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DEGRADATION BY RAT TISSUES *IN VITRO* OF ORGANOPHOSPHORUS ESTERS WHICH INHIBIT CHOLINESTERASE

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Abstract—Hydrolytic "A"-esterase activities of various tissues of rat (plasma, liver, kidney, brain and intestinal mucosa) against selected OP esters of diverse structure as potential substrates (paraoxon, di-*n*-propyl paraoxon, di-*n*-butyl paraoxon, chlorpyrifos oxon, di-(4-phenyl butyl) phosphorofluoridate and the chiral isomers of ethyl 4-nitrophenyl phenylphosphonate) were studied. We have developed a sensitive and widely applicable assay depending on measuring decline in residual inhibitory power of any chosen OP against horse serum cholinesterase: for seven compounds examined so far I_{50}s against BuChE ranged from 0.07 to 70 nM, and it is easy to monitor loss of OP starting from an initial 25 μM concentration. Progressive destruction rates were always highest in liver and plasma with activity sometimes detectable in kidney, brain but not in intestinal mucosa, but the ratios of activity between tissues differed for different substrates. At 25 μM/37°/pH 7.2 hydrolysis rates ranged from 8500 nmol/min/g liver for di-(4-phenylbutyl) phosphorofluoridate down to 0.8 nmol/min for the butyl analogue of paraoxon; the rate for L(−) isomer of EPN oxon (23 nmol/min/g liver) was >2× that for the D(+) isomer and for paraoxon. From our data we conclude that several OP hydrolases exist whose identity may be further characterised by use of selective substrates.

The first demonstration of an "A"-esterase was made by Mazur [1], who showed in 1946 that rabbit sera hydrolyse paraoxon and DFP (di-*iso*propylphosphorofluoridate). Aldridge [2, 3] defined "A"-esterases as a group of enzymes which hydrolyse organophosphorus (OP) esters and are not inhibited by them in contrast with "B"-esterases which are inhibited by OP compounds. Much of the early work was summarized and reviewed by Aldridge and Reiner [4]. Although numerous studies of OP hydrolases have been published, they mostly focused on hydrolysis of paraoxon or related phosphate esters. It has been generally believed that there may be more than one "A"-esterase in plasma or in liver (the tissues most studied) but that they are all soluble and depend absolutely on the presence of calcium ion for expression of their activity.

Our interest in OP-hydrolysing enzymes started during studies of neuropathy target esterase (NTE), an OP-sensitive esterase tightly bound to neural membranes. We tried to inhibit NTE of brain microsome samples which had been prepared from homogenates in buffer containing EDTA and which were then suspended in a similar buffer and found that the concentration of several OP esters required to inhibit NTE increased markedly (more than 100× in some cases) as the concentration of the tissue increased. It seemed probable that some OP-destructive process was occurring which required neither added metal ions nor other cofactors. Also, we have found some compounds with anticholinesterase and anti-NTE activity in the region of 1 nM *in vitro* but which were virtually non-toxic *in vivo* [5]. We wished to explore these discrepancies further and have started to investigate the destruction of a rather broader range of structural variants than have been reported by other workers.

In this work we have studied the destructive activity catalysed by a range of rat tissues, only in the presence of Ca^{2+} ions, against selected OP esters of diverse structure as potential substrates using a sensitive and widely applicable assay.

A preliminary report was given at the International Meeting on Esterases hydrolysing Organophosphorus Compounds, Dubrovnik, April 1988.

MATERIALS AND METHODS

Chemicals

The chiral isomers of EPN oxon (ethyl 4-nitrophenyl phenylphosphonate) were gifts from Sumitomo Chem. Co., as used previously [6]; chlorpyrifos oxon (diethyl 3,5,6-trichloro-2-pyridyl phosphate) was a gift of Dr. C. H. Walker (University of Reading, U.K.); di-*n*-propyl paraoxon (di-*n*-propyl 4-nitrophenyl phosphate), di-*n*-butyl paraoxon (di-*n*-butyl 4-nitrophenyl phosphate) and di(4-phenyl-*n*-butyl) phosphorofluoridate (PBPF) were the same samples as used previously [5, 7].

Paraoxon (diethyl 4-nitrophenyl phosphate), horse serum butyryl cholinesterase (EC 3.1.1.8) (BuChE), acetylthiocholine iodide and 5-5′-dithiobis-(2-nitrobenzoic acid) (DTNB) were purchased from Sigma Chemical Co. (Poole, U.K.). Other reagents were of analytical grade. The substrates were dissolved in isopropyl alcohol or acetone to give a 10 mM stock solution. Dilutions were freshly

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made for assays in 50 mM sodium phosphate buffer, pH 7.2 containing Triton X-100 (0.01%).

Enzyme preparations

Male Porton Wistar rats (Strain LACP), weighing 180–200 g at the time of sacrifice, were used. Animals were killed by decapitation and blood was collected in heparinized tubes. Plasma was separated by centrifugation and used immediately or stored at −20°.

Brains, livers and kidneys were quickly removed, rinsed in distilled water, weighed and homogenized as described below. The small intestine of the rat was removed and washed through with running tap water and then with distilled water. After the intestine had been cut open, the mucosa was scraped off with a blunt spatula and then homogenized.

Tissues were homogenized in 10 vol. of ice-cold 50 mM sodium phosphate buffer (pH 7.2 at 25°) using a power-driven close-fitting Perspex/glass homogenizer. Homogenates were used without further dilution or were diluted in the same buffer.

Determination of "A"-esterase activity

We monitored decrease in the concentration of OP compound by a decrease in the inhibitory power against BuChE, a typical OP-sensitive (B) esterase [8].

(a) *Inhibition of BuChE by OP compounds.* BuChE activity was determined accordingly to the method of Ellman *et al.* [9] but using acetylthiocholine as substrate. A mixture of enzyme (0.1 unit) in 100 mM sodium phosphate buffer pH 7.4 (0.1 ml) with 3 ml of DTNB (0.374 mM) was preincubated for 5 min at 37°. Inhibitor solution (0.2 ml) or solvent only was then added and the mixture was maintained at 37° for chosen times (0–30 min). At the end of the inhibition period 0.05 ml of 35 mM acetylthiocholine was added. The addition of substrate stopped the inhibitory reaction and the residual BuChE activity was determined from the rate of increase in absorbance at 410 nm over the next 5 min.

(b) *Calculation of rate constants for BuChE inhibition.* Percentage inhibition was calculated with reference to the activity of the sample preincubated with solvent only. Log % activity remaining was plotted against time and the slopes of each semi-log plot (see Fig. 1A for an example) were calculated by a linear regression procedure applied to each set of data points. The rate constant values ($k' = 2.303 \times$ slope) were then plotted against inhibitor concentration [I] and the slopes of this line (= the second order rate constant, k_a) was derived by linear regression as above.

(c) *Calibration curves.* A calibration curve (see Fig. 1B for an example) was constructed for the effect of each OP compound on BuChE under fixed conditions (37°, 20 min of incubation with all the inhibitors except PBPF where the preincubation time was 10 min) (see below).

(d) *Degradation of OP esters by rat tissues.* The assay medium contained usually 50 mM sodium phosphate buffer, pH 7.2, 0.01% Triton X-100, calcium acetate (0.1 mM) and tissue sample (0.25 ml of plasma or 1 ml of tissue homogenate containing up to 0.1 g fresh tissue) in a final volume of 3.9 ml. When the tissues were very active against the substrate used, 2.5–50 μl of plasma were used and the tissue homogenates were diluted 10–100-fold with the homogenization buffer shortly before use.

The tissue mixture was preincubated for 5 min at 37° and the reaction was initiated by the addition of substrate solution in buffer pH 7.2, 0.01% Triton X-100 to give a final substrate concentration of 25 μM. At intervals between 0 and 180 min, aliquots of 0.5 ml (at least three) were removed and mixed with 0.5 ml of $HClO_4$ (0.35 M)/sodium acetate (0.36 M) to precipitate protein and to stop enzyme-catalysed and non-enzymic hydrolysis: the final pH was about 4.5. After thorough mixing of the contents the tubes were kept in an ice bath for 30–90 min and then centrifuged. The cold clear supernatant was diluted and assayed for content of OP ester as described above: the inhibitory power of the compounds controlled the size and dilution of sample taken for the assay. Dilute solutions of PBPF were not very stable and assays were performed immediately after dilution and with only 10 min allowed for incubation with BuChE, since the semi-log progress lines ceased to be linear after such a time; the other esters tested were more stable and the dilute solutions could be kept for several hours without loss. Dilutions were made to give an inhibition of BuChE between 20 and 80%. Non-enzymic hydrolysis was determined in each experiment by running a buffer-only (no tissue) in parallel with the tissue containing samples. In all the degradation experiments, samples from two different animals were processed simultaneously, and three or four time points were determined, so that rates were calculated from at least six to eight data points.

(e) *Calculation of hydrolysis rates.* The first order rate constants for the hydrolysis of the OP compounds studied were calculated using the plot of the logarithm of the percentage remaining OP versus time using linear regression curve fitting (cf. Fig. 2). These values ($k' = 2.303 \times$ slope) were corrected for non-enzymic hydrolysis by subtracting for each compound the corresponding k' of the control. The corrected initial rates ($V_0 = k'[S_0]$) were converted to rates per g or per ml of tissue.

A comparison test was used to establish the existence of significant differences between the slopes for control and test samples in each case. The standard error was calculated for the corrected slopes (generating the standard error of V_0) as well as for the ordinate at zero time.

RESULTS

Inhibitory activity of OP compounds against horse-serum BuChE in vitro

Using the procedures described in the Materials and Methods section, semi-log plots of BuChE inhibition vs time of preincubation were obtained for each OP ester, and Fig. 1A shows the plot for paraoxon. In many cases the progress lines for higher concentrations of esters intercepted the ordinate below log 100% activity but the slopes (k') were approximately proportional to concentration so that k_a could be calculated from the combined data and,

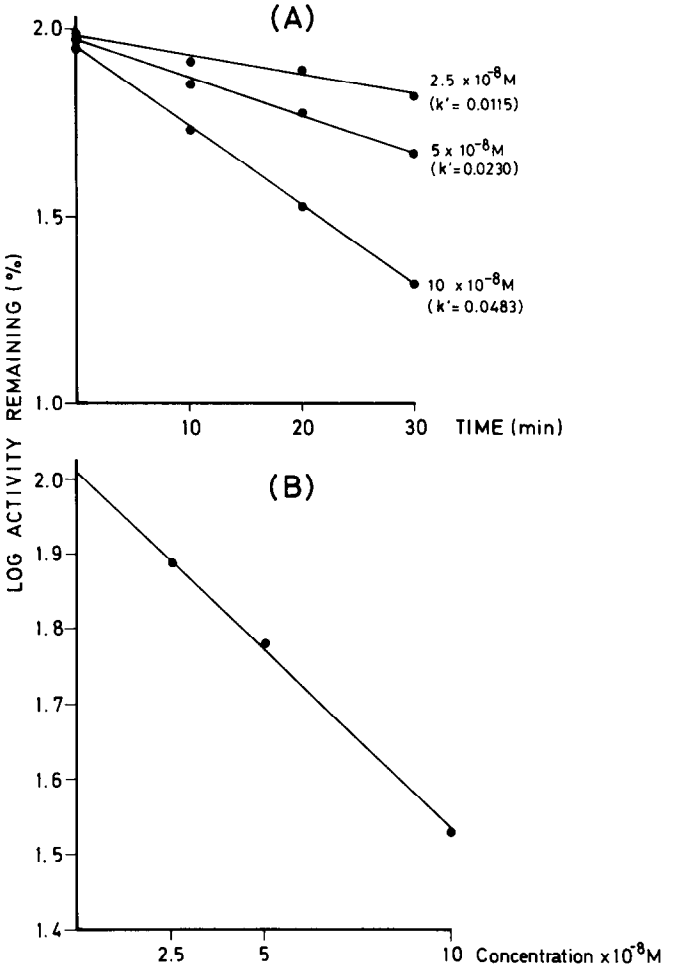

Fig. 1. (A) Semilog plot of progressive inhibition of horse-serum BuChE by paraoxon at concentration shown and at pH 7.4 and 37° (k': pseudo-first order rate constant). Slope of the progress lines were measured and used to derive k_a values given in Table 1. (B) Calibration curve for paraoxon. The degree of inhibition of BuChE (37°, 20 min of incubation) is plotted on a logarithmic scale against the concentration on a linear scale. Similar plots, (A) and (B), were obtained with all the others inhibitors studied except PBPF (see text).

also, a linear calibration line for concentration of ester against log % BuChE activity remaining after fixed-time incubation (usually 20 min: see Materials and Methods) could be obtained as shown in Fig. 1B. The intercepts below log 100% in some progress plots indicated that non-progressive as well as progressive inhibition was occurring but this does not invalidate the calibration procedure.

Table 1 shows the values of k_a and the derived calculated values of I_{50} (concentrations of inhibitor which would give 50% progressive inhibition in 20 min). The inhibitory activity of OP compounds studied against horse-serum BuChE *in vitro* ranged from 0.07 to 70 nM. The most potent inhibitors were chlorpyrifos oxon, PBPF and di-*n*-butyl paraoxon, the I_{50} values of which were below 1 nM.

Degradation of OP compounds by rat tissues

Figure 2 has time-course data for four compounds; similar lines were obtained for the other compounds tested. The figure shows that three distinct destructive processes occur when OP esters are incubated with tissue. These are: (1) a rapid loss of OP identified by an intercept below log 100% on the ordinate at zero time; (2) a progressive non-enzymic degradation seen in some of the buffer-only controls; (3) a tissue-catalysed progressive reaction. Reactions (1) and (3) are typically considered to represent irreversible organophosphorylation of OP-sensitive ("B") esterases (tissue binding) and "A"-esterase activity respectively.

(1) *Initial rapid loss of OP.* Figure 2 shows that when incubation mixtures containing tissues were quenched within a few seconds of additions of OP, recovery of the compound varied between 40 and 100%. Moreover, when the lines linking quantities of OP recovered at various intervals were plotted and extrapolated back to zero-time, the intercept on the ordinate was sometimes, but not always, lower than the directly measured zero-time value. The zero-time intercepts may indicate irreversible binding (covalent organophosphorylation) or non-progressive binding not easily reversed by denaturing the tissue at pH 4.5: this could well be partition

Table 1. Inhibitory activity of organophosphorus compounds against horse-serum BuChE

Compound	Second-order rate constant k_a (/M/min)	I_{50}† (nM)
A. Phosphates		
1. Paraoxon	$(4.9 \pm 0.1)^* \times 10^5$	70
2. Di-*n*-propyl paraoxon	$(1.3 \pm 0.1) \times 10^7$	2.7
3. Di-*n*-butyl paraoxon	$(4.7 \pm 0.2) \times 10^7$	0.73
4. Chlorpyrifos oxon	$(5.0 \pm 0.2) \times 10^8$	0.069
5. PBPF	$(7.3 \pm 1.0) \times 10^7$	0.47
B. Phosphonates		
1. D-(+)-EPN oxon	$(5.2 \pm 0.1) \times 10^5$	66
2. L-(−)-EPN oxon	$(9.5 \pm 0.2) \times 10^5$	36

* The SEM values of k_a derived from each family of lines (Fig. 1A) are indicated in each case. Inhibition of BuChE was determined at pH 7.4 and 37° as described under Materials and Methods.

† Values of I_{50} refer to 20 min preincubation and are derived from k_a.

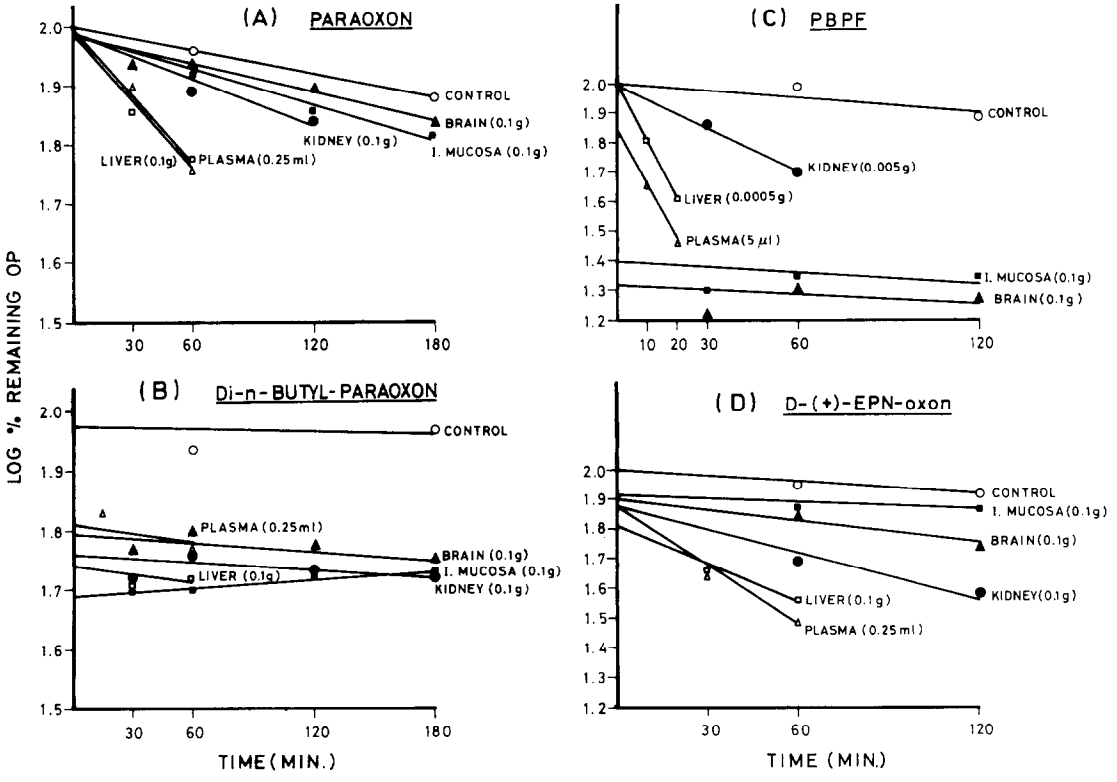

Fig. 2. Semilog plots of rate of disappearance of OP compounds in the presence of different amounts (in brackets) of rat tissue. Experiments were made with 25 μM initial concentration of OP compound in 4 ml of medium at pH 7.2 and 37°. The maximum quantity of tissue was limited by the non-progressive destruction for the conditions used in these assays: ○, buffer-only control; △, plasma; □, liver; ●, kidney; ▲, brain; ■, intestinal mucosa. The points are the average of data from at least two animals, within a range less than ±10%.

into lipid. We have insufficient data to distinguish between these effects with precision.

(2) *Non-enzyme-catalysed hydrolysis.* It is shown in Fig. 2 that non-enzymic hydrolysis of the different compounds in phosphate buffer, pH 7.2 occurs at different rates. The range of rates for all compounds tested was 0–0.0021 nmol/min in the conditions of the assays and could be ranked as PBPF > L-(−)EPN oxon ~ D-(+)-EPN ~ paraoxon > chlorpyrifos oxon > di-*n*-butyl paraoxon > di-*n*-propyl paraoxon.

(3) *Progressive tissue-catalysed degradation.* Figure 2 shows the semi-log plots of disappearance of several OP compounds studied in presence of different amounts (in brackets) of rat tissue homogenates and Table 2 summarizes the tissue-catalysed

progressive destruction rates calculated after correction for non-enzymic hydrolysis. An approximate correction was also made to the observed rates to allow for the fact that, when tissue binding occurred, some OP was removed from the medium so that the effective initial concentration was less than the stated 25 μM. Initial experiments (not shown) showed that, at this comparatively low OP ester concentration, a simple correction was possible since the first-order rates of hydrolysis were approximately proportional to concentration.

In several cases, tissue-catalysed hydrolytic activity was not detected. The limit of activity detectable varied between compounds depending on their differing non-enzymic rates (see above and Fig. 2). Also, it was not possible in every case to increase the quantity of tissue in order to detect possible low levels of activity since the disposal of the compound by tissue binding became substantial (for example see intercepts on Figs 2B–D).

Table 2 shows that plasma and liver had the highest hydrolytic activity against the OP compounds assayed. Lower rates were found with kidney and brain, while in intestinal mucosa no significant enzymic effect was detected.

For plasma and liver the ranking of rates of hydrolysis of the compounds was the same: PBPF ⩾ chlorpyrifos oxon ⩾ L-(−)-EPN oxon > D-(+)-EPN oxon > paraoxon ⩾ di-*n*-propyl paraoxon ~ di-*n*-butyl paraoxon. However, this ranking was not the same in every tissue. Thus, in kidney the hydrolytic rate for chlorpyrifos oxon was much lower in proportion to PBPF and actually lower than the rates for EPN oxon isomers.

Table 2. Progressive destruction rates (nmoles/min/ml or g) for rat tissues against different OP compounds

Compound	Non-enzymic rate*	Enzymic rate in rat tissues†				
		Plasma	Liver	Kidney	Brain	Intestinal mucosa
Paraoxon	$(1.49 \pm 0.03) \times 10^{-3}$	3.06 ± 0.24‡	7.88 ± 0.36‡	1.77 ± 0.47	0.35 ± 0.33	0.56 ± 0.46
Di-*n*-propyl paraoxon	N.D.	0.77 ± 0.01‡	0.77 ± 1.63	0.27 ± 0.47	0.02 ± 0.03	0.27 ± 0.15
Di-*n*-butyl paraoxon	$(0.23 \pm 0.79) \times 10^{-3}$	0.27 ± 0.97	0.77 ± 1.92	0.25 ± 0.67	0.32 ± 0.66	N.D.
Chlorpyrifos oxon	$(0.28 \pm 0.12) \times 10^{-3}$	200 ± 68	843 ± 538	3.61 ± 0.62‖	1.84 ± 0.52‖	N.D.
PBPF	$(2.09 \pm 1.03) \times 10^{-3}$	815 ± 90‖	8520 ± 900‖	175 ± 37‖	N.D.	N.D.
D-(+)-EPN oxon	$(1.53 \pm 0.17) \times 10^{-3}$	5.57 ± 0.55‡	8.94 ± 1.27‖	4.45 ± 0.93‖	1.11 ± 0.54	N.D.
L-(−)-EPN oxon	$(1.68 \pm 0.79) \times 10^{-3}$	9.06 ± 0.66‡	23.2 ± 1.5‡	9.23 ± 1.74‖	1.57 ± 0.84	1.57 ± 0.80

* First-order rate constants (/min; $K = 2.303 \times$ slope) calculated as indicated below but in absence of tissue.
† The initial rate (mean ± SE) was estimated for 25 μM initial concentration of OP compound in 4 ml of medium at pH 7.2 and 37°. The values presented were derived from the first-order rate constants (k') corrected for non-enzymic rates as indicated in Materials and Methods.
Statistical comparison was made between the actually observed rates in absence and presence of tissue.
Different from the non-enzymic control: ‖$P < 0.05$; ‡$P < 0.01$; §$P < 0.001$.
N.D., not detected.

DISCUSSION

Advantages and disadvantages of the use of BuChE monitor

The main advantage of this method is its almost universal applicability for OP esters since many of these compounds are able to inhibit BuChE. This method has enabled us to test a wider range of chemical structures that has been examined previously, but it has two limitations. (i) The method determines disappearance of OP ester rather than appearance of product. Therefore it is not possible to detect accurately the disposal of less than about 20% of the substrate, but the rates of reaction decline when a large proportion of substrate is disposed of. For this reason the procedure is more suited to semiquantitative screening as desired in this study than to rigorous kinetic analysis. (ii) Some OP compounds, such as ethyl-*S*-(4-chlorobenzyl) phenylphosphonothiolate in which we are interested are poor inhibitors of BuChE and it is not possible to use the method for such OPs. It may be that other commercially available "B"-esterases (pig liver carboxyesterase or trypsin) would be suitable adjuncts to BuChE in further screening of unusual structures.

Tissue-catalysed hydrolysis

We have not obtained proof that the progressive disposal of substrates observed in these studies was actually hydrolytic but, since reactions were cata-

lysed by low quantities of tissue and in the absence of added cofactors, the assumption seems reasonable. Also no attempt has been made to obtain maximum rates which would require concentrations far higher than those we chose which seemed more relevant to the possible situation in intoxicated animals. Also, since it seems that more than one enzyme is involved in hydrolysing some OP esters no universally optimum pH could be chosen.

From our results (Table 2) it is evident that the extent of OP hydrolysis by intestinal mucosa is negligible. Aldridge [8] found "B"-esterase but negligible OP-resistant esterase activity in extracts of rat intestinal mucosa assayed with phenyl acetate or butyrate. Also, the "A"-esterase activity of brain tissue was only significant against chlorpyrifos oxon. Aldridge [3] did not detect paraoxonase activity in brain tissue, but Chemnitius *et al.* [10] report rat brain paraoxonase about 6% of that in whole blood.

Our results with paraoxon are broadly compatible with those of Brealey *et al.* [11] and Mackness *et al.* [12], who, respectively, found "A"-esterase activity towards paraoxon of 57.3 ± 3.9 and 61 ± 4 nmol/min per ml of rat serum using a substrate concentration of 2 mM and monitoring the generated *p*-nitrophenol colorimetrically. In a single experiment using our monitoring assay with paraoxon 2 mM we found a rate of about 89 nmoles/min/ml plasma (details not given). This 30× increase in hydrolytic activity for an 80× increase in substrate concentration from 25 μM used in our other experiments suggests K_m for paraoxon is significantly less than 2 mM. Aldridge and Reiner [4] report a K_m value about 0.5 mM for rabbit liver paraoxonase. Chemnitius *et al.* [10] measured paraoxonase rates of several tissues from a range of species with only 1 mM substrate and at 25°: they report a mean rate of 326 nmol/min per ml whole blood, 718/g liver and 34–51 for kidney, spleen and lung.

The ratios liver/plasma and liver/kidney for paraoxonase activity found by us (cf. Table 2) were in the same order of magnitude of those reported by Aldridge [3]. The data of Becker and Barbaro [13] on the enzymatic hydrolysis of *p*-nitrophenyl ethyl phosphonates (EPN oxon and its analogues) are difficult to compare with our results because they used rabbit serum samples and differences in "A"-esterase activity between species are well known [3, 4].

Structure/activity relationships

The results presented here show that there are remarkable differences in hydrolysis of different OP esters. For the hydrolysis of dialkyl 4-nitrophenyl phosphates by plasma and liver, the activity decreased markedly when increasing the length of the alkyl group (Table 2). This may, in part, be a reflection of the decreasing ease of non-enzymic hydrolysis of these esters: the first-order rate constants for this reaction derived from Fig. 2 are 1.5×10^{-3}/min for paraoxon and $0\text{–}0.2 \times 10^{-3}$/min for the higher analogues. However the differences in enzyme-catalysed breakdown between paraoxon and the isomers of EPN oxon cannot be explained in this way since the non-enzymatic rates are very similar (Table 2).

Paraoxon and chlorpyrifos oxon are both diethyl phosphates. They differ in the structure of the leaving group and have a high difference in their rate of hydrolysis by plasma and liver. The ratio of activities chlorpyrifos oxon/paraoxon in plasma was about 65, in accordance with that reported by Mackness and Walker [14]. Table 2 shows that chlorpyrifos oxon is more easily hydrolysed non-enzymatically also.

We found that all tissues hydrolysed L-(−)-EPN oxon 1.3–2.6 times faster than D-(+)-EPN oxon. Stereospecific hydrolysis of OPs has been reported previously for Sarin [15], Tabun [16] and Soman [17, 18].

Specificity of enzymes

Characterization of the specificity of "A"-esterases is very incomplete [4]. Ratios of activities against two substrates in different tissues has been used as a criterion to detect whether the hydrolysis of a compound is due to one enzyme or more than one enzyme. If two substrates are hydrolysed only by a single enzyme, then the ratio of the activities for these two compounds must remain the same in different tissues of the same individual [4]. Comparison of the ratio for the hydrolytic activity of various tissues against selected pairs of compounds listed in Table 2 show that the ratio for the isomers of EPN oxon varied no more than threefold among all the tissues examined. However, the ratios for either chlorpyrifos oxon/paraoxon or PBPF/D-(+)-EPN oxon differed by 25–50× between liver and kidney. Likewise, differences up to sevenfold exist when ratios for various pairs hydrolysed by plasma are compared with ratios for other tissues. Such variations indicate the presence of more than one "A"-esterase in the tissues studied. The same conclusion has been reached on other grounds in many studies but principally focused on very few compounds [4, 13, 14, 19, 20].

"A"-esterase activity/toxicity relationships

Activity of "A"-esterases has been reported as an important factor in determining the selective toxicity of some organophosphorus compounds in mammals and birds [10, 11, 21–23]. Our finding of the very high hydrolytic rate found for PBPF may be a partial explanation why this compound has an $LD_{50} > 275$ mg/kg in the hen although the I_{50} for hen AChE is less than 2 nM [5].

Although our measurements were inadequate to give accurate measures of covalent binding, the apparent binding capacity of tissues for PBPF was relatively high. However, in spite of such capacity, it can be calculated that *in vivo* the maximum quantity bound would not exceed a very few mg/kg. Therefore, the disposal processes leading to the unexpectedly high LD_{50} may be attributed mostly to "A"-esterase activity rather than to tissue-binding. By contrast, for nerve agents such as soman and sarin, with $LD_{50} < 1$ mg/kg, tissue binding may well be a very significant factor in metabolic disposal as suggested by others [24, 25].

In conclusion, our results suggest that use of a wide variety of structures may assist in the dissection and identification of the, as yet unknown, components of what is often described as tissue "A"-

esterase. Some progress has been made recently in separating several isoenzymes of sheep plasma "A"-esterase with different substrate specificities [14]. Progress in such work can be aided by availability of more sensitive substrates, and PBPF and related fluorides might be very useful. Another asset would be to have one or more selective and irreversible inhibitors. Several sulphonyl fluorides which might, conceivably, have been inhibitory are, in fact, not inhibitors of rat serum paraoxonase (M. K. Johnson, unpublished work).

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