonase activity present in serum of two Brazilian fish species was consistently assayed at pH 8.5 using 7.5 mM paraoxon final concentration. The paraoxonase activity was more activated by 0.5 M NaCl in serum of *Piaractus mesopotamicus* Holmberg, 1887 (pacu) than in serum of *Hypostomus punctatus* Valenciennes, 1840 (cascudo). Apparent values of K_M were 3.3×10^{-3} M for cascudo and pacu paraoxonase activity in the presence of 0.5 M NaCl. Apparent maximum velocity values calculated in the presence of 0.5 M NaCl were 6.1 and 6.5 nmole/min/mL of serum for cascudo and pacu, respectively. V_{max}/K_M ratio values of determinations in the presence and absence of 0.5 M NaCl showed that NaCl had a more evident effect on increasing the affinity of serum paraoxonase for paraoxon in pacu serum. Young specimens of pacu showed a marked decreased paraoxonase serum activity when kept in tanks treated with 0.25 ppm methyl-parathion.
KEY WORDS. Detoxication, fish, organophosphate, serum esterases

Studies on fish enzymes capable of catalyzing the metabolism of pesticides are important to elucidate the processes of intoxication and detoxication in fish exposed to pollutants. Knowledge coming out from such studies can supply the fundamentals to assess intoxication levels yet not macroscopically evident; what is of major utility to keep, during long periods, environmental pollution at compatible levels with life. Indeed, concerning sublethal intoxication of fish, it is noteworthy that pesticides have been used in fish-farming tanks in order to avoid proliferation of predatory insects and parasites which ordinarily attack fish (GRAVE *et al.* 1991a,b).

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Parathion (O,O-diethyl O-p-nitrophenyl phosphorothionate) is still largely used in Brazil to fight agricultural pests. Its original molecule is not toxic, but after undergoing metabolism it renders paraoxon (DIGGLE & GAGE 1951; DUBOIS 1961; NEAL 1967), a phosphate ester (O,O–diethyl O–p–nitrophenyl phosphate), which well known toxic effects come from its ability to inhibit nerve acetylcholinesterase – AChE (BENKE *et al.* 1974; MAIN 1976). Inhibition of AChE by paraoxon results in accumulation of ACh in cholinergic synapses and neuromuscular plates, provoking a steady depolarization of the affected neurons. This might cause death due to respiratory paralysis (NATOFF 1971; MURPHY 1975; BRIMBLECOMBE 1977).

Many living animals are able to get rid of paraoxon through hydrolysis reactions which produce *p*-nitrophenol and diethylphosphoric acid (NEAL & DUBOIS 1965; PLAYFER *et al.* 1976; LI *et al.* 1993, 1995). This hydrolysis can be specifically catalized by an enzyme named paraoxonase (ALDRIDGE 1953). Paraoxonase owes its name to the fact that it shows specificity to paraoxon, albeit being also able to hydrolyze other organophosphate pesticide analogs (REINER *et al.* 1993; WALKER 1993).

We were able to demonstrate that the liver of cascudo, *Hypostomus punctatus* Valenciennes, 1840, a bottom dwelling suckermouth catfish, is capable of biotransforming parathion into paraoxon (CUNHA BASTOS *et al.* 1992). We also showed that brain tissue of cascudo converts parathion into paraoxon (CUNHA BASTOS *et al.* 1988). Livers and brains of pacu, *Piaractus mesopotamicus* Holmberg, 1887, showed to be able to biotransform parathion into paraoxon, as well (data not published). It can be expected that at least a portion of the formed paraoxon in both tissues might be hydrolyzed. Different levels of nerve intoxication may become apparent depending on the promptness with which the *in vivo* formed paraoxon undergoes hydrolysis. In fact, some insects and birds are more sensitive to organophosphates insecticides because they lack the enzymes able to hydrolyze such compounds (BREALEY *et al.* 1980; WALKER & MACKNESS 1987; ECOBICHON 1996).

Attempts to clarify whether serum paraoxonase levels of activity could be involved with nerve intoxication in fish are now in progress in our laboratory. So far no detailed description on paraoxonase determinations in fish serum has been available. Except by trout liver, it has been published that fish tissues showed no appreciable paraoxon hydrolyzing activity (CHEMNITIUS *et al.* 1983).

Kinetic properties of any enzyme can vary among species in such a way that *in vitro* determinations performed without validation of parameters like optima pH, substrate concentration and temperature could be responsible for unreliable results. The results described here were obtained from experiments using specimens of *H. punctatus* and *P. mesopotamicus*. Detailed steps to characterize paraoxonase activity present in serum from both fish and the curves corresponding to the ideal parameters for the determination of this activity *in vitro* are presented. Paraoxonase activity assays using sera of young pacus raised in tanks pretreated with methyl-parathion were also carried as an attempt to understand whether this serum pesticide hydrolytic activity could be related to any possible level of intoxication in fish.

MATERIAL AND METHODS

Fish

Specimens of *H. punctatus* (cascudo) were obtained from the Aquaculture Department of the Rio de Janeiro Federal Rural University (UFRRJ). Sexually mature males and females of cascudo were used. Fish weighed approximately 250 g and measured between 28 and 33 cm. Specimens of *P. mesopotamicus* (pacu) were kindly donated by the National Centre for Research on Tropical Fish (CEPTA) of the Brazilian Environment and Renewable Natural Resourses Institute (IBAMA). Pacus measured around 23 cm and weighed approximately 150 g. After arriving in the laboratory the animals were kept in 500 L aerated tanks equiped with biologic filter containing dechlorinated water. Fish were aclimated for at least 10 days before being used. Eggs of *P. mesopotamicus* were artificially fertilized in November 1995 at CEPTA facilities. Post-larvae pacu specimens were divided into two tanks (350 m^2, 1.2 m deep). After two days one tank received methyl-parathion to 0.25 ppm. Three months later fish from both tanks (10 – 12 cm, weighing 24 – 28 g) were collected to have their serum paraoxon esterase activities compared.

Sera

Cascudo blood was obtained from cardiac puncture. Pacu blood was from dorsal aorta puncture, injecting the needle beneath the lateral line into points located between the tail and the middle of the fish's body. Tubes with blood stayed at 20°C until completely clotted. Then, they were kept in a refrigerator for 20 min. After centrifugation at 3,000 rpm the serum was collected from each tube with a long needle. The sera used for the assays were up to 5 days old (kept under refrigeration).

Reagents

Trichloroacetic acid (TCA), 2-amino-2-(hydroxymethyl-1,3-propanediol (Trizma®), hydrochloric acid (HCl), paraoxon (O,O–diethyl O–p–nitrophenyl phosphate) and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Company, St. Louis, MO, U.S.A. Hexane specific for pesticide analysis was purchased from Grupo Química Industrial Ltda., Rio de Janeiro, Brazil. Methyl-parathion was donated by Centro de Estudos da Saúde do Trabalhador e Ecologia Humana (CESTEH) of Oswaldo Cruz Institute, Rio de Janeiro. All other chemicals were of analytical grade.

Paraoxon repurification

Paraoxon bought from Sigma had an amount of p-nitrophenol (p-NP) contamination which was extracted using hexane. A small volume of paraoxon was mixed with 8 volumes of sodium phosphate buffer 0.1 M, pH 7.4, 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 nitrogen was then bubbled inside this flask till hexane had evaporated. This simple procedure proved to be extremely useful to assure low blank absorbance values.

Paraoxonase activity assay

It was carried out by measuring the p-nitrophenol resulting from serum paraoxon hydrolysis at pH 8.5. The ordinary paraoxonase activity assay was

performed through mixing the following solutions (always prepared by dissolving every solute into a Tris-HCl 0.1 M buffer solution, pH 8.5). Firstly, 50 μL of serum were pipetted into tubes named "assay". In those "assays" depicted to contain salts, 50 μL of a 3 M NaCl and 12 mM $CaCl_2$ solution were added before adding 150 μL of the Tris-HCl buffer. Into tubes without salts 200 μL of the Tris-HCl buffer alone were added. 50 μL of a solution containing 45 mM paraoxon and 45% DMSO into the Tris-HCl buffer were pipetted to start the enzymatic reaction. To measure the activity at different pH values a sodium phosphate buffer 0.1 M, pH 6.5, a Tris-HCl buffer 0.1 M, from pH 7.0 up to pH 9.0, and a glycine-NaOH 0.1 M buffer, pH 9.5 and pH 10.0, were used to sustain the respective pH values. To evaluate the influence of different substrate concentrations over the velocity of paraoxon hydrolysis two solutions of paraoxon were used. A 45 mM solution was made by mixing 12 mg of purified paraoxon with 436 μL of DMSO and 532 μL of Tris-HCl 0.1 M, pH 8.5. A 4.5 mM paraoxon solution was obtained diluting 40 μL of the 45 mM solution with 162 μL of DMSO and 198 μL of Tris-HCl 0.1 M, pH 8.5. Different paraoxon concentrations were used in the range of 0.015 to 7.5 mM. To denature the enzymes 0.9 mL of a 3% TCA (in water) were pipetted into the tubes once elapsed the appropriated time of each reaction. 50 μL of serum were added into the tubes named "blank" (using serum which had been kept along the entire reaction time under the same temperature of the tubes named assay) just after TCA addition. Then, "assay" and "blank" tubes were centrifuged at 4,000 rpm for 15 min and 0.9 mL aliquots of each supernatant were mixed with 0.2 mL of a 0.3 M Tris-HCl buffer solution, pH 8.0. Absorbance measurement in each tube was registered at 400 nm using a Shimadzu UV-160A spectrophotometer in 1 cm optical path cuvettes. Calculations to know the amount of p-nitrophenol formed was done using a p-nitrophenol standard curve (1.5 to 75 nmoles concentration range).

RESULTS

Paraoxonase activity was not lost during the five days sera were kept in the refrigerator at about 5°C. Hexane purified paraoxon gave low blank values up to 6 days after being purified. After this time it had to be repurified to be used as substrate of subsequent enzymatic determinations.

As it can be seen in figure 1 it was possible to assay paraoxonase activity of sera from both species of fish for up to 20 min with no significant loss of linearity.

Figure 2 shows that paroxonase activities from serum of pacu and cascudo were proportional to the amount of serum used in the assay. The volume of 50 μL of serum we have chosen to assay paraoxonase allowed activities ranging from 3 nmoles of p-nitrophenol/min/mL to 6 nmoles/min/mL within 20 min of incubation. Less than 2% of paraoxon was utilized over the 20 min assay time at all substrate concentrations by the 50 μL of serum.

Inferring from the temperature curves showed in figure 3 the optimum temperature to assay the enzyme activity in our conditions was 30°C. In figure 3-B it can be seen that cascudo serum paroxonase levels of activity were low in assay tubes without salts, when assays were run at 20°C and 15°C.

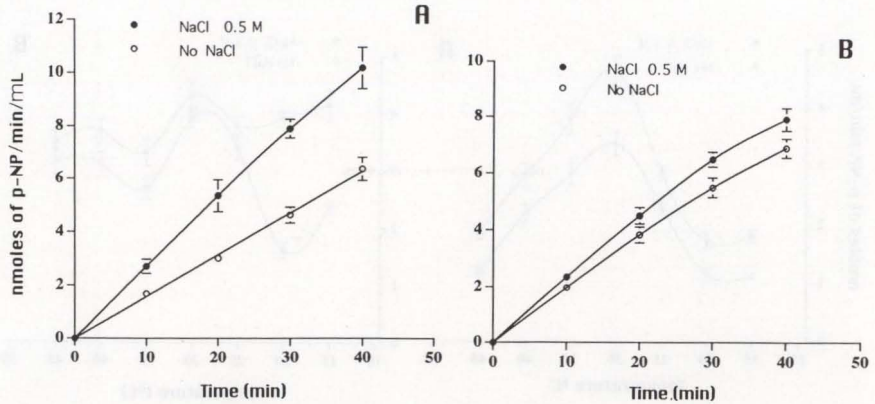

Fig. 1. Paraoxonase serum activity versus incubation time. (A) *P. mesopotamicus*; (B) *H. punctatus*. Assays were performed at 30°C, using 0.1 M Tris-HCl buffer solution pH 8.5, 50 µL of serum in each tube and 7.5 mM paraoxon. Each point is the mean of six assays ± S.E.M. Some S.E.M. are too small to be drew to scale and are not shown.

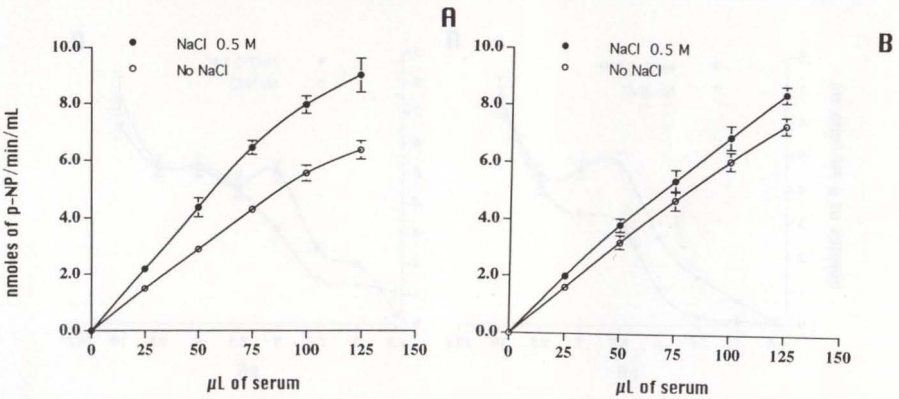

Fig. 2. Paraoxonase activity as a function of serum volume. (A) *P. mesopotamicus*; (B) *H. punctatus*. Assays were performed at 30°C for 20 min, using 0.1 M Tris-HCl buffer solution pH 8.5 and 7.5 mM paraoxon. Each point is the mean of 6 assays ± S.E.M. Some S.E.M. are too small to be drew to scale and are not shown.

As shown in figure 4 serum paraoxonase activity of both fish reached values of activity over 3 nmoles/min/mL at pH 8.5. Higher activity levels were found at pH 10.0.

Data presented in figures 1-A, 2-A, 3-A, 4-A show that 0.5 M NaCl increased the paraoxonase activity of pacu serum. NaCl at the same concentration had no significative effect over the enzyme activity from cascudo serum assayed at 30°C and pH 8.5 (figures 1-B, 2-B, 3-B, 4-B). The double reciprocal plots of 1/v x 1/[S] (LINEWEAVER & BURKE 1934) presented in figures 5 and 6 indicate that pacu and cascudo sera were able to hydrolyze paraoxon with maximum velocities (V_{max}) varying from 5.7 nmoles/min/mL to 7.9 nmoles/min/mL. The substrate affinity constants (K_M) values presented in figures 5A,B show that 0.5 M NaCl provoked a substrate affinity decrease for serum paroxonase activity of pacu serum. Figures

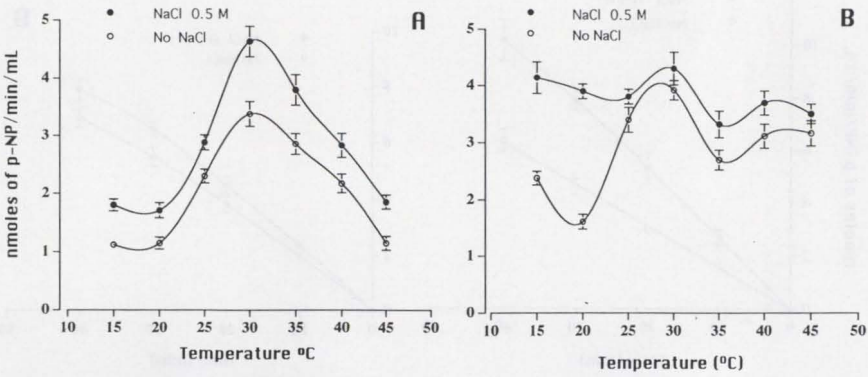

Fig. 3. A typical temperature curve of paraoxonase serum activity. (A) *P. mesopotamicus*; (B) *H. punctatus*. Assays were performed for 20 min, using 0.1 M Tris-HCl buffer solution pH 8.5, 50 μL of serum in each tube and 7.5 mM paraoxon. Each point is the mean of 7 assays ± S.E.M. Some S.E.M. are too small to be drew to scale and are not shown.

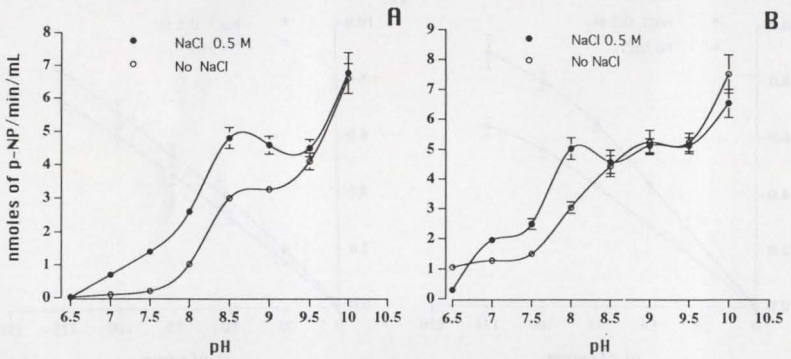

Fig. 4. Influence of pH on enzyme activity. (A) *P. mesopotamicus* serum. (B) *H. punctatus* serum. Assays were performed for 20 min at 30°C using 50 μL of serum and 7.5 mM paraoxon. Buffers used as described in Material and Methods. Each point is the mean of 5 assays ± S.E.M. Some S.E.M. are too small to be drew to scale and are not shown.

6A,B show that 0.5 M NaCl was not so effective in diminishing substrate affinity for serum paraoxonase of cascudo. The K_M values calculated should be taken as minimum values due to the low solubility of paraoxon.

Post-larvae pacu specimens kept in a tank treated with methyl-parathion had their serum paraoxonase activities significantly reduced by 50% at least (Tab. I). This inhibition was more appreciable when enzyme was assayed in the presence of 0.5 M NaCl.

DISCUSSION

Measuring serum paraoxonase activity levels to estimate if fish had been exposed to an organophosphate pollutant led us to the need of establishing a validated method of assaying its activity *in vitro* to avoid misinterpretation of

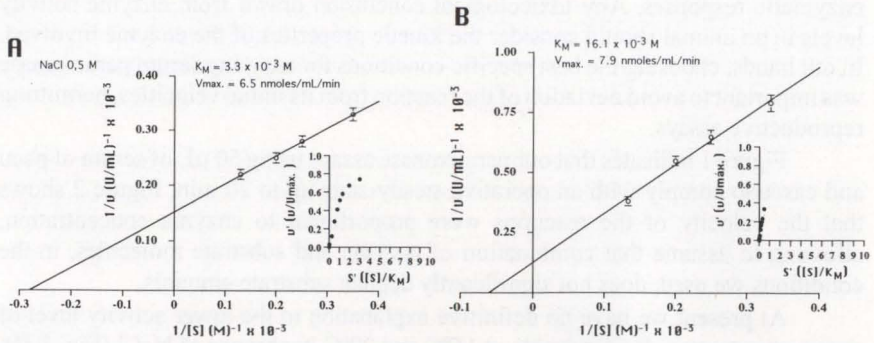

Fig. 5. 1/v versus 1/[S] plot of paraoxonase activity from *P. mesopotamicus* serum. (A) with 0.5 M NaCl; (B) without NaCl. The inserted v' versus [S]' curves show the relantionship between decimals of V_{max} and the apparent K_M of the reaction. Each point is the mean of 6 assays ± S.E.M. Some S.E.M. are too small to be drew to scale and are not shown.

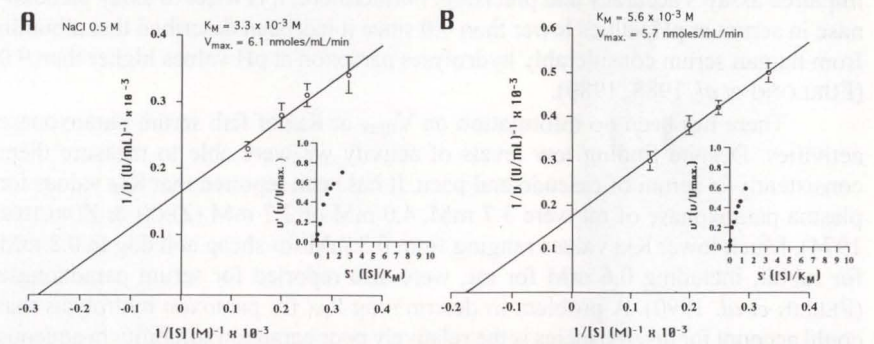

Fig. 6. 1/v versus 1/[S] plot of paraoxonase activity from *H. punctatus* serum. (A) with 0.5 M NaCl; (B) without NaCl. The inserted v' versus [S]' curves show the relantionship between decimals of V_{max} and the apparent K_M of the reaction. Each point is the mean of 6 assays ± S.E.M. Some S.E.M. are too small to be drew to scale and are not shown.

Table I. Serum paraoxonase activity of young *P. mesopotamicus* grown for two months in tanks treated with one 0.25 ppm methyl-parathion dose. Values are expressed as nmoles p-nitrophenol/min/mL of serum.

	Control	Methyl-parathion
0.5 M NaCl	8.79 ± 1.36 (13)	4.82 ± 1.09 (11) *
Without NaCl	4.36 ± 1.54 (11)	2.73 ± 0.81 (8) *

Each value is expressed as the mean ± the standard deviation. Numbers given between parenthesis indicate how many individual serum samples were assayed. (*) Student's t test values of $p < 0.0001$ and (**) $p < 0.04$ related to controls, respectively.

enzymatic responses. Any toxicological conclusion drawn from enzyme activity levels in an animal should consider the kinetic properties of the enzyme involved. In our hands, choosing the best specific conditions for assaying serum paraoxonase was important to avoid deviation of the reaction from its initial velocities, permitting reproductive assays.

Figure 1 indicates that our paraoxonase assays using 50 μL of serum of pacu and cascudo comply with an operative steady-state up to 20 min. Figure 2 shows that the velocity of the reactions were proportional to enzyme concentration, allowing to assume that combination of enzyme and substrate molecules, in the conditions we used, does not significantly deplete substrate amounts.

At present we have no definitive explanation to the lower activity level of serum paraoxonase from cascudo at 15°C and 20°C in absence of NaCl (Fig. 3-B). Such a low activity could be explained on the basis of different molecular structural arrangements of the active site on different paraoxonase molecules.

Accordingly the results shown in figure 4, one could judge more adequate to run paraoxonase assays in serum of both fish at pH 10. However, non-enzymatic hydrolysis of paraoxon is much faster at pH 10 than at pH 8.5. Consequently, we had frequent high blank values when the activity was assayed at pH 10, what impaired assay's accuracy and precision. Furthermore, it is wiser to assay paraoxonase in serum at pH values lower than 9.0 since it has been described that albumin from human serum considerably hydrolyses paraoxon at pH values higher than 9.0 (FURLONG *et al.* 1988, 1989).

There has been no information on V_{max} or K_M of fish serum paraoxonase activities. Despite finding low levels of activity we were able to measure them consistently in serum of cascudo and pacu. It has been reported that K_M values for plasma paraoxonase of rat were 3.7 mM, 4.0 mM or 2.7 mM (ZECH & ZÜRCHER 1974). Much lower K_M values ranging from 0.3 mM for sheep and dog to 0.8 mM for rabbit, including 0.6 mM for rat, were also reported for serum paraoxonase (PELLIN *et al.* 1990). A problem in determining K_M for paraoxon hydrolysis that could account for discrepancies is the relatively poor paraoxon solubility in aqueous buffer solutions. If paraoxon molecules are not available to the active site of the enzyme in the incubaton mixtures a "plateau" of a hyperbolic plot could indicate a false approximation to a maximum velocity reached at non-saturating paraoxon (insoluble) concentrations. This would lead one to calculate a low K_M assuming an apparent saturation of the enzyme. Using 7.5% DMSO in the incubation media allowed paraoxon concentrations up to 7.5 mM in our assays with less than 5% of paraoxonase inhibition. Observing the inserted v' (v/V_{max}) versus S' ($[S]/K_M$) plots in figures 5 and 6 it is noteworthy that 7.5 mM paraoxon did not correspond to 10-fold K_M, which would be the adequate kinetic substrate concentration to ensure the experimental maximum velocity. Nonetheless, 7.5 mM paraoxon caused paraoxonase activity to reach more than 60% of V_{max} in sera of both fish species (Figs 5-A and 6-A, inserts). This made feasible to choose the practical 25% to 70% V_{max} best points of observed velocities related to the experimental substrate concentrations for graphing weighed 1/v versus 1/[S] plots that allowed acceptable approximations for K_M calculation. Therefore, the higher apparent K_M value of 3.3 mM we

found for serum paraoxonase of pacu and cascudo, in the presence of 0.5 M NaCl, agreed with those calculated for rat (ZECH & ZÜRCHER 1974).

Comparison of V_{max}/K_M ratio values of determinations in the presence (1.97×10^{-3} min^{-1} for pacu and 1.85×10^{-3} min^{-1} for cascudo) and absence (0.49×10^{-3} min^{-1} for pacu and 1.02×10^{-3} min^{-1} for cascudo) of 0.5 M NaCl in the assays showed that NaCl had an effect on the affinity of serum paraoxonase for paraoxon in both fish species. These numbers show that paraoxonase of pacu serum was twice more activated than cascudo's activity. Assays of serum paraoxonase activity from individuals are being runned to establish if such a NaCl effect could be concerned with fenotypical differences as described before for human beings (ECKERSON *et al.* 1983).

It was relevant to see that the paraoxonase serum activity we managed to measure changed by keeping fish in a tank with methyl-parathion (Tab. I). At present, there is no detailed explanation for the observed inhibition. In spite of the high K_M observed in pacu and cascudo serum for paraoxon hydrolysis, which must cause the reaction to be operating *in vivo* at a small fraction of the maximum rate, it might be important to investigate if such an inhibition can be useful as a possible sensitive bioindicator capable of informing that fish had been in contact with an amount of organophosphorous pesticide. One can only hypothesize that the protein responsible for paraoxonase fish serum activity would, as several other serum proteins, be produced in liver and that methyl-parathion could be affecting the hepatic synthesis or exportation of paraoxonase in young pacus. Studies on liver and serum paraoxonase activities in intoxicated young pacus specimens are now under way in our laboratory to establish which enzyme activity is more important to sublethal intoxication in pacu. Considering the high enzymatic activity levels observed when sera of non-intoxicated young individuals were assayed in the presence of 0.5 M NaCl we will be also performing assays to learn whether paraoxonase activity is higher in very young fish than in adult ones.

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