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ON THE NATURE OF THE SERUM ENZYME CATALYZING PARAOXON HYDROLYSIS

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SUMMARY

A modified spectrophotometric method for analyzing the enzymatic activity of the serum enzyme which catalyzes the hydrolysis of paraoxon (E-600) has been developed. It was found that the reaction is not subject to either substrate or product inhibition. Neither *p*-aminophenyldiethyl phosphate nor *p*-aminophenylpinacolyl methylphosphonate were substrates, but were competitive inhibitors with K_i values of $1.02 \cdot 10^{-3}$ M and $4.4 \cdot 10^{-4}$ M, respectively. The enzyme active site is proposed to contain a hydrophobic region at the binding site and to require an electron withdrawing group in the substrate to ensure that cleavage occurs through the P–O bond or to stabilize an anionic site at the active site.

INTRODUCTION

The ability of mammalian serum to enzymatically hydrolyze a variety of organophosphorous compounds has been the center of investigation for several decades[1–4]. Aldridge[1,5] noted that serum contains an enzyme capable of hydrolyzing paraoxon (E-600, diethyl-*p*-nitrophenyl phosphate, mintacol) and *p*-nitrophenylacetate, which he called an A-esterase (aryl esterase). Despite the large body of work which has been carried out on the A-esterase which hydrolyzes paraoxon[2,3 6–12], the specificity of the enzyme capable of hydrolyzing paraoxon is still an unsettled issue[4,13]. Previous assay methods[1,2,4,6,14] have required relatively large amounts of serum and have not proved amenable to studying the reaction under conditions of initial rates. The lack of a convenient method for determining paraoxonase activity presents a distinct disadvantage to studying the specificity of the enzyme or purified

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enzyme preparations as evidenced by only one method published for the purification of this enzyme (from sheep)[2].

While investigating methods of mitigating the effects of lethal doses of paraoxon upon experimental animals in our laboratory[15], it came to our attention that basic kinetic information about the enzyme catalyzed hydrolysis of paraoxon was absent. In order to overcome this, we have modified the colorimetric method[14] of analysis such that the initial rates of the enzymatic reaction catalyzed by paraoxonase could be determined in a quick and economical fashion. Results have been obtained which demonstrate a lack of substrate inhibition when paraoxon is the substrate and indicate little or no product inhibition. In addition, new insight regarding the nature of the active site and the specificity of the enzyme, paraoxonase† has been gained using closely related substrate analogues.

EXPERIMENTAL

Materials

The paraoxon, *O*-*p*-aminophenyl-*O'*,*O''*-diethyl phosphate (Compound I) (m.p. 52–53 °C) and *O*-*p*-aminophenyl-*O'*-pinacolyl methylphosphonate·HCl (Compound II) (m.p. 138–140 °C) were all purchased from Ash-Stevens, Inc., Detroit, Michigan and were used without further purification. The paraoxon was found to be 97% pure, as determined by ultraviolet absorbance and gas chromatography; both the diethylphosphate and the pinacolyl methylphosphonate were checked for purity by NMR and were found to contain no detectable impurities*.

The *p*-nitrophenol was a commercial product recrystallized from cold water (m.p. 112–113 °C). The acetonitrile (Eastman) was spectral grade. All buffers were prepared fresh daily from reagent grade materials as were the solutions of paraoxon (0.37 M or $1.8 \cdot 10^{-5}$ M). The serum was collected at convenient intervals from New Zealand White or Flemish Giant rabbits**. The red cells were removed, the serum millipore-filtered (0.45 μm filter No. 04700, Millipore Corporation), and stored in the cold (4 °C) until needed (no longer than 14 days) or frozen if longer storage was required. A recording spectrophotometer was used for the kinetic measurements (either a Cary 14 spectrophotometer or a Cary 16 spectrophotometer equipped with a recorder).

Methods

The assay of the enzyme was carried out as follows: to 2.50 ml of the filtered serum was added 150 μl of $1.8 \cdot 10^{-5}$ M (5 μg/ml) paraoxon in Sörensen's 0.15 M phosphate buffer (pH 7.6)[16]. This solution was diluted up to 25 ml and incubated for

† The authors here use the term paraoxonase to describe the enzyme in sera, that is specific for paraoxon[2] in contrast to the usage of Kojima and O'Brien[4], *i.e.* an enzyme hydrolyzing the cleavage of the *p*-nitrophenyl group.

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** In conducting the research described in this report, the investigator(s) adhered to the Guide for Laboratory Animal Facilities and Care as promulgated by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences–National Research Council.

1 h at 25 °C (longer incubation times, up to 5 h, or preincubation of the sera only with the appropriate amount of $1.8 \cdot 10^{-5}$ M paraoxon and subsequent dilution up to 25 ml after 30 min gave no difference in results). To 9.5 ml of pH 7.6 phosphate buffer (0.15 M) 0.5 ml of the inhibited serum was added. A blank solution was prepared in an identical manner. To initiate the reaction, additions of 20 μl up to 200 μl of paraoxon (or Compound I or Compound II) (0.37 M) in acetonitrile were made to the inhibited serum. The sample was mixed and transferred to a 1-cm cuvette. The reaction was then monitored at 400 nm (or 360 nm for Compounds I or II) for a suitable length of time (2–7 min) at 25 °C to obtain the initial rate. The results for paraoxon were plotted according to Lineweaver and Burk[17] and K_m and V were evaluated by a least squares fit of the data using a program written for the UNIVAC 1108 computer. This procedure was modified for substrate-inhibition studies in that 1.25 ml of serum was incubated with 75 μl of $1.8 \cdot 10^{-5}$ M paraoxon. This serum was then used exactly in the manner described above.

For inhibition studies 0.20 ml of inhibited serum and 0.20 ml of inhibitor at appropriate concentrations were added to 2.0 ml of buffer in a 1-cm cuvette. Substrate was then added in 2 or 5 μl increments from micro-pipettes until 25 μl total volume had been added. After each addition, the samples were mixed and the reaction monitored at 400 nm. A blank was prepared in a similar manner. The reaction mixture without inhibitor served as a control.

RESULTS

Method for determining initial rates and enzymatic activity

The procedure described has been developed to overcome previous objections[1], based on substrate instability, to a spectrophotometric method of determining enzymatic activity.

The rate of hydrolysis of paraoxon has been shown (Fig. 1) to be dependent on

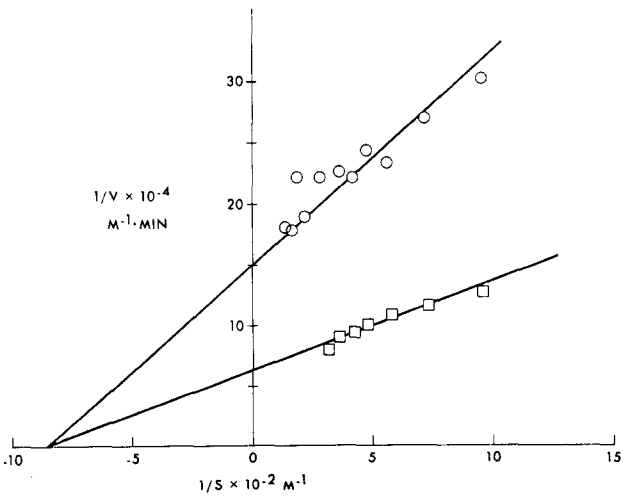

Fig. 1. Typical plot of data obtained from spectrophotometric assay. □—□, 2.5 ml of Pool A serum, inhibited with 150 μl of $1.8 \cdot 10^{-5}$ M paraoxon; K_m $1.17 \cdot 10^{-3}$ M, V $1.49 \cdot 10^{-5}$ M·min^{-1}; ○—○, 1.25 ml of Pool A serum, inhibited with 75 μl $1.8 \cdot 10^{-5}$ M paraoxon; K_m $1.34 \cdot 10^{-3}$ M, V $6.75 \cdot 10^{-5}$ M·min^{-1}.

the concentration of enzyme. The non-enzymatic rate of hydrolysis of paraoxon (determined independently) was found to be $2.54 \cdot 10^{-7}$ moles/l per min at pH 7.6 ($\varepsilon_{400\ nm}$ of *p*-nitrophenol is 12 800 with an initial concentration of paraoxon of $4.6 \cdot 10^{-3}$ M). Since the sera were incubated prior to assay all activity due to B-esterase[1,5] or cholinesterase activity (true or pseudo) has been inhibited, and the observed enzymatic activity was due to a paraoxon-resistant enzyme or paraoxon-specific enzyme. From results obtained with substrate analogues, the latter choice seems to be the correct one.

The results in Table IA show that while the enzyme level varied from one pool of rabbit sera to another, the properties of the enzyme, reflected in K_m did not.

TABLE IA

VARIATION OF ENZYME LEVEL WITH DIFFERENT SERA POOLS

N.A., not assayed.

Sera pool	*V (M·min⁻¹)*	*K_m (M)*	*Sera (ml/25ml)*
A	$1.49 \cdot 10^{-5} \pm 0.026 \cdot 10^{-5}$	$1.17 \cdot 10^{-3} \pm 0.025 \cdot 10^{-3}$	2.5
B	$1.49 \cdot 10^{-5} \pm 0.02 \cdot 10^{-5}$	$1.17 \cdot 10^{-3} \pm 0.025 \cdot 10^{-3}$	2.5
C	$1.09 \cdot 10^{-5} \pm 0.08 \cdot 10^{-5}$	$1.74 \cdot 10^{-3} \pm 0.35 \cdot 10^{-3}$	2.5
D	$2.13 \cdot 10^{-5} \pm 0.15 \cdot 10^{-5}$	$1.72 \cdot 10^{-3} \pm 0.066 \cdot 10^{-3}$	2.5
A	$0.68 \cdot 10^{-5} \pm 0.04 \cdot 10^{-5}$	$1.34 \cdot 10^{-3} \pm 0.046 \cdot 10^{-3}$	1.25
B	N.A.	N.A.	
C	$0.42 \cdot 10^{-5} \pm 0.01 \cdot 10^{-5}$	$0.827 \cdot 10^{-3} \pm 0.13 \cdot 10^{-3}$	1.25
D	$0.85 \cdot 10^{-5} \pm 0.07 \cdot 10^{-5}$	$1.82 \cdot 10^{-3} \pm 0.11 \cdot 10^{-3}$	1.25

TABLE IB

INHIBITION OF PARAOXON HYDROLYSIS

Inhibitor	*Concentration (M)*	*V (M·min⁻¹)*	*K_i (M)*
p-Aminophenyldiethyl phosphate*	$1 \cdot 10^{-3}$	$0.97 \cdot 10^{-5} \pm 0.03 \cdot 10^{-5}$	$1.02 \cdot 10^{-3}$
p-Aminophenylpinacolyl methylphosphate*	$1 \cdot 10^{-3}$	$0.88 \cdot 10^{-5} \pm 0.1 \cdot 10^{-5}$	$4.4 \cdot 10^{-4}$
p-Nitrophenol	$1 \cdot 10^{-4}$	$1.49 \cdot 10^{-5}$	$\geq 10^{-2}$**
p-Nitrophenol	$1 \cdot 10^{-5}$	$1.05 \cdot 10^{-5}$	$\geq 10^{-2}$

* Values for *V* are equal within experimental error.
** See text for discussion.

Enzyme specificity

In order to determine whether the enzymatic activity was due to an enzyme specific for paraoxon (or *p*-nitrophenyl esters) or rather to an enzyme capable of hydrolyzing a variety of aromatic phosphate esters, *p*-aminophenyldiethylphosphate (Compound I) (the reduction product of paraoxon) and *p*-aminophenylpinacolyl methylphosphonate (Compound II) were examined as substrates. The results given in Table IB show that while neither compound was a substrate of paraoxonase, both were competitive inhibitors of the enzyme-catalyzed hydrolysis of paraoxon (Fig. 2) with Compound II having a greater K_i than Compound I.

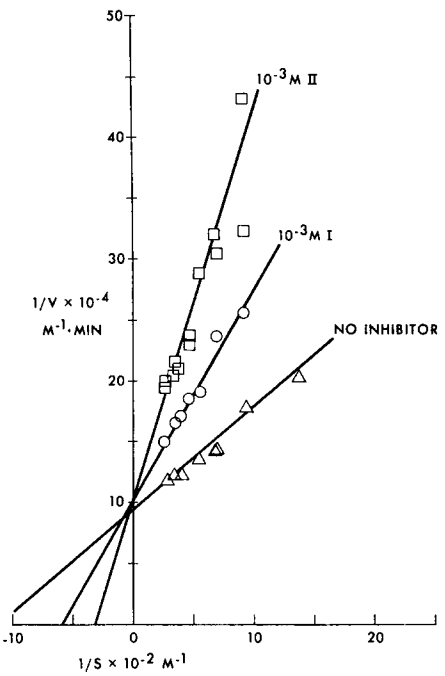

Fig. 2. Competitive inhibition of enzyme catalyzed hydrolysis of paraoxon by $1 \cdot 10^{-3}$ M *O-p*-aminophenyl-*O'*-pinacolyl methylphosphonate (Compound II), □—□, and 1.10^{-3} M *O-p*-aminophenyl-*O',O''*-diethyl phosphate (Coumpond I), ○—○, of pre-inhibited serum. Normal hydrolysis, △—△, of 2.5 ml of serum pre-inhibited with 150 μl of $1.8 \cdot 10^{-5}$ M paraoxon; K_m $8.4 \cdot 10^{-4} \pm 0.5 \cdot 10^{-4}$ M, V $1.05 \cdot 10^{-5} \pm 0.2 \cdot 10^{-5}$ M·min^{-1}.

In contrast to the results obtained with the *p*-aminophenyl derivatives, *p*-nitrophenol, the natural hydrolysis product, produced no effect of product inhibition when used at levels up to $1 \cdot 10^{-4}$ M, (Table IB and Figs 3A and 3B). Examination of possible inhibition at higher levels was precluded due to the high absorbance of the *p*-nitrophenolate ion.

These results suggest that *p*-nitrophenol has a poorer affinity for the enzyme than does paraoxon, that the enzyme exhibits specificity for *p*-nitrophenyl phosphate esters, and has a hydrophobic binding site.

DISCUSSION

The enzyme paraoxonase has been studied in whole serum[1-3,5] and in various organs of mammalian systems[1,4,5]. The previous assay methods have rendered activity data obtained with differing substrates difficult to interpret. Our results at high substrate concentrations ($7.1 \cdot 10^{-3}$ M) and reduced enzyme concentration indicate no substrate inhibition (Fig. 1) suggesting the presence of only one binding site, although multiple, non-interacting sites are also possible.

The inability of *p*-nitrophenol to inhibit the enzyme-catalyzed hydrolysis of paraoxon, even at fairly high ($1 \cdot 10^{-4}$ M) levels allows for an estimate of the lower limit of K_p. Under conditions of initial rate, the expression $v = (V \cdot S)/[K_m(1 + I/$

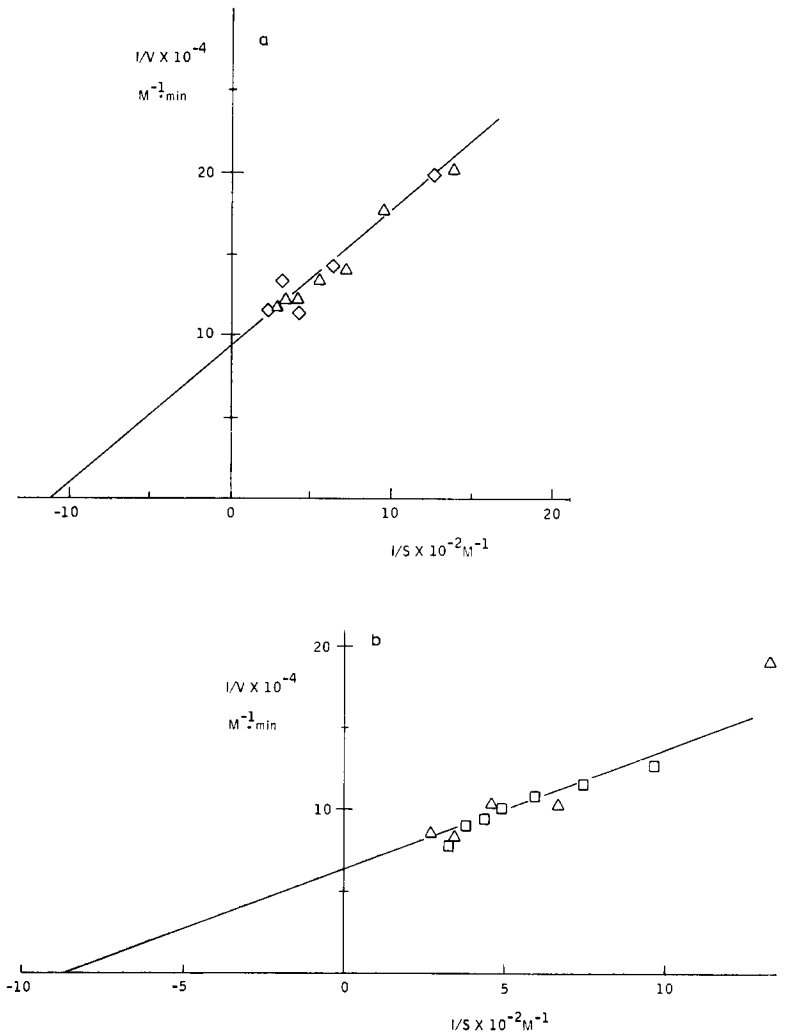

Fig. 3. (A) Plot of enzyme catalyzed hydrolysis of paraoxon in the presence of $1 \cdot 10^{-5}$ M *p*-nitrophenol (◇— ◇) and in the absence of *p*-nitrophenol (△—△). Conditions are those given in text. (B) Same as A except $1 \cdot 10^{-4}$ *p*-nitrophenol (△—△) used; □—□, Results in absence of inhibitor. Different slopes of lines in A and B are due to use of different sera pools for each determination.

$K_i) + S]$ describes the situation adequately. Since $[S] \simeq 1 \cdot 10^{-3}$ M and using the values for V and K_m from Table I and a value for Compound I of $1 \cdot 10^{-4}$, we calculate that $K_p \geqslant 1 \cdot 10^{-2}$ M. This result has the direct implication that the *p*-nitrophenolate anion either binds at some site which does not affect the active site or cannot bind at all.

The specificity of paraoxonase in rabbit serum was investigated using the amine analogue of paraoxon, *p*-aminophenyldiethyl phosphate (Compound I) and a phosphonate ester with the same aromatic ester function as Compound I, *p*-aminophenylpinacolyl methylphosphonate (Compound II).

The lack of enzymatic activity toward the hydrolysis of Compound I or II is

analogous to results observed under conditions of base-catalyzed hydrolysis of Compound I compared to paraoxon[18], and suggests that the transition state in the enzyme-catalyzed reaction leading to *p*-nitrophenol release is quite similar to that occurring in non-enzymatic hydrolysis.

We further investigated possible action of Compound I and II as inhibitors of the hydrolysis of paraoxon. In this role, both compounds behaved as competitive inhibitors as can be seen in Fig. 2 with the K_i values reported in Table IB. Of considerable interest is the fact that the pinacolyl methylphosphonate (Compound II) competes more strongly for the binding site than does the *p*-aminophenyl analogue of paraoxon. We conclude that there exists a hydrophobic region at the binding site which is specific for the aliphatic ester region of the substrate. The inability of the enzyme to catalyze the hydrolysis of Compounds I or II is most likely due to its specificity for phosphates although our results do not demonstrate this unambiguously. Results obtained by other workers[13,19] do, however, support this hypothesis. The most unifying conclusion to be drawn from our results and those previously reported[13,19] is that paraoxonase is specific for *p*-nitrophenylphosphate organic esters (*e.g.* paraoxon).

Our findings, coupled with those of Main[2] for purified paraoxonase from sheep serum, suggest the following picture for the specificity of paraoxonase. Paraoxonase is an enzyme which requires a substrate with an aliphatic ester moiety (not necessarily an ethyl ester) and an electron withdrawing moiety, preferably a *p*-nitrophenyl group (although the cyano group of Tabun seems to fulfill this requirement). The *p*-aminophenyl portion of Compounds I or II is a sufficiently weak electron withdrawing group to fail to satisfy the requirements of the active site of the enzyme. We further postulate that paraoxonase is specific for phosphate esters and not active toward phosphonate esters such as Compound II. This conclusion is supported by several other studies[13,19]. The results have identified a single binding site on paraoxonase, and have not suggested the presence of any allosteric interactions. The requirement of an electron withdrawing group in the substrate is difficult to interpret. The lack of product inhibition suggests a group may be present at the enzyme active site (*i.e.*, an anionic site) which is stabilized in a favorable conformation to aid in catalysis by an electron withdrawing group such as a *p*-nitrophenyl or cyano moiety. Alternatively, or concomitantly, the electron withdrawing group may be necessary to allow for nucleophilic attack by the enzyme at the phosphorous atom of the phosphate ester, rather than attack resulting in C–O cleavage (a result which has not been observed with paraoxonase). The specific mode of action will only emerge in greater detail when purified paraoxonase becomes more readily available.

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