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DIFFERENCES IN THE KINETIC PROPERTIES, EFFECT OF CALCIUM AND SENSITIVITY TO INHIBITORS OF PARAOXON HYDROLASE ACTIVITY IN RAT PLASMA AND MICROSOMAL FRACTION FROM RAT LIVER

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Abstract—The properties of a rat hepatic microsomal enzyme that hydrolyses *O,O*-diethyl-*p*-nitrophenylphosphate (paraoxon) were studied and compared to the paraoxon hydrolase activity found in rat plasma. The pH stability for both enzyme activities was optimum between pH 6.0 and 9.0. An overall analysis of the data showed that the microsomal fraction was less resistant to the effect of the pH than plasma. The kinetic constants for heat inactivation evaluated for paraoxonase in rat plasma and liver microsomal fraction indicate that paraoxonase tends to inactivate faster in rat liver microsomes than in rat plasma. The apparent activation energies of the heat inactivation process were 77.7 and 61.1 kcal/mol for rat plasma and microsomal fraction, respectively. Enzyme activity was lost after both dialysis and incubation with EDTA and partially restored by the addition of calcium. In rat plasma samples the requirement for calcium was absolute (essential activator) while in the microsomal fraction the reaction may occur, to a minimum extent, in the absence of the activator (non-essential activator). Calcium restored 85% activity when added immediately after EDTA; restored activity decreased when the time interval between addition of EDTA and calcium was increased. Other metals were not able to restore activity previously inhibited by EDTA or dialysis. The response to several inhibitors (EDTA, Mn, Co, Zn, Ba, Mg, Cu, La, Hg and *p*-hydroxy-mercuribenzoate) of rat plasma and microsomal fraction was studied, determining the type of inhibition and the inhibition constants. Plasma enzyme was always more resistant than liver sample to the effect of the inhibitors and showed different types of inhibition than the liver microsomal fraction. In general we found more differences than analogies between the rat plasma and liver enzymes which suggests the presence of two enzymes or two different forms of the same enzyme. Furthermore the existence of an EDTA-resistant fraction in rat liver microsomes suggests that more than one enzyme capable of hydrolysing paraoxon is present in the microsomal fraction of rat liver.

Key words: paraoxonase; A-esterase; organophosphates; rat; liver; plasma

Aldridge [1] defined A-esterases as a group of enzymes which hydrolyse OP† esters in contrast with B-esterases which are inhibited by OP compounds. The first demonstration of an A-esterase was made by Mazur [2], who showed that rabbit sera hydrolyse paraoxon and DFP. The hydrolysis of paraoxon was studied in detail by Aldridge [3] and many A-esterases have been identified in different tissues of several animal species. Much of the early work was summarized and reviewed by Aldridge and Reiner [4] and a summary of the present state of knowledge has recently been published [5, 6].

Mammals tend to exhibit high A-esterase activity in the blood and the liver, and this is apparently an important factor in determining the resistance of these organisms to organophosphate toxicity [7]. On the other hand, birds have very low levels of A-esterase activity and are more susceptible to OP compounds [8]. The relationship between serum A-esterases and organophosphate toxicity in man has been reviewed by Lotti *et al.* [9].

The substrate specificity of A-esterases is very complex and not well understood [4, 10, 11] and is further complicated because their physiological role has also not been clarified, since no natural substrates of these enzymes are known [12, 13]. Nevertheless, Mackness and Walker [14] speculated that A-esterases have roles in endogenous metabolism but that particular isoforms, sometimes with very wide substrate specificity, have evolved to metabolize naturally occurring xenobiotics. Another problem is the distinction between A-esterases and their implications for esterase classification [15–18].

In spite of the toxicological significance of A-esterases, relatively little is known about the biochemical properties of these enzymes, even though there is a great interest in these enzymes in toxicology genetics [10, 12] and clinical chemistry

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† Abbreviations: paraoxon, *O,O*-diethyl-*p*-nitrophenylphosphate; OP, organophosphorus; DFP, diisopropylphosphorofluoridate; *p*-OH-MB, *p*-hydroxymercuribenzoate.

[19]. Although numerous studies of OP hydrolases have been reported, they have mostly focused on the hydrolysis of paraoxon or related phosphate esters by serum. However, data on enzymes hydrolysing OP compounds in the liver are scarce [20–24].

In a previous report [25] we stated that rat liver paraoxon hydrolytic activity (paraoxonase) is essentially a microsomal enzyme associated with vesicles derived from the endoplasmic reticulum. Some biochemical properties were studied and compared to rat plasma paraoxonase showing similarities in optimum pH, K_m and calcium requirement, but differences in the response to several inhibitors. It has been suggested [20] that rat plasma paraoxonase is similar to the liver enzyme and therefore could be used as a good reflection of the detoxifying activity of the liver enzyme.

In this paper we present a more detailed study in which we have compared the paraoxonase activity present in rat plasma to that in rat liver in an attempt to ascertain whether paraoxonase in plasma or liver might play an important role in organophosphate hydrolysis. To this effect, we have investigated some biochemical properties displayed by these enzymes as well as their sensitivity to and kinetics with different inhibitors. Furthermore, a detailed study of calcium requirement has been performed. On the basis of the results, the identity of both enzymes and their relative importance in the detoxifying role against OP compounds is discussed.

MATERIALS AND METHODS

Chemicals

Paraoxon was purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.) and purified as described [26]. Other reagents were of analytical grade and supplied by Sigma and Merck (Darmstadt, Germany). Milli-Q (Millipore, Bedford, MA, U.S.A.) grade water was used throughout.

Animals

Male Wistar rats weighing 180–200 g at the time of death were used. Animals were maintained on lab chow and tap water *ad lib.* with a 12 hr day/night cycle (light cycle from 7.00 a.m. to 7.00 p.m.). Rats were fasted for 16 hr before decapitation. Fasting was utilized to increase the yield of the microsomal fraction [27].

Sample preparation

(a) Plasma. Blood was collected in heparinized tubes and plasma was separated by centrifugation and used immediately or stored at −40° until analysis. Enzyme activity was unaffected by freezing and storage at −40°.

(b) Preparation of the microsomal fraction. Microsomal fractions were prepared essentially as described previously [28, 29]. Briefly, rat livers were removed, placed in beakers on ice, rinsed with ice-cold homogenization buffer (5 mM Tris–HCl buffer pH 7.4 containing 0.25 M sucrose), minced with scissors and then placed in 4 vol. of ice-cold homogenization buffer. They were then homogenized (6 strokes at 1100 rpm) using a mechanically driven Teflon pestle in a glass homogenizer (Potter–Elvehjem) with 1.02 mm clearance. The homogenate was transferred to a power-driven close fitting (0.045 mm clearance) Perspex/Glass homogenizer and homogenized as before. After diluting the homogenate to 10% (w/v) with homogenization buffer, nuclei and mitochondria were removed by successive centrifugation at 460 *g* for 10 min and 12,500 *g* for 10 min in a Beckman J2-21 refrigerated centrifuge. The post-mitochondrial supernatant fraction was then centrifuged at 105,000 *g* for 60 min in a Beckman 55.2 Ti rotor operated in a Beckman model L8-55. The microsomal pellet derived from 10 g of liver tissue was suspended in 20 mL of 5 mM Tris–HCl buffer pH 7.4. Aliquots of microsomal fraction were used immediately or stored at −40°.

(c) Solubilized microsomal membranes. Paraoxonase was extracted by the addition of Triton X-100 [30]. The microsomal fraction was adjusted to 0.75% Triton X-100, vortexed, stored at 4° for 30 min, and then centrifuged at 105,000 *g* for 60 min. The resultant supernatant fraction was used for enzyme activity assay or kept at −40°.

Protein determination

Protein concentration was estimated by the method of Lowry *et al.* [31] with bovine serum albumin, fraction V (Sigma) as a reference standard. A modification of this procedure [32] was used to assay protein in the presence of Triton X-100.

Enzyme assay

A-esterase activity towards paraoxon (paraoxonase) was quantified spectrophotometrically by a modification of the method described by Reiner and Radic [33] using 100 mM Tris–HCl buffer, pH 7.4, calcium chloride 1 mM. Reaction was initiated by the addition of 50 μL of plasma (1.8 mg of protein) or 100 μL of microsomal fraction (0.3–0.4 mg of protein) and was followed for 2 min at 37° by monitoring the appearance of *p*-nitrophenol at 405 nm in a Perkin–Elmer Lambda 2 automated recording spectrophotometer. All rates were determined in duplicate and corrected for the non-enzymic hydrolysis. The final substrate concentrations during enzyme assay were 2 mM and 1.5 mM for microsomal fraction and plasma, respectively. For pH values below 7.5, when *p*-nitrophenol is poorly dissociated, the *p*-nitrophenol formed in the reaction was monitored by HPLC at 315 nm. Briefly, the reaction was stopped by adding an equal volume of 0.35/0.36 M perchloric acid/sodium acetate, centrifuged and the clear supernatant was injected on a Perkin–Elmer (5μ, 12.5 cm) C-18 reverse phase column, using a mobile phase of methanol/0.05 M perchlorate buffer adjusted at pH 2.5 (40:60) at a flow rate of 1 mL/min.

Effect of pH

The effect of pH was tested over a 5–12 pH range in sodium acetate–acetic acid buffer 0.1 M (pH 4.0, 5.5), phosphate buffer 0.1 M (pH 6.0, 6.5), Tris–HCl 0.1 M (pH 7.0–9.0) and glycine–NaOH buffer 0.1 M (pH 9.5–12.0). Spontaneous hydrolysis was

determined in each case and subtracted from the total enzyme rate.

pH stability was determined by preincubating the enzyme at the desired pH (4.0–11.0) for 0–30 min at 37°. The final pH was checked in parallel control experiments and the deviation from the nominal pH was 0.10–0.45 U in the extreme pHs. Enzyme activity was then measured at pH 7.4 as described under enzyme assay.

Heat inactivation

Aliquots (0.25 mL) of each sample were pre-incubated at 45, 50, 52.5 and 55° in a constant temperature water bath for a set interval in the range 0–75 min. The tubes were then placed on ice and assayed for residual paraoxonase activity using the standard procedure at 37°. The mean protein content (mg/mL) during heat inactivation was 18 for rat plasma and 3–4 for rat liver microsomes.

Calculation of rate constants

For monophasic patterns the equation used was $y = A.e^{-kt}$, where y = per cent remaining activity, A = per cent initial activity, k = apparent rate constant of inactivation and t = time (min). The first order rate constants for enzyme inactivation versus pH or temperature were calculated by plotting the logarithm of the percentage of the original remaining activity versus preincubation time using a computerized program (Enzfitter, Biosoft).

When plots showed a biphasic pattern, the equation used was $y = A_1.e^{-k_1 t} + A_2.e^{-k_2 t}$, where y = per cent remaining activity, A_1 = relative amount of activity (%) inactivating at the fast rate, A_2 = relative amount of activity (%) inactivating at the slow rate, k_1 = apparent rate constant of inactivation for the fast reaction, k_2 = apparent rate constant of inactivation for the slow reaction and t = time (min).

Apparent inactivation energies (E_a) for the heat inactivation were determined from the slopes of the Arrhenius plots [34].

Effect of calcium and inhibitors

Working solutions of the following compounds were prepared from stock solutions in 0.1 M Tris–HCl pH 7.4 free of calcium and added to samples to obtain the desired final concentration in the range showed for each compound as indicated in brackets for the plasma and microsomal fraction, respectively: $CaCl_2$ (0.5–10 mM/0.12–4 mM); EDTA (0.5–3 mM/0.015–1 mM); $MgCl_2.6H_2O$ (0.5–10 mM/0.5–10 mM); $MnCl_2.4H_2O$ (0.62–25 mM/0.62–50 mM); $Co(NO_3)_2.6H_2O$ (0.03–2.5 mM/0.12–3 mM); $ZnCl_2$ (0.5–25 mM/0.12–2 mM); $BaCl_2.2H_2O$ (20–100 mM/5–100 mM); $CuSO_4$ (2–12 mM/0.5–8 mM); $LaCl_3.7H_2O$ (0.25–4 mM/0.25–1 mM); *p*-OH-MB (0.12–1 mM/0.12–1 mM) and $HgCl_2$ (0.1–0.4 mM/0.2–1 mM).

Samples were preincubated at 37° for 5 min except when the effect was time dependent, when the preincubation time before starting the enzyme reaction was 15 min. The resultant paraoxonase activity was determined as described under "*Enzyme assay*", except that no calcium was present in the buffer.

The activation/inhibition kinetics for the different compounds were determined by assaying the effect of the activator/inhibitor in the presence of several substrate concentrations (paraoxon 0.25, 0.37, 0.50, 0.75 and 1.0 mM). The assayed concentrations for each inhibitor were chosen so that the minimum rate measured in the inhibited reaction was not less than 40–50% of the initial activity. Data were fitted by a non-linear computerized fitting method based on the least square principle (Enzfitter, Biosoft).

(a) Determination of the type of inhibition and of K_I. The type of inhibition and K_I were determined by plotting $1/v$ against $1/[S]$ for each inhibitor concentration according to Lineweaver–Burk and $1/v$ against $[I]$ as described by Dixon [35].

(b) Reactivation by calcium and other metals. Enzyme activity in plasma and microsomal fraction was inhibited by adding EDTA at final concentrations of 4 and 0.5 mM, respectively, to complex the endogenous calcium of both samples. After inhibition was achieved, calcium or other metals (manganese, cobalt, barium, zinc, copper and lanthanum) were added to the samples in different concentrations to determine the restoration of paraoxonase activity. The standard assay conditions were used, with the sole exception of a buffer free of calcium. In the calcium reactivation study, calcium was added to the samples 0 min, 30 min and 1, 1.5, 2, 3, 5, 6, 7 and 21 hr after inhibition by EDTA.

RESULTS AND DISCUSSION

Effect of pH

The pH profile of both plasma and microsomal fraction showed optimum activity at 8.5 as previously reported [25].

Preincubation of the plasma samples at pH 6.0 or 9.0 had no effect on the activity measured at pH 7.4. However, when preincubated at pH >9.0 or pH <6.0 activity was inhibited and full activity was not regained at pH 7.4. Broadly speaking, a similar behaviour was observed with the microsomal fraction assayed. The decline in activity between pH 7.5 and 9.0 and between pH 7.5 and 6.0 observed in the microsomal fraction might result from the formation of an improper ionic form of the enzyme and/or substrate. Moreover, part of the decline in activity above pH 9.0 and below pH 6.0 could result from irreversible enzyme inactivation [34]. Study of the pH-dependence of the enzyme-catalysed reactions can provide important information about the identity of the prototropic groups at the active site of the enzyme [34, 36]. An estimation of pK_a values was made according to Wilkinson [37] and showed similar values for plasma and liver enzyme. The pK_{a1} values calculated for plasma and microsomal fraction were 7.02 and 6.83 and pK_{a2} were 9.55 and 10.10, respectively. These data suggest that the imidazolium group of histidine residue (pK_a 5.5–7.0) and the ε-amino group of lysine residue (pK_a 9.5–10.6) or the phenolic OH group of tyrosine (pK_a 9.8–10.5) may be responsible for the catalytic activity. Whether these amino acid residues are related or not to the triad of amino acid reported for the catalytic centre of other esterases [38] is still unkown.

The first-order rate constants for enzyme inactivation derived from the slopes of plots of logarithm

Table 1. Kinetic constants for pH inactivation of paraoxonase in plasma (P) and hepatic microsomal fraction (MF)

		MF			
		Component 1		Component 2	
pH	P (k_1/min)	A_1 (%)	k_1 (min^{-1})	A_2 (%)	k_2 (min^{-1})
3.0	—	61.8	0.231	38.5	0.024
4.0	0.118	34.7	0.274	65.6	0.018
5.0	0.008	98.5	0.017	—	—
6.0	0.001	98.5	0.018	—	—
7.5–9.0	0.002	103.0	0.001	—	—
10.0	0.036	97.5	0.034	—	—
10.5	0.124	78.1	0.923	21.9	0.049

Plasma (P) and microsomal fraction (MF) were incubated in 0.1 M Tris–HCl buffer, pH 7.4 for fixed times in the range 0–30 min at the pH indicated. Each number is the mean value obtained from three experiments. Inactivation reactions for plasma (pH 4.0–10.5) and microsomal fraction (pH 5.0–10.0) showed a monophasic pattern and the apparent first rate constants (k, min^{-1}) were calculated from the slopes of log per cent remaining activity versus preincubation time plots. Apparent rate constants (k_1 and k_2) for the two components (1 and 2) from the inactivation reactions at pH 3, 4 and 10.5 for microsomal fraction were determined from the slopes of the resolved linear components of the biphasic curves using the equation: $A = A_1.e^{-k_1t} + A_2.e^{-k_2t}$. The relative amounts of activity (%) inactivating at the fast and the slow rates (A_1 and A_2) were determined from the intercepts of the linear components on the ordinate.

percentage remaining activity versus time of incubation at different pHs are shown in Table 1. Stability was optimum at pH 7.5. Full catalytic activity was retained for at least 6 months by the two samples when they were placed in 0.1 M Tris–HCl buffer pH 7.4, and stored frozen at −40° (data not presented). An overall analysis of data in Table 1 shows that the microsomal fraction was less resistant to the effect of pH than plasma. Furthermore, a qualitative difference was observed in the pH stability of the microsomal fraction with respect to the plasma. At extreme pH values (pH 3, 4 and 10.5) paraoxonase activity in microsomal fraction showed biphasic kinetics. The kinetic constants for the fast and slow reaction are also included in Table 1.

Microsomal paraoxonase activity is a membrane-bound protein that may be solubilized with Triton X-100 [30] with the retention of about 80% of enzymic activity. pH inactivation studies with preparations of rat liver microsomes solubilized in Triton X-100 showed a monophasic pattern (data not shown). This suggests that the biphasic reaction observed in the microsomal fraction is due to a membrane effect.

Table 2. Kinetic constants for heat inactivation of paraoxonase in plasma (P) and hepatic microsomal fraction (MF)

		Component 1			Component 2		
Sample	Temperature (degrees)	A_1 (%)	k_1 (min^{-1})	E_a (Kcal/mol)	A_2 (%)	k_2 (min^{-1})	E_a (Kcal/mol)
P	47.5	96.1	0.0130				
	50.0	102.8	0.0289				
	52.5	98.9	0.0610				
				77.7			
MF	45.0	99.2	0.0089				
	50.0	61.9	0.0687		37.5	0.0021	
	52.5	58.3	0.1429		41.5	0.0071	
				61.1			101.7

Plasma (P) and microsomal fraction (MF) were incubated in 0.1 M Tris–HCl buffer, pH 7.4 for fixed times in the range 0–75 min at the temperatures indicated. Each number is the mean value obtained from three experiments. Inactivation reactions for plasma (47.5, 50 and 52.5°) and microsomal fraction (45°) showed a monophasic pattern and the apparent first-rate constants (k_1, min^{-1}) were calculated from the slopes of log percent remaining activity vs preincubation time plots. Apparent rate constants (k_1 and k_2) for the two components (1 and 2) from the inactivation reactions at 50 and 52.5° for microsomal fraction were determined from the slopes of the resolved linear components of the biphasic curves using the equation: $A = A_1.e^{-k_1t} + A_2.e^{-k_2t}$. The relative amounts of activity (%) inactivating at the fast and slow rates (A_1 and A_2) were determined from the intercepts of the linear components on the ordinate. Apparent activation energies (E_a) for components 1 and 2 were calculated by the Arrhenius method.

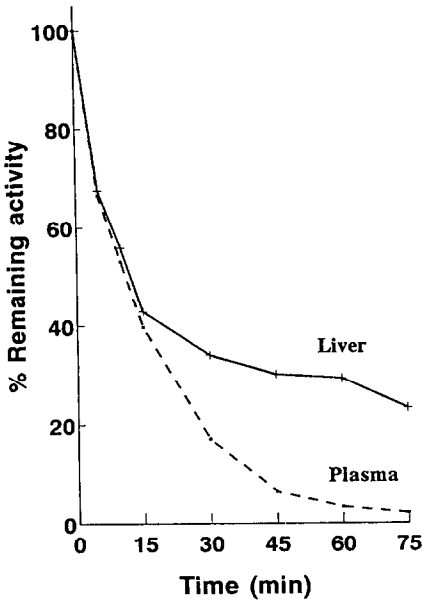

Fig. 1. Thermal stability of paraoxon hydrolase activity (paraoxonase) for plasma and liver microsomal fraction. Enzymes were placed in 0.1 M Tris–HCl buffer, pH 7.4 at 52.5°, over the time periods indicated. At the end of the preincubation period, enzyme suspensions were rapidly cooled in an ice-bath, and enzyme activity was determined as described in Materials and Methods using paraoxon 1.5 and 2 mM for plasma and liver microsomes, respectively, as substrate and $CaCl_2$ (1 mM) as cofactor. Control rates were 554 and 129 nmol/min/mL for the plasma and liver enzymes. Each point is the mean value obtained from three experiments.

Heat inactivation

The kinetic constants evaluated for paraoxonase in rat plasma and liver microsomal fraction are listed in Table 2. An overall comparison of the rate constants from the microsomal fraction with those from the plasma indicated that paraoxonase tended to inactivate faster in rat liver microsomes than in rat plasma.

The time-dependent heat inactivation of rat plasma paraoxonase showed a monophasic pattern (Fig. 1). A similar pattern has been described for rat and human plasma paraoxonase by others [20, 39, 40]. The first order rate constant observed for inactivation at 50° for plasma paraoxonase ($k_1 = 0.029\ min^{-1}$) was similar to those described by Traverso *et al.* [41] for human plasma paraoxonase ($k_1 = 0.024\ min^{-1}$) and Pellín *et al.* [20] for rat plasma paraoxonase ($k_1 = 0.038\ min^{-1}$). At 52.5° the rate of inactivation increases ($k_1 = 0.061\ min^{-1}$) in accordance with data shown by Reiner *et al.* [40] ($k_1 = 0.097\ min^{-1}$ at 53°).

In contrast with plasma paraoxonase, the activity of microsomal fraction paraoxonase was inactivated in a biphasic manner above 45° (Fig. 1). The reaction can be described by the sum of two first-order exponential terms; the kinetic constants are shown in Table 2. Such differences between the inactivation profile of rat plasma and liver paraoxonase were not found by others [20]; however, these studies used crude liver homogenates. Heat inactivation studies in preparations of rat liver microsomes solubilized in Triton X-100 (data not shown) showed a monophasic pattern for the same temperatures, suggesting that, as with pH stability, the biphasic reaction could be due to a membrane effect.

Our values of activation energies (E_a) calculated for rat plasma and microsomal fraction (Table 2) were similar to those reported by Pellín *et al.* [20] and show that the sensitivity of liver paraoxonase is somewhat higher than that of the plasma enzyme. The large magnitude of the E_a is characteristic of heat denaturation of proteins [42] and was observed for both rat plasma and liver paraoxonase. Enzyme denaturation results from an unfolding of a precise tertiary structure leading to a loss in enzymic activity through the formation of at least one enzymically inactive intermediate [43]. These results are consistent with the existence of structural features common to both enzyme activities.

Effect of calcium

As we previously reported [25] plasma and liver paraoxonase exhibit a requirement for Ca^{2+}. In the case of plasma it was absolute, indicating that Ca^{2+} was an essential activator [34]. However, in the liver microsomal fraction the reaction can occur, to a minimum extent, in the absence of the activator so that in this case calcium could be considered as a non-essential activator. Another explanation for the paraoxon hydrolysis in the absence of Ca^{2+} could be the existence of two enzymes in the microsomal fraction having different requirements for Ca^{2+}.

Addition of 2 mM and 0.5 mM EDTA to plasma and liver, respectively, inhibited 100% of paraoxon hydrolysis in plasma and between 60 and 75% in liver microsomes depending upon the substrate concentration (Fig. 2). Activity was restored by addition of free Ca^{2+}. By increasing the free Ca^{2+} concentration in plasma samples a maximum activity was reached at 4 mM and surprisingly higher concentrations of Ca^{2+} exerted inhibitory effects on paraoxon hydrolysis. In the microsomal fraction maximum recovery was achieved with 0.5–1 mM Ca^{2+} and addition of higher concentrations (> 1 mM) of Ca^{2+} had no effect on enzyme activity. In both cases the recovered activity did not reach the maximum rate obtained in the presence of optimum concentrations of calcium (control). The percentage of recovered activity was 72 and 97% when the substrate concentrations were 1.5 and 2 mM for plasma and liver, respectively. Although both enzyme activities require calcium, the kinetics of the Ca^{2+}-induced activation as a function of substrate concentration differ between plasma and liver microsomal fraction as shown in Fig. 3.

The importance of calcium was also seen by dialysis of samples against 0.1 M Tris–HCl pH 7.4 with or without EDTA. The activity of the enzyme decreased in plasma and liver as the calcium concentration diminished when samples were dialysed against 0.1 M Tris–HCl buffer pH 7.4 but was partially restored when calcium was added to the enzyme solution (results not shown). When the

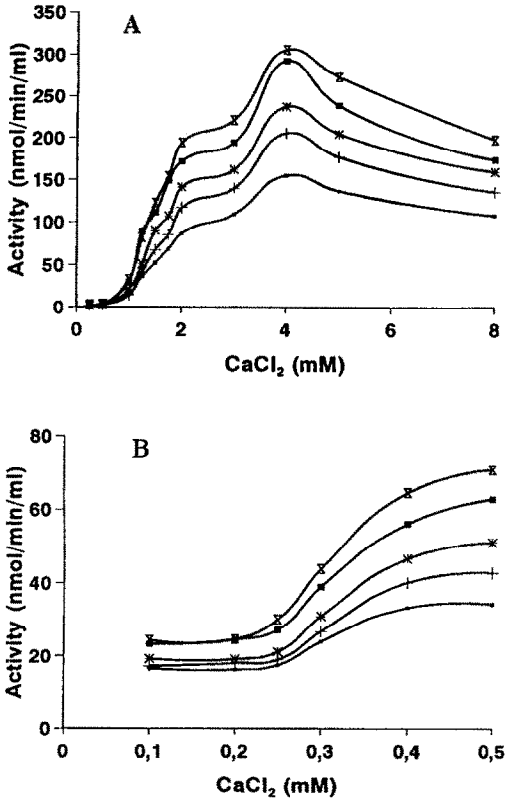

Fig. 2. Plot of paraoxonase activity versus [$CaCl_2$] in plasma (A) and microsomal fraction (B), in the presence of 2 mM (A) and 0.5 mM (B) EDTA. The activity was assayed with 0.25 mM (■), 0.375 mM (+), 0.5 mM (*), 0.75 mM (■) and 1 mM (X) paraoxon and the assay media contained the $CaCl_2$ concentrations indicated on the abscissa. Each point is the mean value obtained from three experiments.

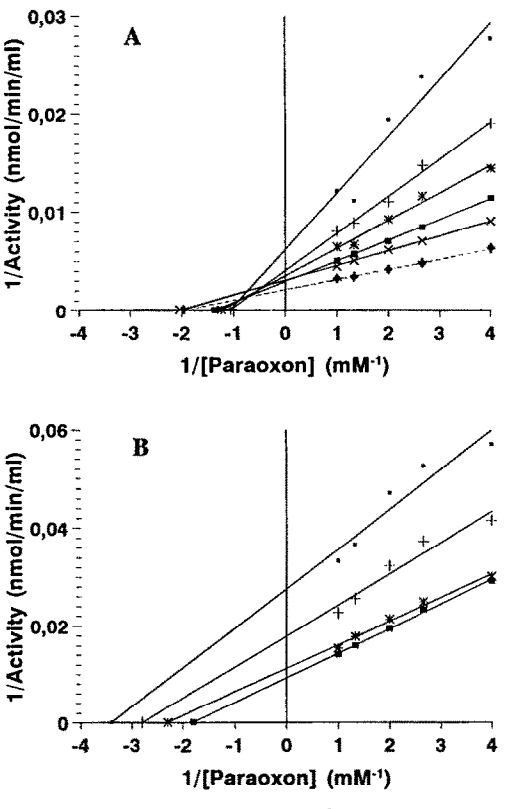

Fig. 3. Lineweaver–Burk plots for Ca^{2+}-induced activation of rat plasma (A) and liver microsomal (B) paraoxonase. The activation of rat plasma paraoxonase was assayed at 1.25 mM (■), 1.5 mM (+), 1.75 mM (*), 2 mM (■), 3 mM (×) and 4 mM (+) $CaCl_2$. The rat liver microsomal paraoxonase activation was assayed at 0.25 mM (■), 0.3 mM (+), 0.4 mM (*) and 0.5 mM (■) $CaCl_2$.

dialysis tube contained EDTA at the beginning of the dialysis, all the activity disappeared and calcium did not reactivate the enzyme. These results suggest that loss of calcium could produce structural changes in the enzyme molecule so that initial activity can not be fully restored. In this regard, it has been stated that several enzymes which hydrolyse macromolecules are activated by calcium which functions to adjust the protein conformation [44].

In agreement with our results the activation of OP hydrolysis by calcium has been reported by others for different substrates: paraoxon in rat liver [22], sheep serum [45] and human serum [41]; dichlorvos in rat liver [46] and human serum [41]; diazoxon in rat liver [22, 23] and methyl-parathion [22] in rat liver; *p*-nitrophenyl-ethyl phosphonate [47] in rabbit plasma and soman in human serum [48].

The reactivation by free calcium after inhibition by EDTA was a time-dependent effect as shown in Table 3. The best reactivation was obtained when calcium was added immediately after inhibition by EDTA. However, when the addition of calcium was delayed the recovery of paraoxonase activity was less efficient, particularly in the microsomal fraction. In this sample, activity was not recovered at all 5 hr after inhibition by EDTA, while in plasma the same effect was obtained after 21 hr. This indicates that the removal of calcium from the protein led to irreversible changes, as was suggested by the dialysis studies.

The reactivation of paraoxonase activity in plasma and liver by metals other than calcium was also studied. After inhibition by EDTA, barium and magnesium were not able to restore the inhibited activity. However, Cu^{2+}, La^{3+}, Mn^{2+}, Co^{2+} and Zn^{2+} were effective in reactivating paraoxonase activity. Maximum reactivation was reached with a metal concentration of 0.5–1 mM and 2 mM for liver and plasma samples, respectively. At higher concentrations metals exhibit inhibitory properties. These results are probably an artefact due to the method used in these experiments. The apparent reactivation by several metals can, therefore, be explained in terms of a displacement of calcium from the Ca–EDTA complex. It is known that, to an extent largely determined by their respective stability constants (K), cations can replace each other in the EDTA complex. If this is so, the apparent activation effect observed with Cu^{2+}, Mn^{2+}, Co^{2+} and Zn^{2+}

Table 3. Reactivation of rat plasma (P) and hepatic microsomal fraction (MF) paraoxonase inhibited by EDTA after different times

Time	Activity (%)	
	P	MF
Control A	100.0	100.0
Control of inhibition	2.4	16.9
0 min	85.3	85.5
30 min	77.9	58.2
60 min	70.6	51.9
90 min	59.5	37.1
2 hr	58.9	34.5
3 hr	51.6	27.6
5 hr	40.9	17.9
6 hr	38.9	20.6
7 hr	33.4	16.4
21 hr	3.9	9.2

EDTA was added to plasma and liver samples at final concentrations of 2 and 0.5 mM, respectively, and their activity was considered as control of inhibition. At times indicated in the table after the inhibitor had been added to the sample, $CaCl_2$ was added to a final concentration of 4 and 0.5 mM Ca^{2+} in plasma and microsomal fraction. Activity was measured using 2 mM (plasma) and 1.5 mM (liver microsomal fraction) paraoxon. Samples were stored in the refrigerator during the experiment. A sample without EDTA assayed in presence of 1 mM Ca^{2+} and stored under the same conditions as inhibited samples, was considered as 100% control activity (Control A).

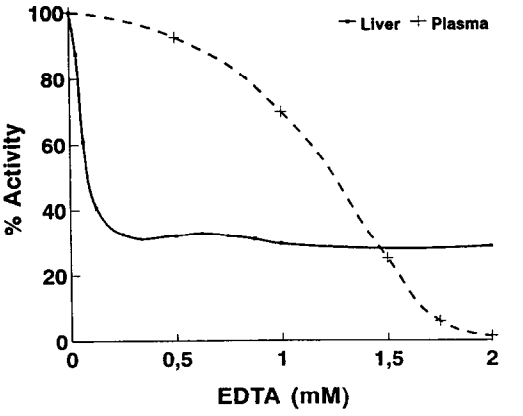

Fig. 4. Effect of EDTA on rat plasma and microsomal fraction paraoxonase activities. Activities were measured with 2 mM (plasma) and 1.5 mM (liver microsomal fraction) paraoxon as substrate and the media contained no $CaCl_2$. The results are the mean of three separate experiments. Activities in the absence of EDTA of rat plasma and liver paraoxonase were 388 ± 14 nmol/min/mL and 71 ± 9 nmol/min/mL, respectively.

can be explained by the higher stability constant of the metal–EDTA complex for those cations with respect to the Ca–EDTA complex. In our experiments the calcium liberated from the endogenous Ca–EDTA complex is the actual reactivator of paraoxonase activity. Maximal activity is reached when the metal concentration is sufficient to release all the calcium complexed by EDTA. Higher concentrations of metals result in the presence of free metal in the medium after which the normal inhibitory effect of the metal was observed. We can conclude that none of the metals assayed can replace calcium in the activation of rat plasma and liver paraoxonase.

Effect of inhibitors

In a previous paper [25] we reported that rat plasma and liver microsomal paraoxonase activities are inhibited by different metal ions (Mn^{2+}, Co^{2+}, Zn^{2+}, Ba^{2+}, Cu^{2+}, La^{3+}, Hg^{2+} and Mg^{2+}) as well as by EDTA and *p*-OH-MB. In the present study we performed inhibition experiments in more detail to determine the type of inhibition and the inhibition constants for the different effectors.

In the experiment shown in Fig. 4 we studied the effect of EDTA on paraoxonase activity in rat plasma and liver microsomal fraction. EDTA was found to inhibit the hydrolysis of paraoxon in both samples, with differing patterns of inhibition. Plasma paraoxonase was totally inhibited by 2 mM EDTA. Liver microsomal paraoxonase was inhibited at different concentrations of EDTA, reaching approx. 75% inhibition at 0.5 mM EDTA. Higher inhibitor concentrations did not change the residual activity suggesting the presence of an EDTA-resistant paraoxonase fraction. Another important EDTA-resistant fraction has been also described in hen brain particulate fraction by Reiner *et al.* [49].

The other inhibitors tested also inhibited the hydrolysis of paraoxon in both plasma and microsomal fraction. The results of the inhibition kinetic studies are summarized in Table 4. Magnesium, barium, manganese, cobalt and zinc were non-progressive inhibitors showing an immediate effect after addition to the assay medium. For these metal ions plasma paraoxonase was always more resistant to inhibition than the liver enzyme, especially with magnesium, barium and manganese which showed the highest values of K_I. Lanthanum, *p*-OH-MB, mercury and copper progressively inhibited paraoxonase activity as a function of the preincubation time with the enzyme.

Analysis of the inhibition curves generated with rat plasma and liver paraoxonase indicates that different inhibitors exhibit different inhibition patterns (competitive, non-competitive and acompetitive) showing differences between plasma and liver (except for copper, mercury and *p*-OH-MB) as well as among the different inhibitors for the same sample. It has previously been indicated that calcium is an essential activator for paraoxonase activity that could promote a conformational change in the enzyme molecule so that substrate can bind enzyme. In the case of competitive inhibition, our results can be explained if it is assumed that the specific site for the activator (calcium) on the enzyme could be occupied by other metals; in this case the structural change would not be produced impeding the binding of substrate to enzyme resulting in an inhibition of activity. In the case of non-competitive and

Table 4. Inhibition of rat plasma (P) and hepatic microsomal fraction (MF) paraoxonase activity by different effectors

	P			MF		
Inhibitor	Type	K_I (mM)	IC_{50} (mM)	Type	K_I (mM)	IC_{50} (mM)
EDTA	C	0.27	0.89	A	0.04	0.05
Magnesium	NC	47.51	47.51	A	0.86	1.27
Barium	NC	5.58	5.58	A	0.19	0.29
Manganese	NC	3.74	3.74	A	0.11	0.17
Cobalt	C	0.71	2.33	NC	0.09	0.09
Zinc	C	0.32	1.06	NC	0.13	0.13
Lanthanum	C	0.35	1.16	NC	0.28	0.28*
Mercury	NC	0.52	0.52*	NC	0.32	0.32*
p-OH-MB	NC	0.43	0.43†	NC	0.84	0.84†
Copper	C	0.05	0.17*	C	0.28	0.85*

* IC_{50} at 5 min; † IC_{50} at 15 min. C: competitive inhibition; NC: non-competitive inhibition; A: acompetitive inhibition.

acompetitive inhibition, our results can be explained according to the classical models described elsewhere [34]. Inhibition by EDTA is mediated by the sequestration of calcium.

The inhibition of paraoxon hydrolysis by EDTA, metals and other compounds has been reported by others. Sheep serum paraoxonase was inhibited by EDTA, Ba and Mn, while the rabbit serum enzyme was inhibited by Mg, Hg, Ni, Cu, *p*-chloromercuribenzoate [50] and Ba and EDTA [47]. Inhibition by EDTA, Cd, Hg and Ag has also been reported in human serum [41, 51]. The only available data on rat paraoxonase inhibition were reported by Kojima and O'Brien [22]. These authors found that rat liver microsomal enzyme was inhibited by Co, Hg, Cd, Ni and Cu but that Mn, Mg, Zn and *p*-chloromercuribenzoate had no effect on enzyme activity. These results differ from the data obtained in our study. These discrepancies could be explained by differences in experimental design. Kojima and O'Brien [22] used only one concentration (1 mM) for the inhibition studies and as shown in Table 4 several inhibitors tested by us required very high concentrations to exhibit their inhibitor power. However, the results related to inhibition by Zn and *p*-chloromercuribenzoate are not comparable.

Inhibition by Hg and EDTA has also been reported for the hydrolysis of dichlorvos by human serum [41]. Shishido and Fukami [23] reported the inhibition of diazoxon hydrolysis in rat liver microsomes by Ba, La, Co, Cu, Hg, Zn, EDTA and *p*-chloromercuribenzoate in agreement with our results, although Mg was ineffective. The similar behaviour of diazoxonase and paraoxonase suggests that both enzyme activities are very closely related proteins.

The inhibition of OP hydrolysing activity by low concentrations of mercuric salts, mercurials and copper and nickel salts has been interpreted to mean a thiol group is in or very near the catalytic center [3, 52]. Our data are in accordance with this assumption. Mercury and *p*-OH-MB are non-competitive inhibitors that could be bound to thiol groups out of the catalytic centre and probably related to the maintenance of an active configuration of enzyme molecule.

Erdös *et al.* [53] extensively analysed the effect of calcium and inhibitors on the hydrolysis of phenylacetate by human plasma. Certain lines of evidence suggest that A-esterase (OP-hydrolase) activity and arylesterase (phenylacetate-hydrolase) activity are different enzymes [13, 15, 45]. However La Du and Eckerson [54] described paraoxon and phenylacetate as two substrates for the same enzyme. In the light of the controversy about the identity between arylesterase activity and OP hydrolases it is interesting to compare the results from Erdös *et al.* [53] with our data of rat plasma and liver paraoxonase activities. In both cases Ba and Mg show the highest IC_{50} values. However, there are strong differences in the inhibitor potency of La, which is the most potent inhibitor of arylesterase activity ($IC_{50} = 0.06\ \mu M$) although it exhibits an intermediate effect on paraoxonase activity (Table 4). On the other hand, copper and cobalt are the best inhibitors of rat serum and rat liver paraoxonase, respectively, while these metals are poor inhibitors of arylesterase. In any case, arylesterase activity [53] and rat paraoxonase activity show strong quantitative differences in their sensitivity to inhibitors. Arylesterase was always much more sensitive than paraoxonase suggesting that both activities may be expressed by different enzymes as has been proposed by Walker and Mackness [15].

The purpose of the present work was 2-fold. First, to obtain more detailed information about paraoxon hydrolase activity in rat plasma and liver in order to establish their biochemical characterization as well as to ascertain different properties of liver enzyme that might be used in the purification process. Secondly, to establish the identity between plasma and liver enzyme activities regarding their relative importance as a detoxication mechanism against OP compounds. In summary, we observed differences in pH stability (Table 1), heat inactivation (Table 2) and effect of inhibitors (Table 4) and analogies in optimum pH, K_m [25] and calcium requirement (Table 3 and Fig. 2). In general we found more

differences than analogies between rat plasma and liver enzymes that could be interpreted as the presence of two enzymes or two different forms of the same enzyme.

Pellín *et al.* [20] reported that rat plasma paraoxonase was similar to the hepatic one and represented 50% of total paraoxonase activity and suggested that plasma activity could be used as a good mirror for detoxifying activity. From our data rat plasma paraoxonase activity represented only 25% of total activity. Plasma activity could be a good indication of total activity as suggested by Pellín *et al.* [20].

With respect to the relative importance of both enzymes as a detoxifying mechanism it is difficult, given the available data, to establish a clear difference between the role of both enzyme activities *in vivo*. A protective role of paraoxonase, at least in chronic exposure, has been proposed by several authors [54, 55]. *In vitro* studies suggest that serum paraoxonase activity could determine individual sensitivity to paraoxon assuming that the OP circulates in serum after exposure to parathion. However, as pointed out by Lotti *et al.* [9], the results are controversial. The unknown physiological role of these enzymes is another difficulty for the correct interpretation of their role in the detoxication of OP compounds.

An interesting difference found in our study between rat plasma and liver paraoxonase activity is that we have not found evidence that supports the presence of more than one enzyme in rat plasma, while in rat liver microsomes the existence of an EDTA-resistant fraction suggests that more than one enzyme capable of hydrolysing paraoxon is present in the microsomal fraction of rat liver.

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