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HUMAN SERUM "A"-ESTERASES

HYDROLYSIS OF *O,O*-DIMETHYL-2,2-DICHLOROVINYL PHOSPHATE*

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Abstract—Some characteristics of the hydrolysis of *O,O*-dimethyl-2,2 dichlorovinyl phosphate (DDVP) by human serum are reported and compared with the hydrolysis of *O,O*-diethyl-4-nitrophenyl phosphate (paraoxon) which is a substrate for Paraoxonase, a known "A"-esterase of human serum. When incubated with human serum, DDVP was losing its inhibitory power toward acetylcholinesterase (AChE). The loss of DDVP followed first order kinetics and was proportional to serum dilution. The disappearance of DDVP after incubation with human serum was not due to protein binding. Apparent K_m and V_m for the hydrolysis of DDVP were 7.1 mM and 143 nmol·min^{-1}·ml^{-1}. The pH sensitivity, EDTA inhibitory and Ca^{2+} requirements of DDVP-ase were similar to those of Paraoxonase. DDVP inhibited the Paraoxonase activity and paraoxon inhibited the DDVP-ase activity. Ca^{2+}, Ag^{+} and Hg^{2+} were better inhibitors of the Paraoxonase than the DDVP-ase. The rate of heat inactivation was also different; at 55° Paraoxonase inactivated almost completely within 10 min, while DDVP-ase lost only about 10% activity over 1 hr. Consequently, DDVP-ase and Paraoxonase can be differentiated by means of heat sensitivity. The DDVP-ase was normally distributed in a population of 60 individuals, while Paraoxonase is known to show a marked polymorphism.

The esterases which interact with organophosphorus compounds (OP)‡ have been divided in two classes: "A" and "B" esterases, the former being a group of enzymes which hydrolyse OPs much faster than the latter [1]. Thus "A"-esterases are thought to be involved in the detoxification of OPs whereas "B"-esterases represent, as in the case of acetylcholinesterase (AChE), the target of OP toxicities.

The first description of an "A"-esterase was given more than 40 years ago by Mazur, who described the hydrolysis of paraoxon (*O,O*-diethyl 4-nitrophenyl phosphate) and DFP (diisopropyl phosphorofluoridate) by rabbit serum [2]. The hydrolysis of paraoxon was then studied in detail [3] and a number of "A"-esterases have been further identified in different tissues of several animal species [1, 4]. The substrate specificity of "A"-esterases is very complex and far from understood [1]. Their physiological role has also not been clarified since no natural substrates of these enzymes are known [5]. Nevertheless it was recently speculated that particular isoforms of these enzymes, with very wide substrate specificity, might have evolved to metabolize naturally occurring xenobiotics [6].

"A"-esterases are present in the sera of several mammalian species [3] and much of the activity has been found in the high-density lipoprotein (HDL) fraction [7, 8]. However, relatively little information on the biochemistry and pharmacology of "A"-esterases is available, even though there is now a great interest for these enzymes in toxicology, genetics and clinical chemistry. The possible role of "A"-esterases in determining the selective toxicity of OPs has been reviewed [9, 10] and studies reported on the use of "A"-esterases as markers for some genetic disorders [11, 12] and for myocardial infarct [13].

We report in this paper some biochemical characteristics of an "A"-esterase in human serum which hydrolyses *O,O*-dimethyl-2,2-dichlorovinyl phosphate (DDVP). This OP is a widely used pesticide and an anthelmintic drug whose mode of action is due to inhibition of AChE. The metabolism of DDVP was investigated extensively in mammalian plasma and tissues [14–18], and some characteristics of its enzymatic hydrolysis by human plasma already reported [19].

MATERIALS AND METHODS

Tissues. Pools of human sera were formed with at least 30 samples of serum collected from the clinical chemistry laboratory of a general hospital. Pooled sera were then subdivided in small aliquots and stored at −20° until used.

Human nucleus caudatus and cerebral cortex were obtained during post-mortem examinations performed within 36 hr after death, from the bodies of patients who died of extranervous causes. Samples were then subdivided into fragments of about 300 mg and stored at −20°. This procedure of sampling and storage does not affect the activities of AChE in the nucleus caudatus and of Neuropathy Target Esterase

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‡ Abbreviations used: AChE, acetylcholinesterase; DDVP, *O,O*-dimethyl-2,2-dichlorovinyl phosphate; DFP, diisopropyl phosphorofluoridate; NTE, neuropathy target esterase; OP, organophosphorus esters.

(NTE) in the cortex [20]. Sometimes the nucleus caudatus was homogenised in 50 mM Tris/HCl pH 8.0 (7.7 mg/ml wet weight) and stored in aliquots at −20°; AChE activity was maintained up to three months.

Chemicals. *O,O*-dimethyl-2,2-dichlorovinyl phosphate (DDVP) was purchased from Societa' Italiana Chimici (Roma, Italy). Paraoxon (*O,O*-diethyl-*p*-nitrophenyl phosphate) was purchased from Sigma Chemical Co. (St Louis, MO) and purified as already described [21]. Mipafox (*N,N*-diisopropyl phosphorodiaminofluoridate) and phenyl valerate were synthesized and purified as previously described [21]. All other chemicals were of the highest analytical grade available.

DDVP-ase assay. Hydrolysis of DDVP was followed by measuring the loss of DDVP inhibitory power on human AChE after incubation with human serum.

Ten microlitres of DDVP (final concentration 10 mM) in anhydrous acetone were incubated with 0.5 ml undiluted or 50 mM Tris/HCl buffer diluted serum (1:2, 1:4), for 40–60 min at 37° (pH 7.6–8.0). The hydrolysis was stopped by adding 3 ml of ice-cold 0.35 M perchloric acid (PCA)/0.36 M sodium acetate (pH 3.0) and allowing the tubes to stand at 0° for 10–20 min. After centrifugation (500–1000 *g* for 15 min) the supernatant was diluted as required with PCA/Na acetate and 100 μl added to 2 ml of nucleus caudatus homogenate (7.7 mg/ml in 50 mM Tris/HCl, pH 8.0). The mixture was incubated for 20 min at 37° and then AChE activity was measured kinetically according to the method of Ellman *et al.* [22] (E_{412} = 13,600). PCA/Na acetate does not affect AChE assay. The DDVP concentration remaining after incubation with the serum was calculated from the activity of AChE; this concentration was derived from an AChE inhibition semilog plot with a range of DDVP (eight concentrations between 0.1 and 0.9 μM) which underwent the same assay procedure. AChE inhibitions were always measured in the range 20–60%, using appropriate dilutions. The activity of human serum DDVP-ase was expressed as nmoles of DDVP lost per min per ml of serum. In summation experiments, the loss of DDVP inhibitory power after incubation with serum was calculated from human brain cortex NTE inhibition, as measured according to Johnson [21]. The reason for using NTE instead of AChE is because remaining paraoxon (see below) would not interfere with NTE, an esterase which is known to be almost insensitive to relatively low concentrations of paraoxon (<1 mM) [23].

The DDVP-ase activity of the pooled sera used throughout all the experiments was 111 ± 9 nmol·min^{-1}·ml^{-1} (mean ± SD, N = 6). DDVP-ase activity was not affected by the storage at −20° for a period up to three months.

Paraoxonase assay. The hydrolysis of paraoxon was followed by measuring the formation of *p*-nitrophenol according to the method of Playfer *et al.* slightly modified [24] (E_{405} = 13,000). Tris/HCl buffer (50 mM, pH 7.4) was used instead of 100 mM glycine pH 10.5; the reaction was stopped with PCA/Na acetate and supernatant diluted with equal volumes of 0.5 M Tris/HCl, pH 10.0. The activity is expressed as nmoles of *p*-nitrophenol formed per min per ml of serum. Paraoxonase activity decreases slightly (10–15%) over a period of more than a year at −20°, but its capacity to be restored by salt addition is unaffected [25].

In one experiment (pH sensitivity, Fig. 2) Paraoxonase activity of pooled sera was unusually low. However this low activity does not influence its sensitivity to pH, as suggested by a separate experiment where a serum with normal Paraoxonase activity was diluted with 100 mM Tris/HCl and the sensitivity to pH found similar.

RESULTS

When DDVP is incubated with human serum, a progressive loss of its inhibitory power on AChE is observed. DDVP loss follows a first order kinetics and is proportional to serum dilutions (Fig. 1).

The existence of reversible non-specific binding-sites for DDVP was tested by incubating 50 mM Tris/HCl pH 7.4 diluted serum (1:2) with DDVP (20 mM) at pH 7.4 at 37°. Paired samples were taken at 0, 5, 10, 15, 20, 40 and 60 min to measure DDVP-ase activity. The reaction was stopped either with 1 vol. of PCA/Na acetate or with 30 vol. of cold buffer. Volumes were then adjusted and the residual inhibition power tested as usual. The two time–course lines drawn as in Fig. 1 (not shown) were not significantly different, suggesting that reversible bindings are not involved in the disappearance of DDVP from human serum. The possibility of an irreversible unspecific binding as the cause of the disappearance of DDVP was tested preincubating the serum for 14 hr with DDVP (70 μM) at 28°, pH 7.4 in phosphate buffer. Two time-course semilog plots were drawn comparing the hydrolysis of DDVP by DDVP-preincubated serum and acetone-preincubated serum, and found to be similar (not shown). Incubation of DDVP with bovine serum albumin (BSA: 40 mg/ml of 50 mM Tris/HCl, pH 7.4, 37°) up to 120 min causes 10–15% loss of inhibitory power. This loss is negligible with lower concentrations of BSA (10 and 20 mg/ml) and is not time dependent. It should be concluded that the loss of DDVP inhibitory power after incubation with human serum is enzymatic and that unspecific bindings do not contribute substantially.

Kinetic analysis of DDVP hydrolysis, according to Lineweaver–Burk, was performed with substrate concentrations between 0.1–5 mM incubated with undiluted or 50 mM Tris/HCl diluted sera for 40–60 min at 37°. A double reciprocal plot was obtained with data belonging to seven different sets of concentrations assayed on separate experiments (four pools of sera). Apparent K_m was 7.1 mM and V_m was 143 nmol·min^{-1}·ml^{-1}. These values agree with the Michaelis constants reported by Reiner *et al.* [19] for the hydrolysis of DDVP by human plasma but not with those reported by Blair [17].

Some biochemical characteristics of DDVP-ase were compared with a known "A"-esterase activity, the serum Paraoxonase. DDVP-ase sensitivity to pH is shown in Fig. 2 together with that of Paraoxonase. The pH sensitivity pattern is similar for both enzymes. As for most "A"-esterases there is no pH optimum [1]. A notable exception, however, is the

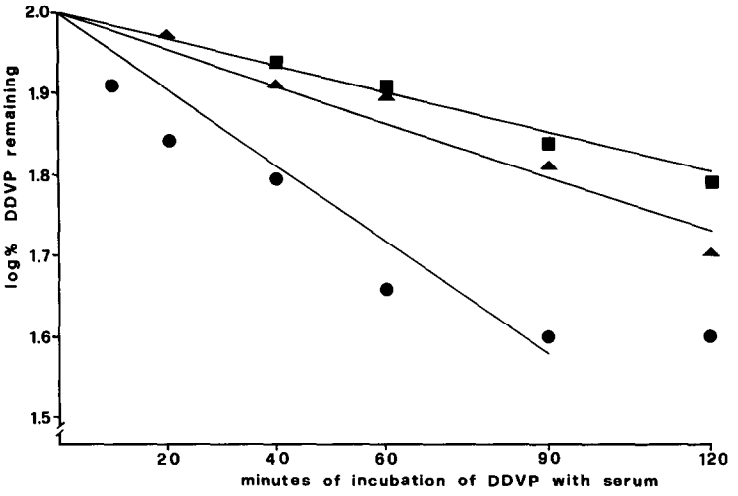

Fig. 1. Time-course of the loss of DDVP after incubation with human serum. DDVP (10 mM) was incubated at 37° with different dilutions of serum as follows: ●, undiluted serum; ▲, 1:2 diluted serum with 50 mM Tris/HCl at pH 7.6; ■, 1:4 diluted serum with 50 mM Tris/HCl at pH 7.6. AChE inhibition at time 0 (54–70%) was obtained after incubation of AChE with appropriate dilutions of the supernatant. Reported data are the average of two separate experiments in which the slopes (k) were similar. k was a linear function of serum concentrations. v was calculated according to the equation $v = k(a - x)$. The final concentration was derived from a calibration semilog plot of dichlorvos concentrations and AChE inhibition, as described under Material and Methods.

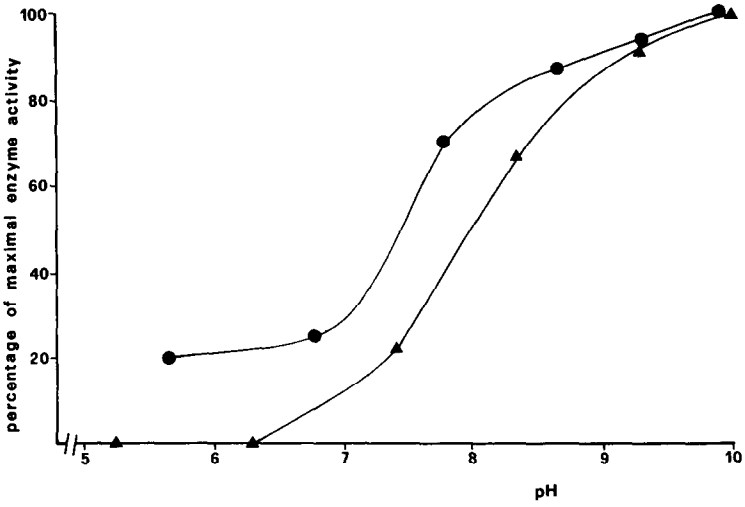

Fig. 2. Sensitivity to pH of DDVP-ase (●) and Paraoxonase (▲) DDVP (10 mM) was incubated in serum diluted (1:2) with universal buffer (Britton and Robinson [26]) for 60 min at 37°. No spontaneous hydrolysis of DDVP occurred in these conditions. Reaction was stopped with PCA/Na acetate and DDVP-ase activity calculated as usual. Paraoxon (1 mM) was incubated with serum diluted 1:10 with the same buffer for 30 min. Reaction was stopped with PCA/Na acetate, the supernatant diluted in 0.5 M Tris pH 10.0 and p-nitrophenol red at 405 nm. Some spontaneous hydrolysis (15%) of paraoxon occurs at pH 10.0. The maximal activities of the pooled sera were 141 and 25 $nmol \cdot min^{-1} \cdot ml^{-1}$ for DDVP-ase and Paraoxonase, respectively.

sheep serum paraoxonase which shows an optimum pH at about pH 8.5 [27].

DDVP-ase was assayed after incubation of undiluted serum with DDVP (10 mM) for 40 min and Paraoxonase after incubation of phosphate buffer diluted serum (1:10) with paraoxon (1 mM) for 30 min. Under the above conditions DDVP-ase and Paraoxonase activities increased between 22° and 40°. Heat inactivation of DDVP-ase was noted at 55° and of Paraoxonase at 43°.

Heat inactivation at 50° followed the kinetics of a first order reaction when paraoxon was used as substrate and the first order rate constant of inactivation ($k/min^{-1} = 0.024$) was similar to that already reported at 53° ($k/min^{-1} = 0.026$) [19]. Heat inactivation of DDVP-ase started at 55° but kinetics analysis was prevented by coagulation of serum ($t > 55°$) (Fig. 3). These results differ from those

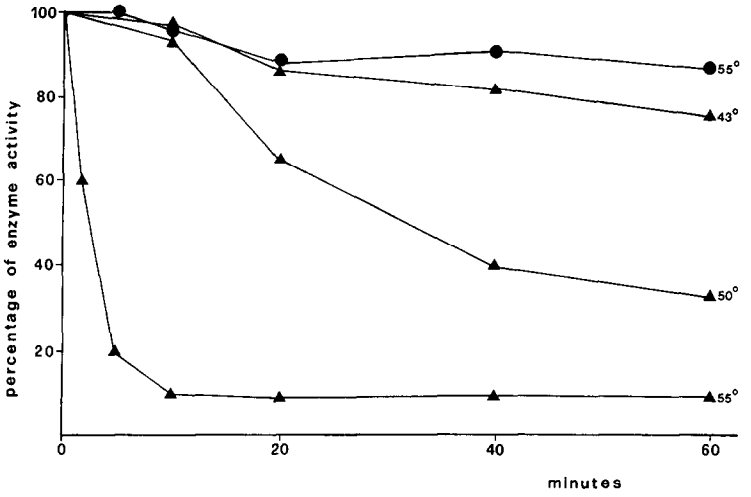

Fig. 3. Time-course of heat inactivation of DDVP-ase (●) and Paraoxonase (▲). Undiluted serum was incubated at the given temperatures and then cooled at 0° for 15 min before assayed for DDVP-ase and Paraoxonase. DDVP-ase and Paraoxonase were not affected by incubation with serum for 60 min at 50° and 37° respectively. Similar results for DDVP-ase heat inactivation were obtained with serum diluted 1:2 with 50 mM Tris/HCl pH 7.4 at 53°. Coagulation of serum occurs at temperatures >60°. The activities of the pooled sera at zero time were 113 and 53 nmol·min^{-1}·ml^{-1} for DDVP-ase and Paraoxonase, respectively.

Table 1. Serum DDVP-ase and Paraoxonase activities as influenced by EDTA, cations and competitive substrates

	% Activity in presence of†:											
	EDTA (mM)‡				Cations (1 mM)§			Paraoxon (mM)‖		DDVP (mM)¶		
Substrate (mM)*	1	2	5	10	Cd^{2+}	Hg^{2+}	Ag^{+}	1	50	0.1	1	10
DDVP (10)	100,67	42	11,24	0,0	78	58	69	46,66	65	—	—	—
Paraoxon (2)	—	—	—	—	8	4	4	—	—	—	—	64,51
Paraoxon (1)	95	20	6	3	—	—	—	—	—	100	76	56,46

* Enzymes activities were measured as described in Materials and Methods.

† Percentages of DDVP-ase and Paraoxonase activities of appropriate controls. When two numbers are reported it means two experiments.

‡ Equimolar concentrations of Ca^{2+} restore completely the inhibited activities (tested for both enzymes when inhibited with 5 and 10 mM EDTA).

§ The metal was incubated with serum for 10 min at 37°. The following salts were found to be inhibitors: $CdSO_4$, $HgCl_2$, $AgNO_3$. Several other cations were tested without effects on DDVP-ase including: $Pb(NO_3)_2$, $BaCl_2$, $ZnSO_4$, $NiCl_2$, $CuSO_4$, $MnCl_2$, $FeSO_4$, $MgCl_2$.

‖ DDVP-ase activity was assayed after 60 min incubation with serum at pH 7.4 in 100 mM phosphate buffer at 37°. The method endpoint was NTE inhibition instead of AChE.

¶ Serum was incubated in the same conditions as ‖ and *p*-nitrophenol was measured after 30 min incubation.

reported by Reiner *et al.* [19], where they found a rate constant of inactivation similar for both substrates and concluded that the same esterase hydrolysed both paraoxon and DDVP.

Table 1 shows the comparative effects of EDTA, cations and competitive substrate on DDVP-ase and Paraoxonase. EDTA inhibits the two enzymes to a similar extent. Among several cations which have been tested only Cd^{2+}, Hg^{2+} and Ag^{+} inhibit DDVP-ase; these metals are, however, more potent inhibitors of Paraoxonase. Summation tests were performed to investigate the substrate specificity of DDVP-ase and Paraoxonase. The results shown in this Table indicate that since the rate of hydrolysis of the mixture is less than the sum, either only one enzyme is present or the two enzymes show overlapping specificity [1].

DDVP-ase activity was measured incubating DDVP (10 mM, 37°, 40 min) with fresh undiluted sera from a white caucasian population of 60 volunteers (34 males and 26 females), age 45 ± 24 years (x ± SD, range 3–91) and found to be 122 ± 22 nmol·min^{-1}·ml^{-1} (x ± SD). These values substantially agree with those reported by Reiner *et al.* of 11 μmoles·hr^{-1}·ml^{-1} of plasma [19]. DDVP-ase activity was normally distributed among these

individuals (Skewness and Kurtosis $P < 0.05$). No sex or age related differences were detected.

DISCUSSION

The enzymatic hydrolysis of DDVP leads to the formation of dichloroethanol and dimethylphosphate [14]. Limited *in vivo* data indicate that dichloroethanol is present in human urine after exposure to DDVP vapours [16]. Urinary dialkylphosphates are usually measured as an index of exposure to organophosphates, including dichlorvos [28]. These data suggest that the hydrolysis of DDVP is likely to be an important route of detoxification in man [18]. In certain species the main tissue where OP hydrolysis takes place is plasma [3], and human plasma is known as able to hydrolyse DDVP [17, 19].

Our data confirm that the disappearance of DDVP after incubation with human serum is due to enzymatic hydrolysis, and the kinetic constants are comparable to those reported by Reiner *et al.* [19] but not to those reported by Blair *et al.* [17]. Contribution of non enzymatic hydrolysis was irrelevant and suggestions of reversible non-specific binding of DDVP to plasma components [29] cannot be confirmed. Irreversible protein binding in serum is known to occur for several OPs, particularly at low concentrations including sarin [30], soman [31] and paraoxon [32]. Covalent binding does not contribute to the removal of DDVP from the blood, probably because of the relatively low concentration of covalent binding sites as it can be judged from the preincubation and albumin experiments.

There are considerable problems in the nomenclature of "A"-esterase because the substrate specificity of these enzymes is complex and far from being understood [1, 4, 7]. The results of the summation test are not conclusive in this regard since two interpretations are possible [1]: only one enzyme hydrolysing both substrates or two enzymes with an overlapping specificity for both paraoxon and DDVP. Our results suggest, however, that human serum Paraoxonase is different from DDVP-ase; the time-course of heat inactivation at 55° is obviously different for DDVP-ase and Paraoxonase, suggesting that different enzymes hydrolyse DDVP and paraoxon. Furthermore, there is a residual activity of Paraoxonase which is thermostable (about 10% of total activity) indicating that two enzymes might hydrolyse paraoxon. The sensitivity to temperature was also the criteria used by Chemnitius *et al.* to differentiate Paraoxonase from DFP-ase of hog liver [4]. The difference between serum and liver enzymes is also noteworthy. Hog liver Paraoxonase is for instance inactivated to 50% after 24 hr at 25° [4]. Results obtained by Dr. Maricruz Pellin in our laboratory suggest that human liver Paraoxonase is also rapidly inactivated at room temperature, while serum Paraoxonase is stable for 5 days at 37° (unpublished results).

The distribution of serum DDVP-ase in a caucasian population shows that the values are normally distributed, yet another difference with the serum Paraoxonase activity which shows a marked genetic polymorphism [24].

OP detoxicating esterases are thought to play an important role in the selective toxicity of these chemicals [4, 10, 33–35].

The presence in human serum of esterases which hydrolyse OPs suggest the possibility to monitor human exposure to OPs and to predict whether subjects with low serum activity are more susceptible to the toxic effects of a given OP [32, 36]. However, biomonitoring will be possible when other information becomes available [10]. Thus the organ distribution of "A"-esterases in man should be investigated, bearing in mind that it differs among species [3, 4]; the blood enzyme activity should be compared to that in other organs, and consequently the relevance of the blood enzyme established. The relative importance of this hydrolytic pathway versus other metabolic pathways for each OP has to be determined in man: it will be characteristic for a given OP, but other factors like the dose might be critical [37].

In conclusion studies in man on distribution of DDVP-ase and its comparison with other metabolic pathways will better enable conclusions on the role of "A"-esterases in determining DDVP toxicity to man.

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