Kinetics of substrate hydrolysis and inhibition by mipafox of paraoxon-preinhibited hen brain esterase activity

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For the purpose of assessing the neurotoxic potential of organophosphorus compounds, it has been determined that paraoxon-preinhibited hen brain has both neurotoxicant (mipafox)-sensitive (neurotoxic esterase; NTE) and -insensitive esterase components. Several experiments designed to investigate the kinetic parameters governing the reaction of these esterases with two substrates and one organophosphorus inhibitor are presented. First, kinetic parameters for the hydrolysis of phenyl valerate and phenyl phenylacetate were measured. At 37 °C, the K_m values of NTE for phenyl valerate and phenyl phenylacetate were found to be about 1.4×10^{-3} and 1.6×10^{-4} M respectively. At 25 °C, the K_m of NTE for phenyl valerate was determined to be about 2.4×10^{-3} M. Secondly, the kinetic constants of NTE for mipafox were measured at both 25 °C and 37 °C. With either phenyl valerate or phenyl phenylacetate as substrate, the K_m at 37 °C was determined to be about 1.8×10^{-4} M, and the phosphorylation constant (k_2) was about 1.1 min^{-1} . For phenyl valerate only, the K_m at 25 °C was found to be about 6×10^{-4} M, and the k_2 was about 0.7 min^{-1} . The data obtained at 25 °C were analysed by using a two-component model without formation of Michaelis complex, a two-component model with formation of Michaelis complex on the second component (NTE), or a three-component model without formation of Michaelis complex. The fact that the Michaelis model fit the data significantly better than either of the other two models indicates that the higher apparent K_i values that occur with low concentrations of mipafox are due to formation of Michaelis complex at high concentrations, rather than because of the presence of two NTE isoenzymes, as has been suggested by other investigators.

INTRODUCTION

Many organophosphorus compounds are capable of inducing a delayed neuropathy (Abou-Donia, 1981). The neurotoxic esterase (NTE) assay, in which the paraoxon-insensitive mipafox-sensitive portion of hen brain esterase activity is measured (Johnson, 1977), is commonly used to assess the ability of an organophosphorus compound to act as a neurotoxicant. The inhibition of NTE *in vivo*, which is usually measured with PV as a substrate, has been found to be well correlated with the development of organophosphorus-compound-induced delayed neurotoxicity. NTE is known to be a membrane-bound protein (Richardson *et al.*, 1979), and the major NTE-like binding protein has an apparent M_r of 150 000–178 000 as determined by either polyacrylamide-gel electrophoresis (Williams & Johnson, 1981; Carrington & Abou-Donia, 1985a) or by gel filtration (Williams, 1983). In the present paper we report the results of some experiments concerned with the kinetics of substrate hydrolysis and the kinetics of inhibition by mipafox of the paraoxon-resistant esterases in a membrane fraction of hen brain.

First, the Michaelis constants (K_m) and the maximum velocities (V_{max}) for PV of the mipafox-sensitive (NTE) and inhibitor-resistant (IRE) portion of a hen brain preparation preinhibited with paraoxon were measured at 37 °C and 25 °C. In addition, the V_{max} and K_m values were measured for the mipafox-sensitive and -insensitive components for PPA at 37 °C.

Secondly, the kinetic parameters for the inhibition of the paraoxon-resistant esterase activity by mipafox at 37 °C were measured with either PV or PPA as substrate. Although these parameters have been estimated previously by Soliman *et al.* (1982), we have employed a different assay method and means of data analysis to obtain somewhat different results.

Finally, the kinetic parameters for the inhibition of the paraoxon-resistant esterase activity at 25 °C were measured with a wide range of mipafox concentrations and incubation periods. The data were analysed by using several different models in order to determine whether the apparently higher relative rates of inhibition observed with low concentrations of mipafox (Chemnitius *et al.*, 1983; Chemnitius & Zech, 1983; Carrington & Abou-Donia, 1985b) are due to the presence of two isoenzymes of NTE, as suggested by Chemnitius & Zech (1983), or are instead of a consequence of Michaelis–Menten kinetics (see Main, 1973).

MATERIALS AND METHODS

Animals

White Leghorn hens, 18 months old, were obtained from Featherdown Farms, Raleigh, NC, U.S.A.

Chemicals

Paraoxon (*OO*-diethyl *p*-nitrophenyl phosphate) was obtained from Sigma Chemical Co., St. Louis, MO,

Abbreviations used: NTE, neurotoxic esterase; IRE, inhibitor-resistant esterase; PV, phenyl valerate; PPA, phenyl phenylacetate.

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U.S.A. Mipafox (*N,N'*-di-isopropyl phosphorodiamidic fluoride) was synthesized by the Midwestern Research Institute (Kansas City, MO, U.S.A.). PV and PPA were synthesized from phenol and the corresponding acyl chloride (Aldrich Chemical Co., Milwaukee, WI, U.S.A.) as described by Johnson (1977). PV was purified by vacuum distillation, and PPA was crystallized from ethanol.

Tissue preparation

Fresh hen brain was homogenized in buffer (50 mM-Tris/HCl buffer, pH 8.0, containing 0.5 mM-EDTA) for 15 s in a Polytron homogenizer. Paraoxon was then added to achieve a concentration of 40 μ M (PV at 25 °C and three replications of PV at 37 °C) or 100 μ M (PPA and three replications of PV at 37 °C, and all the mipafox inhibition experiments). The homogenate was then spun at 1000 *g* for 10 min. The supernatant was removed and spun at 100000 *g* for 30 min. The resulting pellet was resuspended in fresh buffer (Polytron homogenizer for 5 s). The two centrifugation procedures were carried out at 25 °C and took about 40 min. Protein concentrations were measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Mipafox preinhibition

For substrate kinetics, the protein (about 0.75 mg/tube) was preincubated either with or without 50 μ M-mipafox for 40 min at 25 °C in 2 ml of Tris/HCl buffer, pH 8.0 (three replications of PV at 37 °C, and PV at 25 °C), or about 0.4 mg of protein was preincubated with 50 μ M-mipafox for 20 min at 37 °C in 1 ml (PPA and three replications of PV at 37 °C). For the experiments concerned with inhibitor kinetics, 0.375 mg of protein was preincubated in a volume of 50 μ l. For the experiments at 37 °C, four to six different mipafox concentrations ranging from 4 to 80 μ M and 12 different preincubation periods ranging from 0 to 20 min were used. For the experiments at 25 °C, nine to 12 mipafox concentrations (range 3–400 μ M) and 12–15 time points (range 0–60 min) were employed. For the zero-time points, the inhibitor was added immediately after the substrate. In order to make the experiments at 25 °C technically simpler to perform, some of the time points were omitted at the shorter time points. Nonetheless, each repetition of the experiment included over 120 data points.

Substrate addition

For substrate kinetics, the substrate concentration was varied. Substrate solutions containing PV or PPA were prepared in 0.03% Triton X-100 and 3.3% (v/v) *NN*-dimethylformamide. Equal volumes of the substrate solutions were added to the preinhibited protein to result in substrate concentrations ranging from 0.1 to 1 mM. Blanks containing no protein were incubated with substrate as well. A substrate incubation period of 15 min was used for PV at 37 °C, whereas the incubation period for PPA at 37 °C and PV at 25 °C was 40 min. For the inhibitor kinetic experiments, the substrate solution was diluted 1:1 with Tris buffer immediately before the addition of the substrate to form a 2 mM substrate solution. A 1.95 ml volume of the substrate/buffer solution was then added to the 50 μ l of preinhibited protein to start the reaction. For the experiments at 37 °C, the tissue homogenate was incubated with PV for

7.5–10 min or with PPA for 20–30 min. In the experiments at 25 °C, the protein was incubated with PV for 20 min at 25 °C. All experiments were performed with 1 mM-NiCl₂ (final concentration) included with the substrate to lower the activity of the mipafox-resistant component (Johnson, 1982).

Measurement of phenol concentration

The reactions were stopped and phenol concentrations were measured as described by Johnson (1977).

Calculations

All data were analysed by using weighted least-squares non-linear regression (Rodbard *et al.*, 1976). An Apple II computer was programmed with a direct search method (Hooke & Jeeves, 1961) to find the parameters that best fitted the model employed to the data.

For the experiments concerned with substrate kinetics, velocities were calculated for both NTE (velocity without mipafox minus velocity with mipafox) and IRE (velocity with mipafox minus blank) for each substrate concentration. For the calculation of the kinetic constants for NTE, the substrate concentrations were corrected for substrate depletion by assuming the mean concentration to be equal to the average of the initial and final substrate concentrations in the absence of mipafox. The final concentration was calculated by subtracting the phenol concentration from the final concentration. The data were then fitted to the Michaelis-Menten equation (Michaelis & Menten, 1913):

$$v_{\text{obs.}} = V_{\text{max.}} \frac{[S]}{K_m + [S]}$$

where $V_{\text{obs.}}$ is the observed velocity, $[S]$ is the substrate concentration and the other variables are as previously described.

For the mipafox inhibition experiments, the data were analysed by using one of two submodels for each of two or three components. The first submodel used only a second-order inhibition constant to describe the phosphorylation reaction (Aldridge & Reiner, 1972):

$$v_{\text{obs.}} = v_0 \cdot e^{-t[I]K_i}$$

where v_0 is the velocity in the absence of inhibitor, t is the time of preincubation, $[I]$ is the inhibitor concentration during preincubation, K_i is the bimolecular rate constant and the other variables are as described previously. The second submodel was based on the equation derived by Main & Iverson (1966) designed to account for irreversible esterase inhibition by organophosphorus compounds when there is a significant amount of formation of Michaelis complex:

$$v_{\text{obs.}} = v_0 \cdot e^{-tk_2/(1+K_m/[I])}$$

where k_2 is the first-order phosphorylation constant, K_m is the Michaelis constant of the enzyme for the inhibitor and the other variables are as described previously.

Since the 40-fold dilution and the addition of substrate were not able to stop the reaction of the inhibitor with the enzyme completely, it was necessary to correct within the submodels for inhibition taking place after the addition of the substrate. Post-substrate inhibition was particularly evident in the fourth experiment, where very high concentrations of mipafox were used. Although the amount of inhibition occurring after the addition of substrate was significant, the percentage inhibition was

small enough (< 10%) so that the velocity could be approximated by a model in which the activity is assumed to decrease linearly over the substrate incubation period (i.e. zero-order instead of first-order). Consequently, the average amount of activity was calculated to be equal to the amount of activity at the midpoint of the substrate incubation period. Therefore the following equations were used for the non-Michaelis and Michaelis components respectively.

Non-Michaelis submodel:

$$v_{\text{obs.}} = v_0 \cdot e^{-K_1 t_p [I] - K_1 (t_s/2) \{ [I] / (df \times sc) \}}$$

Michaelis submodel:

$$v_{\text{obs.}} = v_0 \cdot e^{-t_p k_2 / (1 + K_m / [I]) - (t_s/2) k_2 / \{ 1 + (K_m \times df \times sc) / [I] \}}$$

where t_p is the preincubation period, t_s is the substrate incubation period, df is the dilution factor and sc is the substrate competition factor. The substrate competition factor is equal to the reciprocal of the fraction of the sites unoccupied by the substrate and was calculated from the results of the first and second experiments (range 1.5–8.1). With the non-Michaelis submodel, the correction factor translated into the equivalent of an extra few seconds of preincubation. For instance, for NTE inhibition at 25 °C, where $t_s = 20$ min, $df = 40$ and $sc = 1.5$, the equation could be transformed into:

$$v_{\text{obs.}} = v_0 \cdot e^{-(t+0.164)[I]K_1}$$

In other words, the submodel assumes an extra 0.164 min, or about 10 s, of preinhibition at each time point. The Michaelis model cannot be transformed so simply.

For the data obtained at 37 °C, the non-Michaelis submodel was used (assigned by the initial estimates for the parameters) for the low-affinity components (IRE) and the Michaelis submodel was used for the high-affinity component (NTE), so that $v_{\text{obs.}}$ represents IRE + NTE activities. The data obtained at 25 °C were analysed by using three different models. The first model used the non-Michaelis submodel for both IRE and NTE. The second model was the same as used for the 37 °C data. The third used three non-Michaelis components (i.e. IRE and two NTE isoenzymes). The second and third models were statistically compared with the first by using the extra sum-of-squares principle (Munson & Rodbard, 1980; Draper & Smith, 1981).

Table 1. Kinetics of the hydrolysis of PV at 25 °C and 37 °C and of PPA at 37 °C

The values are averages ($\pm$ S.E.M.) from three replications for the maximum velocities ($V_{\text{max.}}$) and Michaelis constants (K_m) for PV of the mipafox-sensitive (NTE) or -insensitive (IRE) portions of a paraoxon-preinhibited hen brain membrane fraction. The velocity units are nmol of phenol liberated/min per mg of protein.

	PV		PPA
	25 °C (<i>n</i> = 3)	37 °C (<i>n</i> = 6)	37 °C (<i>n</i> = 3)
$V_{\text{max.}}$ (nmol/min per mg)	63 $\pm$ 8	128 $\pm$ 14	5.4 $\pm$ 0.9
$10^3 \times K_m$ (M)	2.43 $\pm$ 0.18	1.36 $\pm$ 0.09	0.16 $\pm$ 0.01

Fig. 1. Lineweaver-Burk plot for the hydrolysis of either PV (○) or PPA (●) by paraoxon-preinhibited chicken brain homogenate

The velocity units (v) are nmol of phenol liberated/min per mg of protein. The lines used to fit the data were generated by non-linear least-squares fits of the untransformed data.

Table 2. Kinetics of inhibition of the esterase activity of a paraoxon-preinhibited hen membrane preparation by mipafox at 37 °C with either PV or PPA as substrate

The velocities (nmol of phenol liberated/min per mg of protein) and kinetic constants are the averages ($\pm$ S.E.M.) from three replications.

	PV	PPA
Velocities (nmol/min per mg)		
NTE	44 $\pm$ 3	3.6 $\pm$ 0.5
IRE	13 $\pm$ 3	2.8 $\pm$ 0.2
Kinetic constants		
K_1 of IRE ($\text{M}^{-1} \cdot \text{min}^{-1}$)	350 $\pm$ 180	13.2 $\pm$ 34
k_2 of NTE (min^{-1})	1.37 $\pm$ 0.19	0.92 $\pm$ 0.11
$10^4 \times K_m$ of NTE (M)	2.45 $\pm$ 0.85	1.26 $\pm$ 0.33
K_1 of NTE ($\text{M}^{-1} \cdot \text{min}^{-1}$)	5.6×10^3	7.3×10^3

RESULTS

The K_m and $V_{\text{max.}}$ values of the mipafox-sensitive component (NTE) are listed in Table 1 for both PV at 25 °C and 37 °C and for PPA at 37 °C. A Lineweaver-Burk plot of experiments with either PV or PPA is given in Fig. 1. The K_m and $V_{\text{max.}}$ values for PV were both considerably higher than those for PPA. Thus, although the hydrolysis of PV proceeds considerably faster, the number of sites occupied by a saturated solution (about 1.2 mM) of PV is considerably less (about 48% at 37 °C and 34% at 25 °C) than those filled by PPA (about 89% at 37 °C). The inhibitor-resistant component (IRE) is probably composed of multiple enzymes that are not distinguished by mipafox (Johnson, 1982), and that may have different K_m values. However, K_m values were calculated for use in analysing the mipafox inhibition data. At 37 °C the K_m values of IRE were

Fig. 2. Plot of the data from five of nine concentrations of mipafox from one of the four mipafox inhibition experiments at 25 °C

The concentrations plotted were 3 μM (*), 10 μM (◆), 30 μM (◇), 100 μM (●) and 400 μM (○). The lines denote the predicted values at each of the five plotted concentrations when the data (all nine concentrations) were fitted by using non-linear regression and one of three models: -----, two-component non-Michaelis model; —, two-component Michaelis model; ·····, three-component model.

Table 3. Estimates of the kinetic constants governing the inhibition of the esterase activity of a paraoxon-preinhibited hen membrane preparation by mipafox at 25 °C with PV as a substrate with three different models

See the text for description of the models. $V(1)$, $V(2)$ and $V(3)$ respectively indicate the first, second and third velocity component in a model. The values are the averages ($\pm$ S.E.M.) from four replications. The unit for the K_i determinations is $\text{M}^{-1} \cdot \text{min}^{-1}$, the unit for the k_2 determination is min^{-1} , and the K_m determination is in M.

Model	Velocities (nmol/min per mg)			Kinetic constants			Sum of squares (% of model 1)
	$V(1)$	$V(2)$	$V(3)$	$K_i(1)$	$K_i(2)$ or $k_2(2)$	$K_i(3)$ or $10^4 \times K_m(2)$	
1	4.9 ± 0.6	14.9 ± 0.9	—	42 ± 10	1058 ± 110	—	—
2	4.0 ± 0.4	15.0 ± 0.7	—	28 ± 9	0.67 ± 0.15	5.9 ± 1.5	74.2 ± 7.6
3	4.4 ± 0.5	11.8 ± 1.2	3.5 ± 0.7	30 ± 10	797 ± 145	5012 ± 2570	94.2 ± 1.9

$3.2 \times 10^{-4} \pm 1.6 \times 10^{-4} \text{ M}$ ($n = 3$) for PV and $4.3 \times 10^{-4} \pm 1.0 \times 10^{-4} \text{ M}$ ($n = 3$) for PPA. The K_m of IRE for PV at 25 °C was $9.5 \times 10^{-4} \pm 2.6 \times 10^{-4} \text{ M}$ ($n = 3$).

Although the velocities of the two components in the experiments at 37 °C differed between the two substrates, the estimates of the k_2 and the K_m of NTE and mipafox were very similar (Table 2). Although the estimates for the K_i of the resistant components for mipafox did differ, the concentrations of mipafox and time points employed in the third experiment were too low to provide an accurate estimate of this constant. There did not appear to be any particular advantage to using PPA over PV with regard to stopping the reaction of the inhibitor with the esterases. Although a saturated solution of PPA will

occupy a greater percentage of the active sites of NTE than will a saturated solution of PPA (about 80% compared with 40%), the fact that a longer incubation period is required to generate the same signal negates this advantage.

For the inhibition of the hydrolysis of PV at 25 °C (Fig. 2 and Table 3), the Michaelis model consistently fitted the data significantly better than did the non-Michaelis two-component model ($P < 0.05$ in all four replications). In only two of four replications did the three-component model fit significantly better than the two-component model. Furthermore, in each replication, a better fit of the data was afforded by the two-component Michaelis model than by the three-component model in spite of the

fact that the former model used only five variables instead of six. Although the phosphorylation rate of NTE was only slightly less at 25 °C than at 37 °C, the K_m was much higher, and the K_i much lower.

DISCUSSION

Without presenting any data, Johnson (1980) reported that the K_m of NTE for PV is about 10 mM. Our results indicate that it is actually between 1 and 2 mM. However, our measurement of the K_m of NTE for PPA is close to that reported by Johnson (1980).

Although our measurements of the kinetic constants for NTE and mipafox at 37 °C with PV and PPA are similar, they differ from the values reported by Soliman *et al.* (1982). We estimated the phosphorylation constant (k_2) to be about 3-fold lower (about 1.1 min⁻¹ instead of 3 min⁻¹) and the K_m to be about 3-fold higher (about 1.8×10^{-4} M instead of 6.7×10^{-5} M). The results reported by Soliman *et al.* (1982) are suspect for several reasons. First, they were using an assay method (Soliman *et al.*, 1981) that does not take the inhibition of IRE by mipafox into consideration. Secondly, they used a substrate (*p*-nitrophenyl valerate) whose rate of hydrolysis is very dependent on the pH of the buffer and for which the specificity of NTE is very low. Assays with this substrate have been found to be difficult to reproduce. Thirdly, their estimate for the bimolecular rate constant ($K_1 = 4 \times 10^4 - 5 \times 10^4$ M⁻¹·min⁻¹) for NTE is in severe disagreement with most of the literature on NTE. The IC_{50} for mipafox at 37 °C and with a 20 min preincubation has repeatedly been found to be in the range 5–10 μM (Johnson, 1970; Dudek & Richardson, 1982; Caroli & Lotti, 1982), which would correspond to K_1 of $3.5 \times 10^3 - 7.0 \times 10^3$ M⁻¹·min⁻¹. These latter values are close to the estimates that result from our data (5.6×10^3 M⁻¹·min⁻¹ with PV, 7.3×10^3 M⁻¹·min⁻¹ with PPA). The estimate of K_1 obtained by using the Michaelis model should be somewhat higher than the apparent K_1 (Main, 1973), but the apparent K_1 should be at least half the true K_1 when mipafox concentrations below the K_m are employed.

The experiments at 25 °C were performed in order to determine the verity of the NTE isoenzymes reported by Chemnitius *et al.* (1983). Our initial doubts sprang from the fact that, although we have found that a three-site model is significantly more appropriate for a mipafox concentration curve (Carrington & Abou-Donia, 1985b), we have not been able to obtain similar results with an incubation curve, where the time of incubation with a single concentration of mipafox is varied. Since our present results indicate that the three-site model is made unnecessary by the two-site Michaelis model, we now conclude that no NTE isoenzymes can be distinguished by mipafox. The improved fit gained by adding the third component when fitting a concentration curve probably results from the greater apparent K_1 with low concentrations of mipafox than for concentrations of the inhibitor that approach the K_m .

It is noteworthy that with both PV and mipafox the K_m increased when the temperature was decreased. This observation is in contrast with the observation by Main & Iverson (1966) that although the k_2 of di-isopropyl phosphorofluoridate and acetylcholinesterase decreases

with temperature the K_m remained relatively unchanged. Higher temperatures might decrease the K_m by facilitating a conformational change that is necessary for binding to the NTE active site.

Michaelis–Menten kinetics might also account for the ability of paraoxon to lower the rate of inhibition of NTE by mipafox (Carrington & Abou-Donia, 1985b). Since a concentration of 640 μM-paraoxon lowers the apparent K_i of mipafox by slightly more than 50%, the K_m of NTE for paraoxon could be estimated to be near 5×10^{-4} M, or about the same as for mipafox. If direct competition with mipafox for the active site of NTE is the mechanism, then the much greater inhibitory power of mipafox must be due to a much greater k_2 for mipafox than for paraoxon.

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