Bull. Environ. Contam. Toxicol. (2004) 72:798–805

Bulletin of Environmental Contamination and Toxicology

Paraoxonase Activity in Sera of Four Neotropical Fish

V. L. F. Cunha Bastos,[1] M. V. Alves,[1] G. Bernardino,[2] P. S. Ceccarelli,[2] J. Cunha Bastos[1]

[1] Department of Biochemistry, Biology Institute, Rio de Janeiro State University, Av. Professor Manuel de Abreu, 444, Rio de Janeiro, RJ, 20550-170, Brazil
[2] National Center for Research on Tropical Fish (CEPTA), Rodovia Prefeito Euberto Nemésio Pereira de Godoy, Km 6,5, Caixa Postal 64, 13630-970, Pirassununga, SP, Brazil

Received: 21 May 2003/Accepted: 6 January 2004

In Brazil, parathion, O,O-diethyl O-p-nitrophenyl phosphorothioate is still in use in many insecticide preparations, not only to protect crops, but also in aquaculture to avoid larvae of predatory insects and some parasites that attack juvenile fish.

It has been demonstrated (Cunha Bastos *et al.* 1992) that fish can metabolize through oxidative desulphurization the non-toxic parathion into paraoxon, O,O–diethyl O–p–nitrophenyl phosphate, the anticholinesterase oxon ester. Considering that before reaching the organs where it causes toxic acetylcholinesterase inhibition paraoxon may undergo hydrolysis catalyzed by serum paraoxonase, which hydrolyses paraoxon into p-nitrophenol and diethyl phosphate (Neal and Dubois 1965; Li *et al.* 1993; Pond *et al.* 1995), it is reasonable to suppose that the more paraoxonase exists in an organism, the less this organism will be affected by these insecticides.

As knowledge of enzymes from fish is still evolving and many significant differences could exist among the great diversity of fish species dwelling in Neotropical waters, we have been investigating whether some native fish of South America would show enough paraoxonase in their sera for paraoxon detoxification. The purpose of this paper is to show a comparison among the paraoxonase activities in sera of four important Brazilian fish species and in mice.

MATERIALS AND METHODS

Sexually mature males and females specimens of fish were used. Cascudo (*Hypostomus punctatus* Valenciennes, 1840, Loricariidae) weighed approximately 230 g and measured between 25 and 28 cm. Pacu (*Piaractus mesopotamicus* Holmberg, 1887, Characidae) measured around 25 cm and weighed approximately 350 g. Matrinxãs (*Brycon cephalus* Günther, 1869, Characidae) measured around 33 cm and weighed approximately 400 g. Dourados (*Salminus brasiliensis* Cuvier, 1816, Characidae) measured around 50 cm and weighed approximately 1,400 g. Fish were maintained with no food in 1,000 L tanks for 10 days, before collecting blood. We collected blood from 10 individuals of each fish species and from 10 adult Swiss mice. Clotted blood was centrifuged at 800 x g for 5 min and the sera samples were collected and kept at 4 °C.

Correspondence to: J. Cunha Bastos

Trichloroacetic acid (TCA), 2-amino-2-(hydroxymethyl-1,3-propanediol), hydrochloric acid (HCl), paraoxon (O,O–diethyl O–p–nitrophenyl phosphate) and dimethyl sulphoxide (DMSO) 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. All other chemicals were of analytical grade.

Paraoxonase activity assay measured the p-nitrophenol resulting from serum paraoxon hydrolysis. Assays were carried out at 30 °C in a Tris-HCl 0.1 *M* buffer solution, pH 8.5, by mixing serum from fishes (50 µL) with 200 µL of the Tris-HCl buffer containing 0.75 *M* NaCl and 6.25 m*M* $CaCl_2$. To determine Swiss mouse serum paraoxonase activity we used 15 µL of serum. Then, 50 µL of a solution containing 45 m*M* paraoxon and 45% DMSO into the Tris-HCl buffer were added. Appropriate volumes of each paraoxon solution were pipetted for different paraoxon concentrations ranging from 0.015 to 7.5 m*M*. To stop the reaction, 0.9 mL of 3% TCA (in water) were pipetted into the tubes after 20 min of reaction. Volumes of 50 µL of fish serum or 15 µL of mouse serum, which had been separately kept during the reaction under the same temperature of the "assay" tubes, were added into the tubes named "blank" immediately after TCA addition. Then, "assay" and "blank" tubes were centrifuged at 2,300 x g for 15 min and 0.9 mL aliquots of each supernatant were mixed with 0.2 mL of a 3 *M* Tris-HCl buffer solution, pH 8.0. Absorbance was determined at 400 nm. One unit of activity (U) corresponded to 1 µmol of p-nitrophenol produced per minute of reaction.

In order to establish how much of the paraoxonase activity measured in dourado serum could be associated with high-density lipoproteins, we carried out a KBr density centrifugation of dourado serum in accordance with what has been reported on pacu (Folly *et al.* 2001).

Data are presented as means ± S.E.M. of triplicate assays carried out using ten different serum samples, each one collected from ten specimens. Calculations were done using GraphPad Prism version 3.0 for Windows, GraphPad Software, San Diego, California, U. S. A.

RESULTS AND DISCUSSION

Toxicological conclusions drawn from enzyme activity levels in an animal should consider the kinetics of the enzyme involved. Regrettably, too often it is seen that many laboratories simply reproduce procedures established elsewhere, for diverse organisms. 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 very reproducible assays. Our paraoxonase assays, using 50 µL of sera from fish and 15 µL of sera from mouse, complied with an operative steady-state up to 30 min, thus enzyme was rate limiting. Less than 5% of paraoxon was utilized over the 20 min assay time at all substrate concentrations.

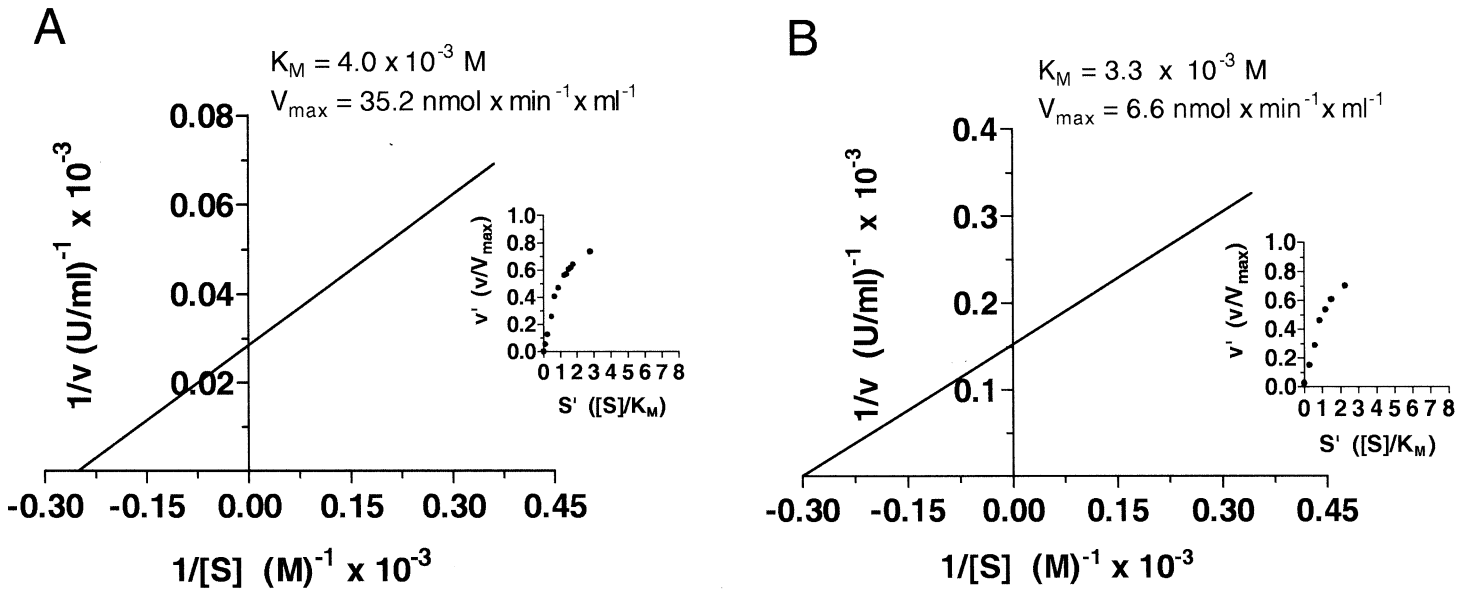

Figure 1. 1/v versus 1/[S] plots of paraoxonase activity from serum of dourado (A) and pacu (B). The assay media contained the following final concentrations of reagents at 30 °C: 0.1 *M* Tris-HCl buffer, pH 8.5; 50 µL of serum; NaCl 0.5 *M*; 2 m*M* $CaCl_2$; 7.5% DMSO and 0.50, 1.00, 1.95, 2.72, 3.51, 5.00, 5.50, 6.00, 6.5, 7.50 m*M* paraoxon. The inserted v' versus [S]' curves show the relationship between decimals of V_{max} and the apparent K_M of the reactions. Each point is the mean of ten assays ± S.E.M. The coefficient of variation of each determination was less than 8%.

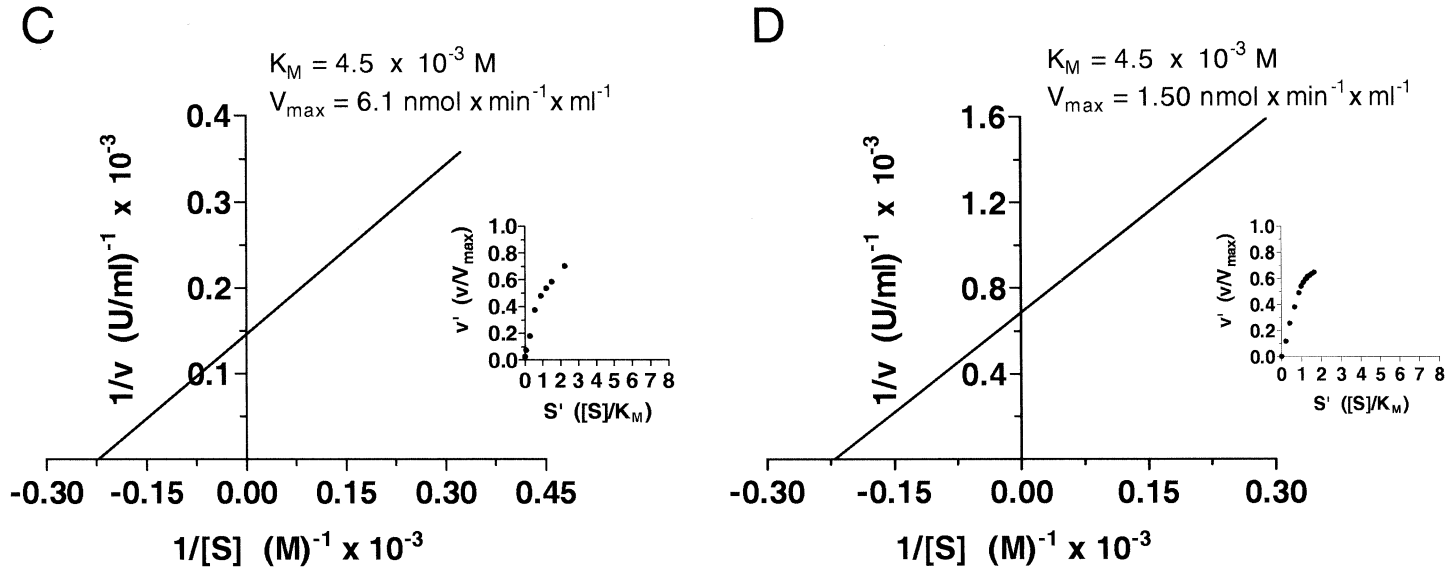

Figure 2. 1/v versus 1/[S] plots of paraoxonase activity from serum of cascudo (C) and matrinxã (D). The assay media contained the following final concentrations of reagents at 30 °C: 0.1 *M* Tris-HCl buffer, pH 8.5; 50 µL of serum; NaCl 0.5 *M*; 2 m*M* $CaCl_2$; 7.5% DMSO and 0.50, 1.00, 1.95, 2.72, 3.51, 5.00, 5.50, 6.00, 6.5, 7.50 m*M* paraoxon. The inserted v' versus [S]' curves show the relationship between decimals of V_{max} and the apparent K_M of the reactions. Each point is the mean of ten assays ± S.E.M. The coefficient of variation of each determination was less than 8%.

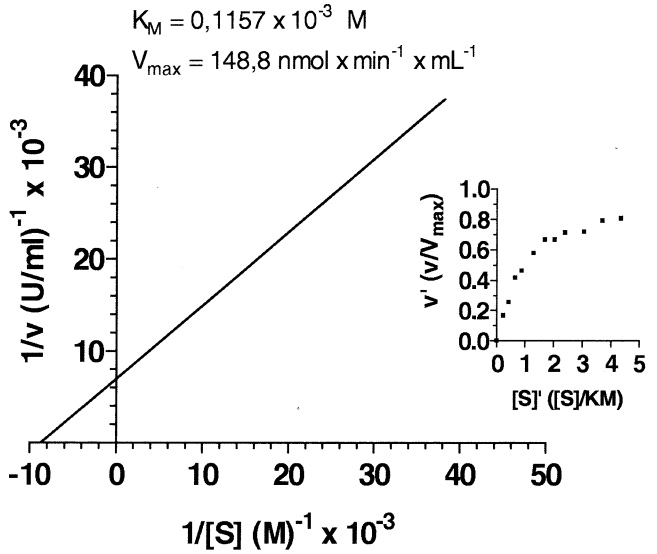

Figure 3. 1/v versus 1/[S] plot of paraoxonase activity from serum of Swiss mouse. The assay media contained the following final concentrations of reagents at 30 °C: 0.1 *M* Tris-HCl buffer, pH 8.5; 15 µL of serum; NaCl 0.5 *M*; 2 m*M* $CaCl_2$; 7.5% DMSO and 0.025, 0.050, 0.075, 0.100, 0.150, 0.200, 0.250, 0.275, 0.350, 0.425, 0.500 m*M* paraoxon. The inserted v' versus [S]' curve shows the relationship between decimals of V_{max} and the apparent K_M of the reaction. Each point is the mean of ten assays ± S.E.M. The coefficient of variation of each determination was less than 8%.

There have been no available V_{max} or K_M values for serum paraoxonase from any fish species. One publication (Chemnitius *et al.* 1983), which used a test concentration of 1 m*M* paraoxon in a pH 7.3 Tyrode buffer at 25 °C, reported that blood of *C. carpio* and *S. trutta* had no appreciable paraoxon hydrolysing activity. In fact, it has been reported that K_M values for plasma paraoxonase of rat were 3.7, 4.0 or 2.7 m*M* (Zech and Zürcher 1974). Lower K_M values, ranging from 0.3 m*M* for sheep and dog to 0.8 mM for rabbit, including 0.6 m*M* for rat and 0.46 m*M* for humans, were also reported for serum paraoxonase (Eckerson *et al.* 1983; Pellin *et al.* 1990). A problem in determining K_M for paraoxon hydrolysis is the relatively poor paraoxon solubility. If paraoxon molecules are not available to the active site of the enzyme, a "plateau" of a hyperbolic plot could indicate a false approximation to a maximum velocity reached at pseudo non-saturating paraoxon (insoluble) concentrations. This could 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 m*M* in our assays with less than 5% of activity inhibition. Observing the inserted v' (v/V_{max}) versus S' ($[S]/K_M$) plots in Figs. 1, 2, 3 and 4 it is seen that 7.5 m*M* paraoxon did not correspond to the 10-fold K_M value, which would be the adequate kinetic substrate concentration to ensure the experimental maximum velocity.

Table 1. Paraoxonase activity of lipoprotein fractions obtained during lipoprotein separation from serum of dourado.

Fraction	Total Paraoxonase (nmol p-nitrophenol x min^{-1} x mL^{-1})	Yield (%)
Serum	37.3	100.0
VLDL density < 1.006 g/mL	0.0	0.0
LDL density 1.02 - 1.06 g/mL	1.42	3.8
HDL density 1.07 - 1.19 g/mL	35.4	95.0

Nonetheless, 7.5 m*M* paraoxon caused paraoxonase activity to reach more than 60% of V_{max} in sera of the four fish (inserts of Figs. 1, 2, 3 and 4). This made it feasible to choose the useful 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, which allowed acceptable approximations for K_M calculation. Therefore, the apparent K_M values ranging from 3.3 to 4.5 m*M* we found for serum paraoxonase of dourado, pacu, cascudo and matrinxã, in the presence of 0.5 *M* NaCl, were calculated from kinetically adequate curves.

Comparing maximal velocities calculated from sera of the four fish with the values found in Swiss mouse serum it is possible to see that serum paraoxonase activity from dourado is at least 24% that of the mouse serum. Paraoxonase activities found in the sera of the other three fish corresponded to less than 5% that of Swiss mouse.

In a previous paper (Folly *et al.* 2001) we showed that paraoxonase was associated with high density lipoprotein (HDL) in serum of pacu. Considering that dourado serum showed an activity sixfold that of pacu serum, we decided to clear up other activities that might also be present in serum of dourado totalling a higher breakdown of paraoxon. Table 1 shows that 95% of the serum paraoxonase activity of dourado serum was recovered in the HDL fraction. Therefore, we concluded that our assays measured major specific paraoxonase activities, which were not due to interference, for instance, from alkaline phosphatase.

Owing to the low values of paraoxonase found in sera of pacu, cascudo and matrinxã, it is unlikely that serum paraoxonase would be protective in these fishes against organophosphates blood concentrations higher than 20 nmol/mL, for such low activities would only destroy very low levels of paraoxon molecules. On the other hand, paraoxonase activity from serum of dourado reached values six times

higher than the values found in pacu and cascudo, and 23 times the values found in matrinxã. Therefore, despite the difficulties in extrapolating our *in vitro* kinetic findings to *in vivo* detoxification events occurring in fish, it is reasonable to suppose that when dourado specimens absorb parathion, biotransform it into paraoxon, and eventually release paraoxon to the blood, their serum should be able to destroy these anticholinesterase organophosphate molecules at a rate sufficient for lowering their concentration. This would diminish the circulating of sublethal concentrations of toxic oxons, helping dourados to cope with incidental absorption of organophosphates.

Acknowledgments. We thank the Rio de Janeiro State Research Support Foundation (FAPERJ) for funding and the National Centre for Research on Tropical Fish (CEPTA) for providing the fish.

REFERENCES

Ashani Y, Rothschild N, Segall Y, Levanon D, Raveh L, (1991) Prophylaxis against organophosphate poisoning by an enzyme hydrolysing organophosphorous compounds in mice. Life Sci 49:367-374

Chemnitius J-M, Losch H, Losch K, Zech R (1983) Organophosphate detoxicating hydrolases in different vertebrate species. Comp Biochem Physiol 76C:85-93

Cunha Bastos J, Cunha Bastos VLF, Rossini A, Fortini H., Castro Faria MV (1992) Activation of parathion by liver of *Hypostomus punctatus*, a Brazilian benthic fish (cascudo). Comp Biochem Physiol 94C:683-689

Diggle, WM., Gage, JC (1951) Cholinesterase inhibition in vitro by O,O-diethyl O-p-nitrophenyl thiophosphate (parathion, E 605). Biochem J 49:491-494.

Dubois KP (1961) Potentiation of the toxicity of organophosphorus compounds. Adv Pest Control Res 4:117-151

Eckerson HW, Romson J, Wyte C, La Du BN (1983) The human serum paraoxonase polymorphism: identification of phenotypes by their response to salts. American J Hum Genet 35:214 - 227

Folly E, Cunha Bastos VLF, Alves MV, Cunha Bastos J, Atella GC (2001) A high density lipoprotein from *Piaractus mesopotamicus*, pacu (Osteichthyes, Characidae), is associated with paraoxonase activity. Biochimie 83:945-951

Li WF, Furlong CE, Costa LG (1993) Serum paraoxonase status: a major factor in determining resistance to organophosphates. J Toxicol Environ Health 40:337-346

Li WF, Furlong CE, Costa, LG (1995) Paraoxonase protects against chlorpyrifos toxicity in mice. Toxicol Lett 76:219-226

Main AR (1976) Structure and inhibitors of cholinesterase. In: Goldberg M, Hanin I. (eds), Biology of Cholinergic Function, Raven Press, New York, p 269

Neal RA (1967) Studies on the metabolism of diethyl-4-nitrophenyl phosphorothionate (parathion) in vitro. Biochem J 108:183-191

Neal RA, Dubois KP (1965) Studies on the mechanism of detoxification of cholinergic phosphorothioates. J Pharmacol Exp Ther 148:185-192

Pellin MC, Moretto A, Lotti M, Vilanova E (1990) Distribution and some biochemical properties of rat paraoxonase activity. Neurotoxicol Teratol 12:611-614

Pond AL, Chambers HW, Chambers JE (1995) Organophosphate detoxication potential of various rat tissues via A-esterase and aliesterase activities. Toxicol Lett 78:245-252

Shih DM, Gu L, Xia Y-R, Navab M, Li W-F, Hama S, Castellani LW, Furlong CE, Costa LG, Fogelman AM, Lusis AJ (1998) Mice lacking serum paraoxonase are susceptible to organophosphate toxicity and atherosclerosis. Nature 394:284-287

Zech R, Zürcher K (1974) Organophosphate splitting serum enzymes in different mammals. Comp Biochem Physiol 48B:427-433