A Heat Stable Paraoxonase (*O,O*-Diethyl *O*-*p*-Nitrophenyl Phosphate *O*-*p*-Nitrophenyl Hydrolase) from Russell's Viper Venom†

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ABSTRACT: Fractionation of Russell's viper venom revealed separate phosphohydrolase activities directed against *p*-nitrophenyl phosphate, bis(*p*-nitrophenyl) phosphate, *p*-nitrophenylthymidylic acid, and *O,O*-diethyl *p*-nitrophenyl phosphate (paraoxon). On gel fractionation, the first two activities are eluted ahead of the latter. They could be resolved further by phosphocellulose cation exchange chromatography. The hydrolytic activities directed against *p*-nitrophenylthymidylic acid hydrolyzing component is heat labile, while the paraoxon hydrolyzing component manifests an unusually high degree of heat stability. Gel filtration yields 9600 for the molecular weight of the "paraoxonase". This enzyme, as all known enzymes of this type, requires the presence of a divalent cation. Maximum activity is obtained in the presence of Ca^{2+}. In the presence of Sr^{2+} the reaction rate is 50% of that of Ca^{2+}; other divalent cations show lower activities. The presence of the enzyme is species specific. Of four species tested, only Russell's viper venom showed significant paraoxonase activity. Enzyme activity is intact following incubation with iodoacetate or *p*-chloromercuribenzoate. Activity is partially preserved even in the presence of 8 *M* urea.

Reactivity of esterases with organophosphate compounds forms the basis of a classification scheme frequently used for these enzymes (Aldridge, 1953a). Enzymes which are inhibited by these compounds can be differentiated from others which are functional in their presence; and a third group is distinguished by the ability of its members to hydrolyze some of these substances. This is the basis of the classification into B, C, and A esterases.

The discovery of a serum enzyme capable of hydrolyzing diethyl *p*-nitrophenyl phosphate (paraoxon) by Aldridge (1953b) and his demonstration of the wide distribution of this enzyme in other tissues led to the extensive purification of the serum enzyme by Main (1960). Subsequently it was shown that the activity apparent in liver homogenates is associated with the microsomal fraction (Neal, 1971).

In an attempt to find other sources for a soluble enzyme of the A esterase class (EC 3.1.1.2) we investigated the effect of several snake venoms on paraoxon. Snake venoms are known to be excellent sources for enzymes acting on different phosphate mono- and diesters. We have subsequently found such activity in some venoms, but there appear to be wide differences in the species examined. The studies reported here were carried out on Russell's viper venom which exhibited the greatest specific activity among those tested.

Materials and methods

Lyophilized Russell's viper venom was obtained from the Miami Serpentarium.

Bis(*p*-nitrophenyl)phosphoric acid and 5′-thymidylyl *p*-nitrophenyl phosphate were products of Calbiochem. AMP disodium salt and *p*-nitrophenyl phosphate were obtained from Sigma. These products were used as supplied. *p*-Nitrophenyl acetate (Eastman) was recrystallized three times from acetone–water.

Paraoxon, 0.1 *M* in propylene glycol, was kindly donated by the E. P. A. Primate Research Center, Perrine, Fla.

The assay conditions for paraoxon hydrolysis were as follows: 0.1 ml of enzyme solution, 50 μl of substrate solution, and 0.85 ml of 50 m*M* Tris-Cl buffer (pH 7.4) containing 5 m*M* $CaCl_2$. Hydrolysis rates were followed at 400 nm or at the isosbestic wavelength of 348 nm (Armstrong et al., 1966) in a Gilford 2000 spectrophotometer.

The venom was dissolved in distilled water, 100 mg in 2.8 ml, and 2 ml of this solution was fractionated on a 38 × 2.5 cm Sephadex G-75 column using 50 m*M* Tris buffer (pH 7.4) without added Ca^{2+}. All fractions were tested for paraoxonase activity, bis(*p*-nitrophenyl) phosphatase activity and *p*-nitrophenyl thymidylase diesterase activity. *p*-Nitrophenol liberation was measured with all *p*-nitrophenyl group containing substrates.

A fraction which elutes close to the void volume, and which does not hydrolyze paraoxon, was reapplied onto a 6-ml phosphocellulose column equilibrated with 0.05 *M* Tris-HCl buffer (pH 7.4). The column was washed with the buffer and the activities were eluted with a solution of the same buffer containing 0.25 *M* NaCl.

p-Nitrophenyl phosphate was employed as substrate at a concentration of 400 μ*M* in a 33 m*M* glycine–NaOH buffer (pH 9.0) containing 10 m*M* $MgCl_2$ (Felix et al., 1960) measured at 400 nm and *p*-nitrophenyl acetate, due to its limited solubility, was used as follows: the substrate, dissolved in Me_2SO, was added to 0.05 *M* Tris-HCl buffer (pH 8.5) in a ratio of 50 μl of Me_2SO solution to 1 ml of buffer giving a final substrate concentration of 1 m*M*. *p*-Nitrophenylthymidylic acid was used in 7 m*M* concentration in 25 m*M* Tris-HCl buffer (pH 9.4) in the presence of 1 mg of albumin/ml (Hurlbert and Furlong, 1967). Bis(*p*-nitrophenyl) phosphate was used as described by Bjork (1963). 5′-Nucleotidase activity was measured using adenylic acid at 5 m*M* concentration in 50 m*M* Tris-acetate (pH 8.5) containing 50 m*M* $MgCl_2$ (Hurlbert and Furlong, 1967).

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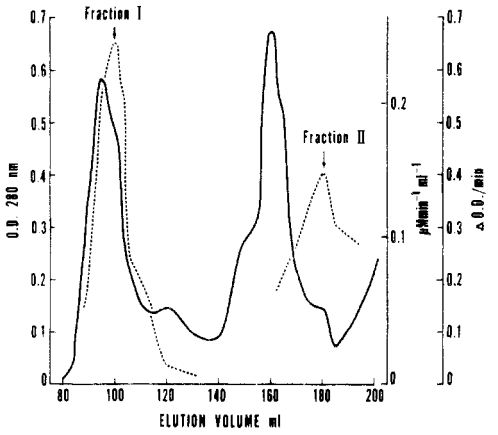

FIGURE 1: Separation of phosphomonoesterase and bis(*p*-nitrophenyl) phosphatase activity (fraction I) from "paraoxonase" and *p*-nitrophenyl thymidylate hydrolyzing activity (fraction II); 71.4 mg of venom in 2 ml of distilled water was applied to a 38 × 2.5 cm Sephadex G-75 column. Elution with 50 m*M* Tris buffer (pH 7.4) without added Ca^{2+}. (—) OD 280 nm; (- - -) enzyme activity. Separation at room temperature, 5-ml fractions, V_0, 75 ml.

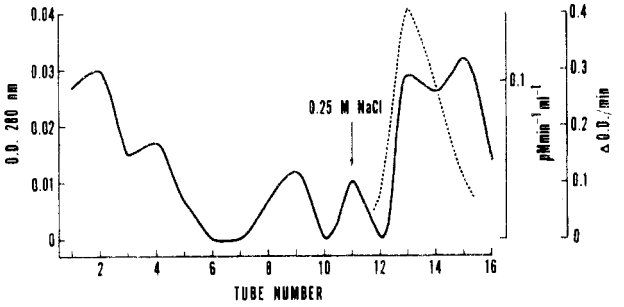

FIGURE 2: Isolation of bis(*p*-nitrophenyl) phosphohydrolase activity on phosphocellulose. Fraction I (Figure 1) was applied to a 6-ml phosphocellulose column equilibrated with 50 m*M* Tris buffer (pH 7.4). Column was eluted first with buffer and then with buffer containing 0.25 *M* NaCl. Fraction size, 5 ml. (—) OD 280 nm; (- - -) enzyme activity.

Table I[a]

Fraction	Enzyme Activity (μM min^{-1} mg^{-1} ml^{-1}) Substrates: Paraoxon	Bis(*p*-nitrophenyl) Phosphate	*p*-Nitrophenyl Thymidylate	*p*-Nitrophenyl Phosphate
A	5.3	3.4	1430	34
B	0	799	214	2010
C	0	4000	1560	0
D	1280	0	49	0

[a] The assays reported were performed employing (A) crude snake venom; (B) fraction with maximum activity against bis(*p*-nitrophenyl) phosphate from fraction I; (C) fraction obtained by rechromatography of fraction I on phosphocellulose and showing maximum activity against bis(*p*-nitrophenyl) phosphate; (D) fraction showing maximum activity against paraxon from fraction II. Protein concentrations were estimated from OD's at 280 and 260 nm.

Table II: Heat Stability of the Crude Snake Venom and Fraction II "Paraoxonase" Activities.

Time (min)	% Activity	Time (min)	% Activity
	Crude[a]		Fraction II[b]
0	(100)	0	(100)
2	63	2	100
5	52.7	4	113.8
10	47.2	8	118.1
14	36.1	15	111.5
20	27.2	30	68
30	17.5		

[a] Assay conditions. 5 mg/ml of distilled H_2O; 0.1-ml aliquots at indicated time interval assayed in 0.05 *M* Tris buffer (pH 7.4), 1 m*M* Ca^+, and 7 m*M* paraoxon. *p*-Nitrophenol liberation was followed at 340 mm. [b] Eluate was treated and assayed as in *a*.

Results

Crude venom solutions hydrolyze all the phosphate ester substrates listed in the Materials and Methods section. Gel filtration separates the bis(*p*-nitrophenyl) phosphatase (phosphodiesterase) activity which emerges slightly behind the void volume. This fraction (fraction I) has no activity on paraoxon, but hydrolyzes bis(*p*-nitrophenyl) phosphate and *p*-nitrophenylthymidylic acid at this stage of separation. 5′-Nucleotidase action is also associated with this fraction, which also hydrolyzes *p*-nitrophenyl phosphate. Paraoxonase activity is eluted in a later peak, emerging at approximately 2 × V_0 and clearly separate from the nearly excluded fractions (fraction II, Figure 1).

Fraction II was also examined for phosphomonoesterase activity using AMP as a substrate. There was no detectable activity. Bis(*p*-nitrophenyl) phosphate was not hydrolyzed by this fraction either, but when *p*-nitrophenylthymidylic acid was used as substrate, liberation of *p*-nitrophenol was noted. The enzyme exhibiting this activity, however, is not responsible for the hydrolysis of paraoxon, because heating an aliquot of this fraction for 2 min in a boiling water bath totally abolishes the phosphodiesterase activity, leaving the paraoxonase activity virtually unaffected. This fraction has no activity on *p*-nitrophenyl phosphate.

Further purification of fraction I was undertaken by chromatography on phosphocellulose. It was applied and washed with 50 m*M* Tris buffer until the OD at 280 nm in the effluent became negligible, and then eluted with 0.25 *M* NaCl in Tris buffer. This fraction contained the phosphodiesterase activity assayed both with bis(*p*-nitrophenyl) phosphate and *p*-nitrophenylthymidylic acid. Phosphomonoesterase activity, however, was no longer found in this fraction when assayed against either AMP or *p*-nitrophenyl phosphate. A clear separation of phosphomonoesterase, diesterase, and paraoxonase activities was thereby accomplished (Figure 2). Activities of the different fractions directed against *p*-nitrophenyl substituted substrates are shown in Table I. No attempt was made to recover the phosphomonoesterase activity which presumably did not elute from the column under the conditions employed.

The paraoxon hydrolyzing enzyme from fraction II is extraordinarily heat stable. Activity is essentially unaffected after prolonged heating in a boiling water bath. On several occasions a slight persistent increase in activity was revealed after heating for 2 min. In contrast to these results obtained on fraction II, heating the crude venom showed a drop in activity over a 0.5-hr period. Here all heat coagulable protein precipitated in the first 2 min accounting for the first 37% loss in activity. The percent activities are shown for a set of duplicate experiments in Table II.

The paraoxon hydrolyzing activity is maximal in the presence of Ca^{2+}, partial in the presence of Sr^{2+}. The diva-

Table III: Cation Specificity of Fraction II "Paraoxonase".[a]

Cation	% Activity	Cation	% Activity
Ca^{2+}	(100)	Ni^{2+}	5
Sr^{2+}	50	Mn^{2+}	5
Mg^{2+}	41.3	Cu^{2+}	<2
Zn^{2+}	13.2	Co^{2+}	<2
Ba^{2+}	12.4		

[a] Assays of *p*-nitrophenol liberation were carried out in 0.05 *M* Tris buffer (pH 7.4). Paraoxon, 7 m*M*. All cations, 5 m*M*.

lent ions tested for activity are shown in Table III. EDTA abolishes enzyme activity, which is fully restored on addition of excess Ca^{2+}.

Molecular weight determinations of paraoxonase were carried out by gel filtration on a 38 × 2.5 cm G-75 column. A value of 9600 was obtained using heated fraction II.

We have examined the activity of paraoxonase in the presence of 5 m*M* iodoacetate following a 1-hr incubation at 37°, and 1 m*M* *p*-chloromercuribenzoate after 0.5-hr incubation. These experiments were carried out using the supernatants of the heated venom as the enzyme source. Medium containing *p*-chloromercuribenzoate was buffered with 50 m*M* Tris at pH 7.4, that containing iodoacetate at 8.0.

Neither reagent inhibited the enzyme activity. We have also measured the activity in the presence of 8 *M* urea. Under these conditions, the enzyme functioned at 30% of its normal rate.

Snake venoms show marked species differences for the presence of paraoxonase. In addition to Russell's viper venom, we have tested the lyophylized venoms of cobra (*Naja naja*), rattlesnake (*Crotalus adamanteus*), and copperhead (*Agkistrodon contortrix contortrix*). These preparations showed only questionable or trace activities, the maximum being exhibited by cobra venom, which showed a specific activity of approximately 6% of Russell's viper venom.

Efforts to achieve saturation kinetics studying this enzyme were frustrated by the limited water solubility of paraoxon. The rate was pseudo-first-order to 10 m*M*, the saturation concentration for the substrate. The K_m thus is greater than the one reported previously (4.5×10^{-4} *M*) for the serum enzyme.

We have attempted to compare the relative activities of the enzyme on *p*-nitrophenyl acetate and paraoxon. We have not been able to show any enzyme activity using the former substrate, which appears to contradict the general classification of this enzyme as an aromatic esterase. It is still conceivable that there is some activity which we were unable to detect, due to the rapid spontaneous hydrolysis of this substrate, but if so it would likely be minimal.

Discussion

Enzymes hydrolyzing organophosphate compounds have been found by many investigators. The first demonstration of the existence of a diisopropyl phosphorofluoridate hydrolyzing enzyme was made by Mazur (1946). Further studies on this enzyme system were carried out by Mounter (1959), and on Tabun hydrolyzing plasma enzymes by Augustinsson (1954). A wide distribution of these enzymes with partially overlapping specificities were recorded.

A paraoxon hydrolyzing enzyme was reported to be present in mammalian serum by Aldrich (1953). He also reported the enzyme to be present in many other tissues with maximum concentrations in the liver. The liver enzyme activity which is apparently microsomal can be solubilized with digitonin (J. Davis, personal communication). An enzyme active in mammalian plasma hydrolyzing *O*-ethyl *p*-nitrophenyl ethyl phosphonates is apparently different from the plasma paraoxonase though chemical structures of the substrates and the cofactor requirements are similar (Becker et al., 1964).

Paraoxonases studied to date have certain characteristics in common. In all cases the presence of Ca^{2+} is required. Ba^{2+} was shown to be an inhibitor of the plasma enzyme by Main who reported 96% inhibition at 5 m*M* ion concentration. Becker et al. observed only a 63% inhibition. The snake venom enzyme reported here is inhibited to 55% at the same concentration, which is reversed on addition of excess Ca^{2+}. The Ca^{2+} requirement could only partially be replaced by Sr^{2+}. Ba^{2+} alone maintains enzyme activity at about 10% of the Ca^{2+} level.

Aldridge made the claim that the serum paraoxonase also acts on aromatic esters, e.g., *p*-nitrophenyl acetate. This activity, as mentioned previously, could not be demonstrated with the semipurified snake venom enzyme. As "paraoxonases" are presumed to be aromatic esterases, this lack of activity deserves reinvestigation. Trace activities with *p*-nitrophenyl acetate as substrate may, however, still be possible. A significantly different characteristic of this enzyme in contrast to the thoroughly studied serum enzymes is the lack of inhibition by sulfhydryl blocking agents.

p-Chloromercuribenzoate at 4 μM produces a 50% inhibition of the serum enzyme, while the snake venom enzyme is not noticeably inhibited when the concentration is 1 m*M*. Iodoacetate is a rather ineffective inhibitor of the serum enzyme (27% inhibition after incubation in 9 m*M* solution), and at a concentration of 5 m*M* it has no measurable effect on the venom esterase. Only EDTA caused a virtually total inhibition of the venom enzyme.

The outstanding characteristic of the enzyme is its extreme heat stability. This is in contrast to the enzymes reported to date with this activity from other sources. The unusual heat stability and a substantial activity in 8 *M* urea may be partially due to the small size of this enzyme. A phosphodiesterase activity which is similar in size, however, is heat labile.

From the data in Table II, a segregation of phosphodiesterase activities directed against bis(*p*-nitrophenyl) phosphate and *p*-nitrophenyl thymidylate is discernible. Whether the activity directed against the latter in fraction II is the same found in fraction I was not investigated. The intent of the fractionations were simply to establish the independent identity of the paraoxonase.

We have not studied the question of what other substrates may be acted upon by the enzyme. Parathion, however, is neither a substrate nor an inhibitor. Clearly the enzyme is species specific and different from other known snake venom phosphohydrolases.

References

Aldridge, W. N. (1953a), *Biochem. J. 53*, 110–117.
Aldridge, W. N. (1953b), *Biochem. J. 54*, 442.
Armstrong, J. McD., Myers, D. V., Verpoorte, J. A., and Edsall, J. T. (1966), *J. Biol. Chem. 241*, 5137.
Augustinsson, K. B. (1954), *Acta Chim. Scand. 8*, 753.

Becker, E. L., and Barbaro, J. R. (1964), *Biochem. Pharmacol. 13,* 1219.
Bergmann, F., and Rimon, S. (1958), *Biochem. J. 70,* 339.
Bergmann, F., Segal, R., and Rimon, S. (1959), *Biochem. J. 67,* 481.
Bjork, W. (1963), *J. Biol. Chem. 238,* 2487.
Felix, F., Potter, J. L., and Laskowski, M. (1960), *J. Biol. Chem. 235,* 1150.
Hurlbert, R. B., and Furlong, N. B. (1967), *Methods Enzymol. 12A.*
Main, A. R. (1960), *Biochem. J. 74,* 10.
Mazur, A. (1946), *J. Biol. Chem. 164,* 271.
Mounter, L. A. (1959), *J. Biol. Chem. 204,* 813.
Neal, R. A. (1971), *Arch Intern. Med. 128,* 118.

Functional Consequences of Modifying Highly Reactive Arginyl Residues of Fructose 1,6-Bisphosphatase. Loss of Monovalent Cation Activation†

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ABSTRACT: Modification of pig kidney fructose 1,6-bisphosphatase with 2,3-butanedione (in the presence of AMP) results in the loss of activation of the enzyme by monovalent cations. Under these conditions about 8 arginyl residues per mole of enzyme were modified. No other residues were modified. No loss of monovalent cation activation occurs when modification with 2,3-butanedione is carried out in the presence of AMP plus the substrate fructose 1,6-bisphosphate and 3.2 less arginyl residues were modified. Since fructose 1,6-bisphosphatase contains 4 subunits, it is suggested that one arginyl residue per subunit plays an essential role in monovalent cation activation of the enzyme. Studies on sulfhydryl group reactivity toward 5,5′-dithiobis(2-nitrobenzoic acid) explain the protection exerted by fructose 1,6-bisphosphate against the loss of monovalent cation activation in terms of an enzyme conformational change induced by substrate, which makes unreactive the essential arginyl residue. The results of the present paper, as well as previous evidence, are discussed in terms of the mechanism of monovalent cation activation of fructose 1,6-bisphosphatase.

Fructose 1,6-bisphosphatase (EC 3.1.3.11, D-fructose-1,6-bisphosphate 1-phosphohydrolase), the enzyme that catalyzes the hydrolysis of fructose 1,6-bisphosphate to fructose 6-phosphate, is a regulatory enzyme that plays a key role in the control of gluconeogenesis (for a review see Pontremoli and Horecker, 1971). The native forms of liver and kidney fructose 1,6-bisphosphatase exhibit maximum activity at neutral pH (Traniello et al., 1971, 1972; Tashima et al., 1972; Colombo and Marcus, 1973), require Mg^{2+} or Mn^{2+} for activity, and are allosterically inhibited by AMP. The enzyme is composed of four presumably identical subunits with molecular weights of approximately 35,000 (Mendicino et al., 1972; Tashima et al., 1972; Traniello et al., 1972). At neutral pH it possesses four binding sites for the substrate (Pontremoli et al., 1968a; Sarngadharan et al., 1969; Kratowich and Mendicino, 1974), four divalent metal ion binding sites (Pontremoli et al., 1969), as well as four allosteric sites for the inhibitor AMP per tetrameric enzyme molecule (Pontremoli et al., 1968b; Sarngadharan et al., 1969; Kratowich and Mendicino, 1974).

Fructose 1,6-bisphosphatase is also activated by monovalent cations, as first mentioned by Hers and Eggermont (1964), and more extensively studied by Hubert et al. (1970). The latter report demonstrated that the enzyme from various vertebrate sources was activated by monovalent cations, potassium or ammonium being the best activators. In addition, it was also shown that the presence of the monovalent cation activator altered other properties of fructose 1,6-bisphosphatases (i.e., AMP inhibition, Mg-saturation curves).

With only a few exceptions (Behrisch, 1971; Black et al., 1972; Gonzalez et al., 1972; Hochachka, 1972; Villanueva and Marcus, 1974), the effect of monovalent cations on fructose 1,6-bisphosphatase has been ignored by most workers in the field, perhaps due to the fact that this property of fructose 1,6-bisphosphatases is one of the properties of the enzyme which is lost upon proteolytic conversion of neutral to alkaline fructose 1,6-bisphosphatase (Colombo and Marcus, 1973; Gonzalez et al., 1974). The present report demonstrates that the monovalent cation activation of fructose 1,6-bisphosphatase can also be abolished by chemical modification of highly reactive arginyl residues of the enzyme with 2,3-butanedione, an arginine-specific reagent which has been successfully used in the past few years for the recognition of the role of arginyl residues in several enzymes (Huang and Tang, 1972; Yang and Schwert, 1972; Riordan, 1973; Daemen and Riordan, 1974; Lange et al., 1974).

Materials and Methods

Fructose 1,6-bisphosphatase activity was determined spectrophotometrically by following the rate of formation of NADPH at 340 nm in the presence of excess phosphoglu-

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