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Formation and disposition of diethylphosphoryl-obidoxime, a potent anticholinesterase that is hydrolyzed by human paraoxonase (PON1)

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Abstract

The potential of pyridinium-4-aldoximes, such as obidoxime, to reactivate diethylphosphorylated acetylcholinesterases is not fully exploited due to the inevitable formation of phosphoryloximes (POX) with high anticholinesterase activity. Mono(diethylphosphoryl) obidoxime (DEP-obidoxime) was isolated for the first time showing remarkable stability under physiological conditions (half-life 13.5 min; pH 7.1; 37 °C). The half-life was considerably extended to 20 h at 0 °C, which facilitated the preparation and allowed isolation by HPLC. The structure was confirmed by mass spectrometry and the degradation pattern. DEP-obidoxime decomposed by an elimination reaction forming the intermediate nitrile that hydrolyzed mainly into the pyridone and cyanide. The intermediates were prepared and confirmed by mass spectroscopy. DEP-Obidoxime was an extremely potent inhibitor of human acetylcholinesterase approaching a second-order rate constant of 10^9 M^{-1} min^{-1} (pH 7.4; 37 °C). The nitrile and the pyridone were still good reactivators. In the presence of human plasma DEP-obidoxime was hydrolyzed into parent obidoxime. Calcium-dependence and sensitivity towards chelators, substitution pattern by other divalent cations and protein-modifying agents all pointed to human paraoxonase (hPON1) as the responsible protein with POX-hydrolase activity. Subjects, probably belonging to the homozygous $_{192}$arginine subtype, were virtually devoid of POX-hydrolase activity while a highly purified hPON1 of the homozygous $_{192}$glutamine subtype exhibited particularly high POX-hydrolase activity. Two parathion-poisoned patients with high and low POX-hydrolase activity responded well and poorly, respectively, to obidoxime treatment although the former patient had higher plasma paraoxon levels than the poor responder. Hence, the POX-hydrolase associated PON1 subtype may be another contributor that modulates pyridinium-4-aldoxime effectiveness.

Keywords: Phosphoryl oximes; Acetylcholinesterase; Paraoxonase; Obidoxime; Paraoxon; Species

1. Introduction

With the curtailed use of organochlorine insecticides, organophosphorus compounds (OPs) became extensively utilized throughout the world. Particularly in the hot climate of developing countries, pest control has been considered indispensable to meet food requirements. OPs have two major advantages over DDT and related organochlorine compounds: they are readily degraded and do not accumulate either in humans or in food chains. However, this advantage is paid for dearly, taking into account the much higher acute toxicity of OPs to mammals. The rate of accidental, suicidal and homicidal intoxications is high and the number of victims worldwide is estimated to exceed 300,000 per year [1].

OPs act primarily by inhibiting acetylcholinesterase (AChE), thereby allowing acetylcholine to accumulate at cholinergic synapses and disturbing transmission at parasympathetic nerve endings, sympathetic ganglia, neuromuscular endplates and certain CNS regions.

Abbreviations: AChE, acetylcholinesterase (E.C.3.1.1.7); DEP-obidoxime, (*O*-(*O*,*O*-diethylphosphoryl)-obidoxime; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); Ery-AChE, erythrocyte AChE; HLö7, 1-[[[4-(aminocarbonyl)-pyridinio]methoxy]methyl]-2,4-bis[(hydroxyimino)methyl]pyridinium dimethanesulfonate; MOPS, 3-(morpholino)propane sulfonate; *p*-OHMB, *p*-(hydroxymercuri)benzoate; PB, phosphate buffer; PMA, phenylmercury acetate; POX, phosphoryloxime; TMB-4, (1,1′-trimethylene bis[4-(hydroxyimino)methyl]pyridinium dibromide

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While atropine represents the mainstay in the treatment of dysfunctions at muscarine sensitive receptors, it fails at nicotine sensitive synapses. Here oximes (OX), acting as specific antidotes, may reactivate the inhibited AChE and improve the clinical picture.

The value of oximes, 4-pyridinium aldoximes in particular, is still a matter of controversy. Enthusiastic reports on outstanding antidotal effectiveness, substantiated by laboratory findings of reactivated AChE and improved neuromuscular transmission, contrast with many reports on disappointing results (for review see [2]). One reason for the partial ineffectiveness of 4-pyridinium aldoximes may be the formation of highly potent and sufficiently stable inhibitors during the process of reactivation. In fact, the reactivation reaction of the phosphylated AChE (EP) by an oxime (OX; Scheme 1) is reversible and the free phosphyloxime (POX) formed is in itself a powerful phosphylating agent of the liberated enzyme (E) (Scheme 1).

The identification of some phosphyloximes was achieved by synthetic means [3–7]. There is, however, a large body of data showing that POXs are also generated in reactivation media, and a recent study confirmed formation of ethoxy methyl-POX, arising during reactivation of the respective AChE conjugate with obidoxime and TMB-4 [8].

A high inhibitory potency of the POXs towards AChE has been demonstrated, which often exceeds the inhibitory potency of the parent OP by one or two orders of magnitude [4,9–12]. This holds particularly true for the product formed in the reaction of obidoxime with paraoxon-inhibited AChE. The resulting diethylphosphoryl-obidoxime (DEP-obidoxime) caused a significant deviation from the expected first-order reactivation kinetics and was particular prominent at high AChE concentrations as found, e.g. on erythrocyte membranes (Ery-AChE) of human blood. Curiously, such a behavior was not found when concentrated, paraoxon-inhibited Ery-AChE was incubated with obidoxime in the presence of one of the author's plasma (DK) [13]. Besides BChE as a scavenger, we identified some POX-sequestering principle in human plasma, which was not inhibited by OPs, was not albumin and required Ca^{2+} ions for enzymatic activity. Such a behavior was reminiscent of paraoxonase/arylesterase. To rule out any interference of BChE, plasma samples were pretreated with soman that inactivates BChE irreversibly [14]. Still, this plasma retained its activity to inactivate DEP-obidoxime.

Isolation of DEP-obidoxime failed until now, since its stability was considered too low [8]. Earlier experiments in this laboratory, however, [13] caused some doubt on this assumption and suggested a half-life in the order of 10 min under physiological conditions. This half-life was expected to be markedly longer at lower temperatures, e.g. 10 °C, where the biphasic reactivation kinetics indicated a half-life of hours. Since we were interested in the POX-destroying activity of human plasma [13], we tried to isolate DEP-obidoxime in order to facilitate the quantitative determination of the enzyme activity. To this end, we took advantage of the supposed stability of DEP-obidoxime at low temperatures and succeeded in isolation, characterization and long-term storage of the desired DEP-obidoxime.

This paper deals with the AChE-inhibiting activity of DEP-obidoxime, its stability and non-enzymatic degradation as well as with the enzymatic disposition by a plasma enzyme that was characterized as a POX-hydrolase, which is most probably identical with the polymorphic $_{192}$glutamine subtype of human paraoxonase 1 (hPON1, AA).

(EP) + (OX) ⇌ (E) + (POX)

Scheme 1. Simplified reaction scheme of reactivation of a phosphorylated enzyme (EP) by an oxime (OX) with formation of free enzyme (E) and the powerful inhibiting phosphoryloxime (POX), leading to a topochemical equilibrium.

2. Materials and methods

2.1. Chemicals

Ion pairing reagents (PIC B7® and PIC A® low UV) for HPLC-analysis were obtained from Waters (Milford, USA). All other chemicals were procured from Sigma (Deisenhofen, Germany), E. Merck KGaA, (Darmstadt, Germany) or from suppliers as mentioned previously [13].

2.2. Enzyme preparations

2.2.1. Acetylcholinesterase (AChE) from electric eel

The AChE stock solution (type III; 950 U/mg protein; Sigma, Deisenhofen, Germany) was diluted with 10 volumes of 0.1 M sodium 3-(morpholino)propane sulfonate (MOPS) buffer, pH 7.4, containing 0.02% gelatine, before inhibition took place with paraoxon ethyl (0.2 μM; Dr. Ehrenstorfer, Augsburg, Germany) at 37 °C for 40 min. The surplus of paraoxon was adsorbed on MeOH-conditioned Baker Bond C18, (Baker, Deventer, The Netherlands) followed by centrifugation.

2.2.2. Paraoxon-inhibited human red cell AChE

Red blood cells in freshly drawn heparinized human blood (blood group 0) were washed 4-times with 0.1 M sodium phosphate buffer, pH 7.4, (PB) to give a final concentration of 150 g Hb/L and incubated with 0.2 μM paraoxon at 37 °C for 35 min, followed by five washings with PB at 6 °C and adjustment to 150 g Hb/L. Hemolysis was achieved by freezing at −20 °C and thawing in ice–water.

2.2.3. Soman-inhibited human plasma

Heparinized human plasma from various donors was incubated with 10 μM soman at 37 °C for 30 min whereupon BChE was completely inhibited. Subsequent dialysis against the desired buffer was performed at 6 °C overnight to remove excess soman and to adjust the pH.

2.2.4. Isolation of paraoxonase and POX-hydrolase by affinity chromatography of soman-pretreated plasma on Cibacron Blue 3GA

According to the method of Gan et al. [15], affinity chromatography on Cibacron Blue 3GA was employed for binding the paraoxonase–HDL-complex of human plasma exhibiting high POX-hydrolase activity (DK), followed by elution with 50 mM Tris–HCl/1 mM $CaCl_2$/5 μM EDTA/ 0.1 M Na-desoxycholate, pH 8.0, at 6 °C within 7 h. Arylesterase activity was determined in the fractions. For determination of POX-hydrolase activity, the fractions with arylesterase activity were pooled, dialyzed against 1 mM PB/1 mM $CaCl_2$ at 6 °C, followed by lyophilization, resolubilization in distilled water and repeated dialysis against PBS, pH 7.4, and $CaCl_2$ (1 mM). The resulting specific activities of paraoxonase and POX-hydrolase were increased about 30fold (yield 14%). Human PON1, type AA was a generous gift from Prof. P. Masson (La Tronche, France). Animal plasma samples were obtained from Charles River (Sulzfeld, Germany) and the German Primate Center (Göttingen, Germany).

2.3. Methods

2.3.1. Enzyme activity determinations

2.3.1.1. Determination of AChE activity in a modified Ellman assay [16]. AChE activity was determined in the presence of 0.45 mM acetylthiocholine and 0.3 mM DTNB in 0.1 M sodium phosphate buffer, pH 7.4, at 37 °C. To reduce hemoglobin interference, determination of the indicator chromophor was performed at 436 nm. To avoid interference with matrix SH groups, the hemolysate was preincubated with DTNB for 10 min, which did not influence AChE activity. This step could be omitted when electric eel AChE was used. If not otherwise indicated, the paraoxon-inhibited, temperature-equilibrated enzyme preparations were incubated with 10 μM obidoxime and plasma samples at pH 7.4 for various time intervals before small aliquots were transferred in the cuvettes to start the modified Ellman reaction at 37 °C.

2.3.1.2. Determination of POX-hydrolase activity in soman-pretreated plasma.

- *Method A: reactivation of AChE*: Paraoxon-inhibited human hemolysates (150 g Hb/L) were incubated with the same volume of soman-pretreated plasma and/or buffer medium along with 10 μM obidoxime before AChE activity was determined. For calculation of POX-hydrolase activity the following equation was employed (for details see [13]): %POX-hydrolase activity = $100 \times (A_+ - A_-)/(A_0 - A_-)$, with A_+ and A_- standing for AChE activity in the presence and absence of plasma, and A_0 for the activity of a diluted AChE sample (1:100), when POX did not interfere with reactivation. This test gives arbitrary activity and can be used for comparisons only. To minimize interference of the reaction media with protein-modifying agents and selected cations, buffers had to be adapted. Hence, buffer solutions, pH values and reaction temperatures were not always consistent, but all comparisons were made under identical conditions each [17].
- *Method B: quantification of generated obidoxime*: DEP-obidoxime (6–8 μM) was hydrolyzed enzymatically at 37 °C and pH 7.4 by incubation with soman-pretreated plasma (dialyzed against 25 mM Tris–HCl/1 mM $CaCl_2$/0.15 M NaCl/0.1% Triton X-100, pH 8.0). Obidoxime formed was quantified by HPLC analysis (method C) and the rate of its generation was used to quantify POX-hydrolase activity (nmol/min).

2.3.1.3. Determination of paraoxonase activity. The release of *p*-nitrophenol from paraoxon (2 mM) was measured at 405 nm ($\varepsilon = 16 \times 10^3\ M^{-1}\ cm^{-1}$). The assay was carried out at 37 °C both in 0.1 M Tris–HCl/ 1 mM $CaCl_2$ (pH 8.5) and in 0.1 M Tris–HCl/ 1 mM $CaCl_2$/1 M NaCl (pH 8.5). The quotient of both paraoxonase-activities was used for determination of the NaCl-factor as a coarse means of phenotyping of paraoxonase [18].

2.3.1.4. Determination of arylesterase activity. The release of phenol from phenyl acetate (4 mM) was determined at 270 nm ($\varepsilon = 1.45 \times 10^3\ M^{-1}\ cm^{-1}$) in 50 mM Tris–acetate/0.3 mM $CaCl_2$ at pH 7.4 and 37 °C [19].

2.3.1.5. Determination of enzyme activities in the presence of Tb^{3+}. To avoid reduction of the availability of Tb^{3+}, phosphate and tryptophan-containing material such as hemoglobin and albumin had to be avoided. Therefore, soman-pretreated, Cibacron-purified plasma was dialyzed against 0.1 M MOPS buffer, pH 7.4, containing 1 mM $CaCl_2$ and reacted with 100 μM $TbCl_3$. POX-hydrolase activity was determined in the presence of 100 μM Tb^{3+} by incubation of paraoxon-inhibited electric eel AChE, stabilized by 0.02% gelatine, with 1 μM obidoxime in 0.1 M MOPS/1 mM Ca^{2+} at 10 °C for 15 min.

2.3.1.6. Determination of POX-hydrolase activity in the presence of carbodiimides. Soman-pretreated, Cibacron-purified plasma was reacted with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (1 mM) or *N*,*N*′-dicyclohexylcarbodiimide (2 mM) at 37 °C, followed by dialysis against 1 mM PB/1 mM Ca^{2+}, pH 7.4 and lyophilization. Finally, this preparation was incubated with paraoxon-inhibited electric eel AChE in the presence of 0.02% gelatine and 1 μM obidoxime at 10 °C for 20 min. Controls without carbodiimide pretreatment were run by the same procedure.

2.3.1.7. Determination of enzyme activities in the presence of phenylmercury acetate (PMA) and p-(hydroxymercuri) benzoate (p-OHMB). Soman-pretreated, Cibacron-puri-

fied plasma (dialyzed against 0.1 M MOPS/1 mM Ca^{2+}, pH 7.4) was incubated with *p*-OHMB (100 μM) or PMA (50 μM) at 37 °C for 30 min. For determination of POX-hydrolase activity, an aliquot of this preparation was incubated with paraoxon-inhibited electric eel AChE and obidoxime (1 μM) at 10 °C for 20 min.

2.3.1.8. Inhibition kinetics of human Ery-AChE by paraoxon and DEP-obidoxime. In these experiments, erythrocyte SH groups were masked by preincubation with DTNB, in order to minimize further inhibition before AChE activity could be measured. To reduce POX-decomposition the inhibition kinetics was followed at 10 °C and not at 37 °C, while AChE activity was determined at 37 °C. Preincubation of AChE with DTNB did not affect the enzyme activity.

2.3.1.9. Generation and isolation of diethylphosphoryl-obidoxime (DEP-obidoxime) and its decomposition products.

- *DEP-obidoxime*: *Caution: DEP-obidoxime is an extremely potent anticholinesterase agent!* Since it was our policy not to prepare and handle hazardous amounts of highly toxic organophosphates, we prepared DEP-obidoxime in a one-pot reaction and agreed upon a route with low yield. To this end obidoxime was allowed to react with equimolar paraoxon that produced DEP-obidoxime at a yield of about 1%. Obidoxime dichloride (50 mM) was diluted in 10 mM sodium veronal buffer, pH 7.5, and adjusted to pH 7.5 with NaOH. Paraoxon (15 mM) was suspended in the same buffer. The final incubation mixture consisted of 10 mM obidoxime and 10 mM paraoxon in 10 mM veronal, pH 7.5, and was allowed to react overnight on ice. Subsequently, 0.1 mL aliquots were separated by HPLC (method A) to collect DEP-obidoxime at Rt 18 min.
- *Obidoxime mononitrile*: For generation of obidoxime mononitrile, isolated DEP-obidoxime (in the mobile phase) was adjusted to pH 6.9 with NH_4HCO_3 and allowed to react at 0 °C for 8.5 h, followed by incubation at 37 °C for 7 min (at the end of the incubation the pH was 8.3). To avoid further decomposition of the nitrile, the pH was adjusted to 5.2 with 1 M HCl, followed by lyophilisation. After dissolution in H_2O, the mononitrile was isolated by HPLC (method B; Rt 6.5 min).
- *Obidoxime monopyridone*: To reduce formation of obidoxime monocarboxamide (see Section 4) conditions were chosen to produce first the mononitrile followed by cyanide elimination and formation of the monopyridone. To this end, the following procedure was adopted: Isolated DEP-obidoxime (in the mobile phase) was brought to pH 7.4 with ammonia and incubated for 16 h on ice and subsequently at 37 °C for 4.5 h, before concentrating the sample (speed vac) and isolating the monopyridone by HPLC (method B; Rt 13.4 min).

2.3.1.10. Quantification of DEP-obidoxime and its decomposition products.

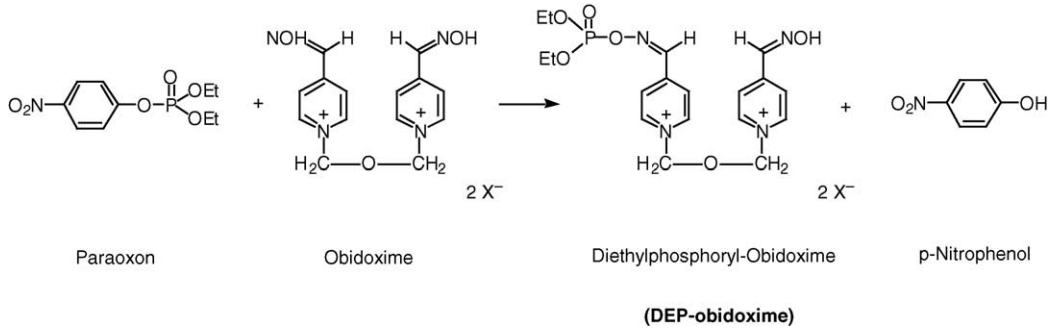
- *DEP-obidoxime*: The concentration of DEP-obidoxime was determined indirectly by the liberation of obidoxime, which was found after complete enzymatic hydrolysis of isolated DEP-obidoxime upon incubation with undiluted soman-pretreated plasma (DK) at 37 °C and pH 7.4. Quantification of obidoxime was achieved by HPLC (method C). Thereby, DEP-obidoxime was quantified and its molar extinction coefficient estimated by UV-spectroscopy [20].
- *Obidoxime*: For identification and quantification of obidoxime in the presence of proteins, the sample (150 μL) was precipitated with 1 M trichloroacetic acid (45 μL), and the supernatant (100 μL) mixed with 50 μL PIC B7-reagent (Waters-Millipore) containing 12% NH_3 (25%). HPLC-analysis with 50 μL aliquots was performed with method C.
- *Obidoxime monopyridone and obidoxime mononitrile*: The UV absorption of these obidoxime derivatives in alkaline medium was assumed to be half the absorption of obidoxime, corresponding to $\varepsilon_{354\ nm} = 23.4 \times 10^3\ M^{-1}\ cm^{-1}$, caused by the remaining monooximate function of the bispyridinium compound. This procedure enabled standardization of the HPLC areas (method D).
- *Cyanide*: This product was determined photometrically after derivatization by the Zincke–Koenig reaction in a modification [21] of the method of Nagashima [22]. The sample was incubated at pH 7.4 and 37 °C in a Fernbach vessel, while cyanide was freed from interfering compounds by micro diffusion into the alkali trap (0.1 M NaOH).

2.3.2. HPLC methods

DEP-obidoxime and the degradation products were analyzed by HPLC using a Hitachi L-6200 pump (Merck). Peaks were quantified by peak integration with an SPD M6A UV-VIS diode-array detector (Shimadzu) coupled with a personal computer Compaq ProLinea 4/66; software Shimadzu Class LC-10 1992. Identification was achieved by comparison of retention times and UV spectra with authentic standards or compounds isolated by HPLC.

For chromatography with ion-pairing, 5% of the ion-pairing reagent was added to the sample to prevent peak broadening.

2.3.2.1. Method A. For the isolation of DEP-obidoxime, chromatography on LiChroCart Superspher 60, RP-select B (3 mm i.d. × 125 mm; 4 μm (Merck)) without ion-pairing reagent was applied. The mobile phase consisted of 10 mM ammonium acetate pH 4.5 (A) and the same buffer containing 50% MeOH (B). Samples were eluted with a linear gradient increasing from 0% B to 40% B in 20 min, 40% B for 10 min, linear increase to 100% B in 10 min, 100% B for 10 min, and return to 0% B in 5 min. The detection wavelengths were set at 260 and 300 nm; the



Scheme 2. Chemical structures of the compounds involved in the preparation of diethylphosphoryl obidoxime (DEP-obidoxime).

retention time of DEP-obidoxime was 18 min at a flow rate of 0.6 mL/min.

2.3.2.2. Method B. To get samples for MS analysis, obidoxime mononitrile and obidoxime monopyridone were chromatographed on LiChroCart LiChrospher 60, RP-select B (4 mm i.d. × 250 mm; 5 μm (Merck)) under the same elution conditions as described for Method A.

2.3.2.3. Method C. For verification and quantification, obidoxime was analyzed on LiChroCart LiChrospher 60, RP-select B (4 mm i.d. × 125 mm; 5 μm (Merck) at a flow-rate of 1.2 mL/min and detection at 285 nm; the mobile phase for ion-pairing chromatography consisted of PIC B7:PIC A low UV:CH_3CN:H_2O = 4:0.35:4:91.65 (v/v).

2.3.2.4. Method D. DEP-obidoxime, obidoxime mononitrile and obidoxime monopyridone were analyzed (detection and quantification) on LiChroCart LiChrospher 60, RP-select B (4 mm i.d. × 125 mm; 5 μm (Merck)) at a flow-rate of 1.2 mL/min with detection at 260 and 300 nm. The mobile phase consisted of aqueous 4% PIC B7, 0.35% PIC A low UV. Gradient elution was started with 2% CH_3CN, which was linearily increased to 12% within 20 min, held at 12% for 5 min and increased to 16% within another 5 min before return to initial conditions within 5 min.

2.3.3. Mass spectrometry

Positive electrospray ionization mass spectrometry (ESI-MS) was performed with a quadruple ion trap tandem mass spectrometer (LCQ DUO, ThermoQuest, Finnigan). The samples were introduced with a syringe pump into the apparatus (flow = 25 μL/min) and analyzed by full-scan MS mode. The parameters were set as follows: capillary temperature of 170 °C and 200 °C, sheath gas flow of 20 (arbitrary units); obidoxime mononitrile was analyzed with LC-MS by elution from LiChroCart LiChrospher 60, RP-select B (4 mm i.d. × 250 mm; 5 μm (Merck) with 10 mM ammonium formiate/50% MeOH, pH 6.0; MS/MS spectra were obtained by ion trap collision-induced dissociation.

3. Results

3.1. DEP-obidoxime

3.1.1. Generation and isolation of DEP-obidoxime

Because the presumably more reactive diethyl phosphorofluoridate was not available to us we tried to generate DEP-obidoxime directly by reaction of paraoxon with obidoxime (Scheme 2). In order to prevent formation of bis-diethylphosphoryl-obidoxime we hesitated to add paraoxon in excess [6]. Since concentrated buffers, especially phosphate buffer, are known to reduce the yield of POX [6,7,23], the assay was buffered only slightly. Best yields of DEP-obidoxime were obtained by reaction of equimolar amounts of obidoxime and paraoxon (10 mM each) in 10 mM veronal at pH 7.5. Lower pH-values retarded the reaction and increased the amount of by-products. Higher pH-values accelerated the reaction by increasing the amount of the oximate–anion, but resulted in more rapid product decomposition (veronal pH 8.25). The stability of DEP-obidoxime was substantially higher at lower temperature, so the incubation was performed at 0 °C over-

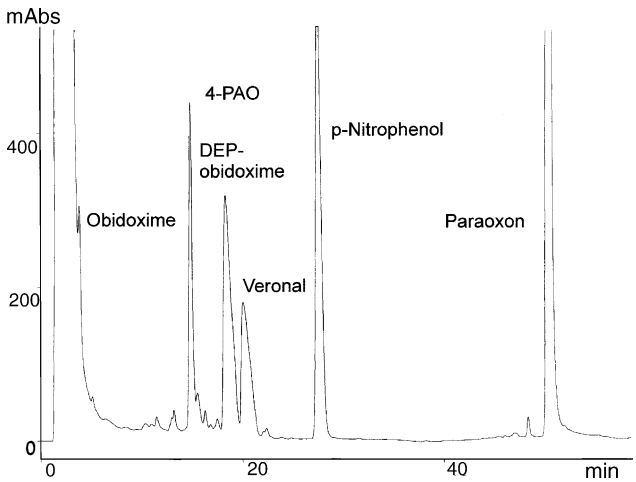


Fig. 1. HPLC-chromatogram of an incubate of obidoxime and paraoxon for the isolation of DEP-obidoxime by method A, detection wavelength 260 nm. 4-PAO (4-pyridine aldoxime) was an impurity of the obidoxime preparation.

night. Subsequently, portions of 0.1 mL of the reaction mixture were separated by HPLC (method A). Fig. 1 shows a chromatogram with the primary products, DEP-obidoxime (Rt 18 min) together with 4-pyridine aldoxime (4-PAO, an impurity of the obidoxime preparation), *p*-nitrophenol and veronal.

The yield of DEP-obidoxime was 1%; it was stable at −70 °C and pH 4.5 for several weeks.

3.1.2. Fate of DEP-obidoxime

DEP-obidoxime disappeared completely during incubation with human plasma at pH 7.4, while diethyl phosphoric acid and obidoxime were generated by hydrolytic cleavage. This mechanism justified the name POX-hydrolase. After incubation of DEP-obidoxime without plasma at pH 7.4, the inhibitor also disappeared, but the degradation pattern was completely different and virtually no obidoxime was generated.

3.1.3. Characterization of DEP-obidoxime and its degradation products

The UV spectrum of DEP-obidoxime at pH 4.5 showed an absorbance maximum at 273 nm (obidoxime 285 nm). At pH 9.2 the absorbance at 273 nm was shifted bathochromically to 354 nm. Such a shift is typical of the transition of 2- and 4-pyridiniumaldoximes into their betaine structure [24,25]. This behavior pointed to a free oxime function as expected for mono(diethylphosphoryl)-obidoxime. In fact, LC-MS confirmed the structure with the molecular peak *m/z* 212 and its fragments of *m/z* 135 (mononitrile) and *m/z* 155 (diethylphosphoric acid). For more details of the MS and MS/MS analysis the reader is referred to Kiderlen [26].

Incubation of DEP-obidoxime at pH 7.4 on ice without plasma resulted in the production of a more hydrophilic product, without detection of cyanide, pointing to obidoxime mononitrile as the primary degradation product. At pH 4.75, this component revealed a maximum at 282 nm, at pH 11 the oximate-absorbance maximum at 354 nm appeared and the complete decomposition of the mononitrile was confirmed by HPLC-analysis. LC-MS analysis of the presumed mononitrile gave the molecular peak *m/z* 135.

During incubation of the obidoxime mononitrile at pH 7.4 and 37 °C the degradation to another hydrophilic product was observed, accompanied by cyanide formation, strongly suggesting a pyridone structure. The supposed monopyridone at pH 4.5 revealed an absorbance maximum at 267 nm with a shoulder at 285 nm that was attributed to an oxime function. At pH 10.5, the oximate absorption at 354 nm appeared, which was reversible after acidification as expected for the obidoxime monopyridone-derivative. LC-MS analysis confirmed the structure with the mole-

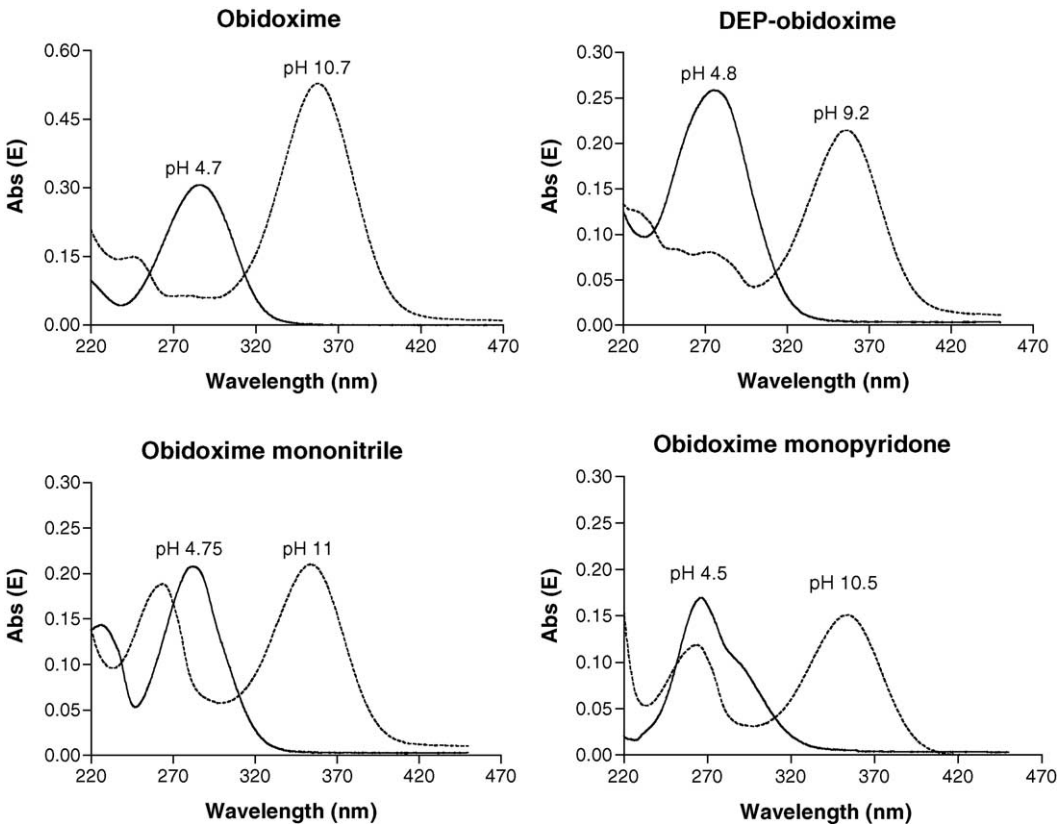
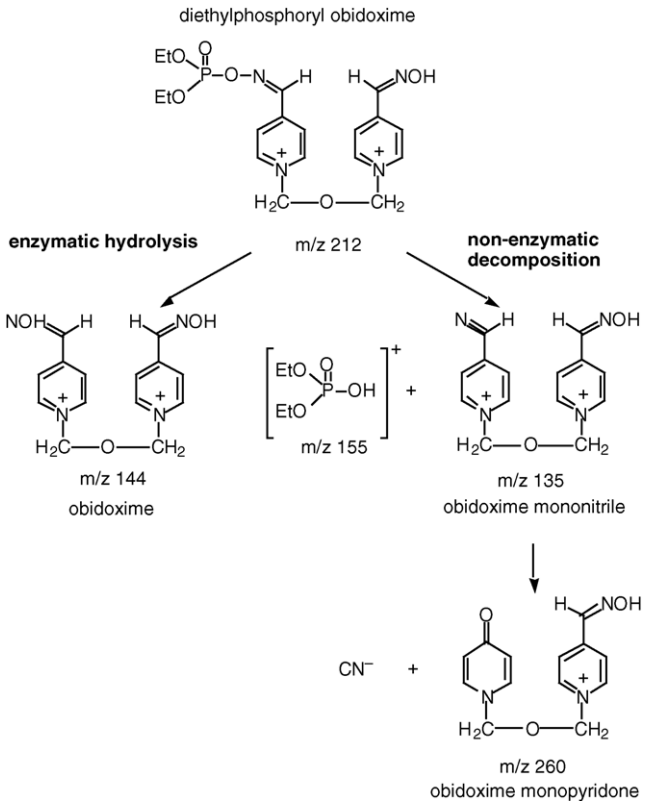


Fig. 2. UV-spectra of obidoxime (10 μM), mono-DEP-obidoxime (9 μM), obidoxime mononitrile (8.9 μM) and obidoxime monopyridone (6.9 μM) at acid and alkaline pH. Spectra were recorded in 10 mM NH_4 acetate containing 20% MeOH (HPLC solvent). Alkaline shift was reversible with obidoxime and obidoxime monopyridone, only.



Scheme 3. Enzymatic and non-enzymatic degradation pathways of diethylphosphoryl obidoxime and the corresponding mass units as confirmed by mass spectrometry.

cular peak *m/z* 260. The UV-spectra of obidoxime and its derivatives are shown in Fig. 2.

Scheme 3 summarizes the observations: incubation of DEP-obidoxime at pH 7.4 resulted in the generation of the mononitrile by an eliminating mechanism, followed by further decomposition into cyanide and the monopyridone. In the presence of soman-pretreated plasma, however, DEP-obidoxime was hydrolyzed enzymatically into obidoxime and diethylphosphoric acid.

3.1.4. Quantification of DEP-obidoxime and its degradation products

For quantification, DEP-obidoxime was incubated with soman-pretreated plasma at pH 7.4 and 37 °C to ensure complete hydrolysis, followed by determination of the obidoxime concentration by HPLC-analysis. The concentration of obidoxime found was equated with the concentration of DEP-obidoxime.

UV-analysis of obidoxime monopyridone at pH 11 was used for its quantification. It was assumed that the UV-absorption of the single pyridinium-4-aldoximate function in obidoxime monopyridone was half the absorption of obidoxime with its two pyridinium-4-aldoximate functions. Hence, its molar extinction coefficient was calculated to be $23.4 \times 10^3\ M^{-1}\ cm^{-1}$. From this the extinction coefficients of the alkali-labile precursors could be derived (Table 1).

3.1.5. Influences on the decomposition pattern of DEP-obidoxime

DEP-obidoxime, incubated in 0.1 M MOPS and 1 mM $CaCl_2$, pH 7.4, at 37 °C decomposed completely, producing 49% cyanide and 80% of the obidoxime monopyridone (percent of theory). Hydrolysis of DEP-obidoxime into obidoxime was not observed. Incubation of DEP-obidoxime in the same buffer at 0 °C produced nearly exclusively obidoxime mononitrile (95%). The half-lives for degradation of DEP-obidoxime and production of obidoxime-mononitrile were comparable. Obidoxime-mononitrile decomposed readily by further incubation at 37 °C into 67% obidoxime monopyridone ($n = 4$; S.D. = 6) and 52% cyanide ($n = 4$; S.D. = 7). Besides, a second hydrophilic product was detected with an estimated yield of 27% ($n = 2$). The UV spectrum at pH 4.5 showed a maximum of absorbance at 282 nm, compatible with a monoisonicotinamide or monoisonicotinic acid derivative of obidoxime. The balance was incomplete, especially with regard to the yield of cyanide.

A completely different degradation pattern resulted after incubation of DEP-obidoxime at 37 °C with the soman-pretreated Cibacron-purified plasma of one of the authors (DK). Obidoxime was generated quantitatively, while cyanide was only detectable in traces. Here, DEP-obidoxime was hydrolyzed before being degraded via the nitrile pathway.

The stability of DEP-obidoxime, approx. 5 μM, was tested under near-physiological conditions in Ringer-phosphate solution, pH 7.1 and 37 °C, followed by HPLC analysis. The compound showed a mono-exponential decay with a half-life of 13.5 min. The stability increased markedly when the reaction temperature was lowered. The Arrhenius plot shown in Fig. 3 indicates an apparent energy of activation of 85 kJ/mol (Ringer-phosphate solution, pH 7.1). Phosphyloximes are known to decompose via two competitive pathways, a cyclic elimination reaction and a

Table 1
Spectroscopic data of obidoxime, DEP-obidoxime, obidoxime mononitrile and obidoxime monopyridone

	Obidoxime[a]	Mono-DEP-obidoxime	Obidoxime mononitrile	Obidoxime monopyridone
λ_{max} (nm) (ε ($mM^{-1}\ cm^{-1}$))				
pH 4–5	285 (34.7)	273 (31.6)	282 (22.4)	267; 285 (S) (24.5)
pH 9–11	354 (46.8)	Degradation to pyridone	Degradation to pyridone	354 (23.4)

If not otherwise stated, spectra were recorded in 10 mM NH_4 acetate, containing 20% MeOH.
[a] In water, from [20]; S: shoulder.

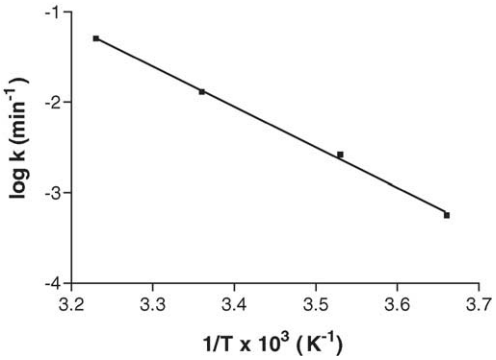


Fig. 3. Arrhenius plot of the decomposition rate constants (min^{-1}) of DEP-obidoxime vs. the reciprocal absolute temperature. DEP-obidoxime, approximately 5 μM, was dissolved in Ringer-phosphate solution, pH 7.1, and incubated at 37, 25, 10 and 0 °C. The decay of DEP-obidoxime was followed by HPLC and exhibited first-order kinetics.

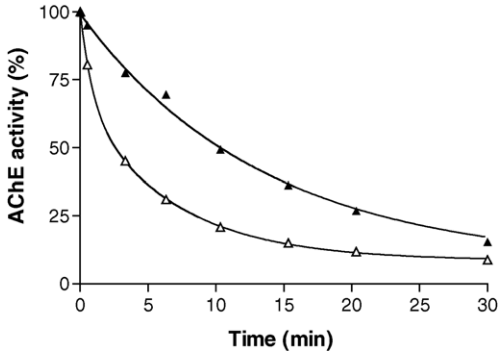


Fig. 4. Inhibition of human Ery-AChE (final concentration 15 g Hb/L) by 200 nM paraoxon (▲) und 2 nM DEP-obidoxime (△) in 0.1 M PB, pH 7.4, at 10 °C. Erythrocyte suspensions were preincubated with DTNB, before the inhibitor was added and incubated at 10 °C. After various time intervals an aliquot was taken and AChE activity determined at 37 °C by the modified Ellman method.

nucleophilic attack on phosphorus to regenerate the oxime. A 4-pyridiniumaldoxime-induced elimination reaction has been described for the conjugate of sarin, whereas the 4-pyridiniumaldoxime-induced nucleophilic attack, leading to re-synthesis of the POX was not observed [23]. In order to get an impression whether therapeutic oxime concentrations could affect DEP-obidoxime degradation, we tested obidoxime and TMB-4, 10 μM each. It turned out that the decay at 25 °C was not accelerated by the presence of obidoxime or TMB-4.

3.1.6. Inhibitory potency of DEP-obidoxime on human Ery-AChE

In order to prevent the rapid degradation of DEP-obidoxime, the reaction temperature was lowered to 10 °C. Human Ery-AChE (15 g Hb/L, approximately 0.3 nM AChE) in 0.1 M PB, pH 7.4, was pretreated with DTNB to mask matrix SH groups and was incubated at 10 °C with either 2 nM DEP-obidoxime or 200 nM paraoxon before determination of AChE activity. (By diluting the mixture in the cuvette (1:32), further inhibition of the enzyme during measurement was sufficiently retarded to allow correct determination.)

As shown in Fig. 4, the inhibition of AChE by DEP-obidoxime (2 nM) proceeded faster than by paraoxon (200 nM). The bimolecular inhibition rate constants were calculated at 1.2×10^8 M^{-1} cm^{-1} for DEP-obidoxime and 3.5×10^5 M^{-1} cm^{-1} for paraoxon; this difference by a factor of 350 corresponds to the report of De Jong and Ceulen, who generated DEP-obidoxime in situ and determined the inhibition kinetics on bovine Ery-AChE [11].

3.1.7. Reactivating potencies of obidoxime mononitrile and obidoxime monopyridone

The reactivating properties of the degradation products, obidoxime mononitrile and obidoxime monopyridone, were compared with obidoxime. Paraoxon-inhibited electric eel AChE in 0.1 M MOPS/1 mM Ca^{2+}, pH 7.4, was reacted with the compounds at 37 °C. Fig. 5 illustrates that

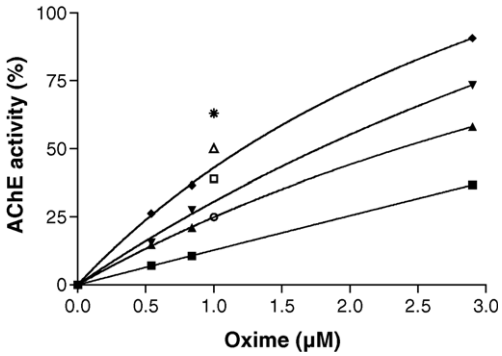


Fig. 5. Reactivation potency of obidoxime mononitrile. Paraoxon-inhibited electric eel AChE was incubated with various concentrations of obidoxime mononitrile (closed symbols) and with 1 μM obidoxime (open symbols), respectively, in 0.1 M MOPS/0.02% gelatine, pH 7.4, at 37 °C. After various time intervals, AChE activity was determined in 0.1 M MOPS/0.02% gelatine, pH 7.4, at 37 °C. Obidoxime mononitrile 2 min (■), 6 min (▲), 10 min (▼), 15 min (◆); obidoxime 2 min (○), 6 min (□), 10 min(△) and 15 min (*).

for comparable reactivation the incubation time with obidoxime mononitrile was 2.5–3 times longer than with obidoxime. Equieffective reactivation with obidoxime monopyridone required four times longer reaction times than with obidoxime.

3.2. POX-hydrolase

3.2.1. Isolation of POX-hydrolase

Paraoxonase/arylesterase as an HDL-associated glycolipoprotein was isolated by affinity-chromatography on Cibacron Blue 3GA, whereby the paraoxonase-HDL-complex was first adsorbed on Cibacron Blue 3GA, resulting in removal of albumin and other proteins, before desorption of the paraoxonase-HDL-complex by Na-desoxycholate ensued [15]. This method was exploited for isolation of POX-hydrolase in soman-pretreated plasma, with complete recovery of POX-hydrolase activity in the fractions containing paraoxonase/arylesterase activity. The fact of

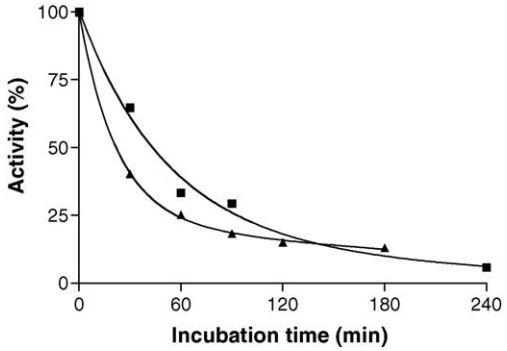


Fig. 6. Influence of calcium deprivation on the activities of POX-hydrolase and arylesterase. Purified plasma was lyophilised, dialyzed against 10 mM PB/1 mM Ca^{2+}/0.9% NaCl, pH 7.4, and incubated with EDTA (2 mM) at 37 °C for the time intervals indicated. Thereafter, aliquots were incubated with 1 mM excess Ca^{2+} (over EDTA) at 10 °C for 20 min before POX-hydrolase (method A; ■) and arylesterase (▲) determination.

the simultaneous elution of paraoxonase/arylesterase and POX-hydrolase raised the question of a common protein. For this purpose the effect of various modifiers on the Cibacron-purified preparation was examined based upon known characteristics of paraoxonase/arylesterase [27–29].

3.2.2. EDTA and the influence of various cations

Ca^{2+}-ions are necessary for both catalytic activity to hydrolyze arylesters and for enzyme stability [30]. As shown in Fig. 6, incubation with EDTA (2 mM) resulted in a progressive inhibition of arylesterase and POX-hydrolase and also in the inhibition of paraoxonase (not shown). It appeared that the inactivation proceeded in a biphasic way.

In order to characterize the cation sensitivity, soman-pretreated plasma was dialyzed against 0.1 M MOPS buffer/1 mM Ca^{2+} pH 7.4 at 4 °C. Calcium ions were removed by flash chromatography of the dialyzed material (2 mL) on chelex 100-resin (1 mL) equilibrated with Ca^{2+}-free MOPS buffer (4 °C) immediately before incubation of samples with various ions for 1 h at 37 °C. Aliquots (1/30 and 1/100) were assayed for POX-hydrolase and arylesterase activity.

Table 2 shows that the activities of arylesterase and POX-hydrolase are influenced comparably: Sr^{2+} did stabilize both activities, Ba^{2+} and Mn^{2+} did stabilize arylesterase but not POX-hydrolase. Mg^{2+} and Ni^{2+} did slightly inhibit both enzyme activities which remained stable over 1 h. Incubation with Zn^{2+}-ions resulted in strong inhibition, which was reversed by addition of EDTA (under conditions: $[Zn^{2+}] < [EDTA] < [Ca^{2+}]$). This effect pointed to reversible inhibition. (It should be noted that the final concentrations of the salts examined was 1.5 μM in the arylesterase and 400 μM in the POX-hydrolase assay. While AChE was not affected by these salts, their influence on POX-hydrolase in the assay cannot be excluded.)

The lanthanide and Ca^{2+}-analogue Tb^{3+} was suitable to detect the involvement of tryptophan, which may play some role in the active site of paraoxonase/arylesterase [28]. Paraoxonase/arylesterase was reversibly inhibited by Tb^{3+} (100 μM) even in the presence of Ca^{2+} (1 mM). This inhibition could be reversed by EDTA as long as Ca^{2+} was in excess. Also the enhanced reactivation of paraoxon-inhibited electric eel AChE by Cibacron-purified plasma was nearly completely abolished by the addition of 100 μM Tb^{3+}.

3.2.3. Influence of protein modifying reagents

Preincubation of Cibacron-purified enzyme with 0.2 mM *N*-bromosuccinimide, another reagent for tryptophan-modification [29,31], in 0.1 M MES/0.1 mM Ca^{2+}, pH 6.5 at 37 °C for 15 min, followed by addition of 0.2 mM tryptophan to scavenge free NBS, resulted in a decrease of arylesterase-activity to 7% and of POX-hydrolase to 11%.

Carbodiimides like dicyclohexylcarbodiimide (DCC) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) are reagents for modification of peptide-bonded aspartate and

Table 2
Influence of various salts (1 mM) on arylesterase and POX-hydrolase activities

	$CaCl_2$	$MnCl_2$	$SrCl_2$	$BaCl_2$	$MgCl_2$	$NiSO_4$	$ZnSO_4$
Arylesterase	Stable	Stable	Stable	Stable	Slightly inhibited	Slightly inhibited	Inhibited
POX-hydrolase	Stable	Inactive	Stable	Inactive	Slightly inhibited	Slightly inhibited	Inhibited

Table 3
Influence of various modifiers on paraoxonase/arylesterase and POX-hydrolase activity

Inhibitor	% Paraoxonase/arylesterase	% POX-hydrolase	Reaction with
EDTA	No activity	No activity	e.g. Ca^{2+}
Tb^{3+}	No activity	No activity	Tryptophan
N-bromosuccinimide (NBS)	7%	11%	Tryptophan
Dicyclohexylcarbodiimide (DCC) or 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide (EDC)	30%	30%	Aspartate glutamate
Phenylmercury acetate (PMA) or *p*-(hydroxymercuri)benzoate (*p*-OHMB)	No activity	No activity	SH groups

Table 4
Arylesterase and paraoxonase activities in different subjects

Subject	Aryl-esterase (U/mL)	Paraoxonase (mU/mL) (without NaCl)	Paraoxonase (mU/mL) (with NaCl)	Paraoxonase (with NaCl)/ arylesterase × 1000	paraoxonase (without NaCl)/ arylesterase × 1000	NaCl-factor
PE	213.5	502.5	1256	5.88	2.35	2.5
GH	118.5	241	615.6	5.19	2.03	2.55
DK	198	253	570.3	2.88	1.28	2.25
hPON1 AA	210.4	149.7	290.5	1.38	0.71	1.9

The NaCl-factor (activity with1 M NaCl vs. without) of paraoxonase may be used for phenotyping the various plasma samples [18].

Table 5
Influence of POX-hydrolase of various subjects on the degradation of DEP-obidoxime

Subject	Arylesterase (U/mL)	Obidoxime (μM)	Obidoxime-nitrile (μM)	DEP-obidoxime (μM)	Σ (μM)
PE 1:10	9	–	–	7.1	7.1
PE conc.	90	1.3	–	6.1	7.4
GH conc.	48	0.4	–	6.8	7.2
DK 1:10	8	1.3	–	6.3	7.6
DK conc.	80	7.6	–	–	7.6
hPON AA 1:10	9	2.3	–	5.2	7.5
hPON AA conc.	90	5.6	–	1.5	7.1
Dilution buffer		–	0.9	8.1	9

DEP-obidoxime (8 μM) was incubated in the presence of various plasma sources in 25 mM Tris–HCl, 1 mM Ca^{2+}, 0.15 M NaCl, 0.1 % Triton X-100 at pH 7.4 and 37 °C for 60 s.

glutamate [32]. Addition of 0.6 mM DCC or 1 mM EDC resulted in the inactivation of arylesterase, its extent being dependent on the incubation medium. In order to avoid some inhibiting effect on AChE activity during the determination of POX-hydrolase activity, Cibacron-purified enzyme was first incubated with the carbodiimide at 37 °C, followed by dialysis against 1 mM PB/ 1 mM Ca^{2+}, pH 7.4, before arylesterase and POX-hydrolase activities were determined. Both activities were reduced to 30% of the expected value. Phenylmercury acetate (PMA) and *p*-(hydroxymercuri)-benzoate (*p*-OHMB) completely blocked arylesterase and POX-hydrolase, indicating free SH groups being important for catalytic activity of plasma for both reactions. Table 3 gives an overview on the influence of the protein-modifying reagents on paraoxonase/arylesterase and POX-hydrolase activity.

3.2.4. Experiments with soman-pretreated plasma of various donors and purified human PON1

The potency of soman-pretreated plasma of various donors and of purified human PON1, type AA, to degrade DEP-obidoxime was investigated. Table 4 characterizes the plasma activities towards phenyl acetate and paraoxon as well as the stimulation of paraoxonase by NaCl [18]. For determination of POX-hydrolase activity, DEP-obidoxime was incubated with enzyme preparations of various arylesterase activities at pH 7.4 and 37 °C for 60 s, followed by analysis of the degradation products by HPLC. Table 5 presents the quantities of the degradation products after DEP-obidoxime incubation at 37 °C in the presence of various enzyme sources. It is apparent that subjects PE and GH have low POX-hydrolase activity compared to subject DK and to the purified hPON AA, when related to their arylesterase activities.

3.2.5. Paraoxonase, arylesterase and POX-hydrolase distribution in different species

Paraoxonase, arylesterase and POX-hydrolase activities were determined in soman-pretreated plasma samples of different species and compared with human values. As shown in Fig. 7 marked species differences in the activities of paraoxonase and arylesterase were observed. Interestingly, stimulation of paraoxonase by NaCl was only observed in the two human plasma samples. POX-hydrolase activities in plasma samples of identical arylesterase activity (Fig. 8) were calculated by following the decrease of DEP-obidoxime after correction for non-enzymatic hydrolysis. Highest POX-hydrolase activities were found in primates and humans.

4. Discussion

4.1. DEP-obidoxime

To the best of our knowledge, mono(diethylphosphoryl)-obidoxime (pyridinium, 1-[[[[4-(diethoxy-phosphoryloxy)imino]methyl]pyridinio]methoxy]methyl-4-[(hydroxyimino)methyl)]-diacetate) was isolated and characterized for the first time. Notwithstanding, the existence of this intermediate has already been postulated several times [7,11,13,33], because the reactivation of diethylphosphorylated AChE by obidoxime followed biphasic kinetics in the case of highly concentrated enyzme. On

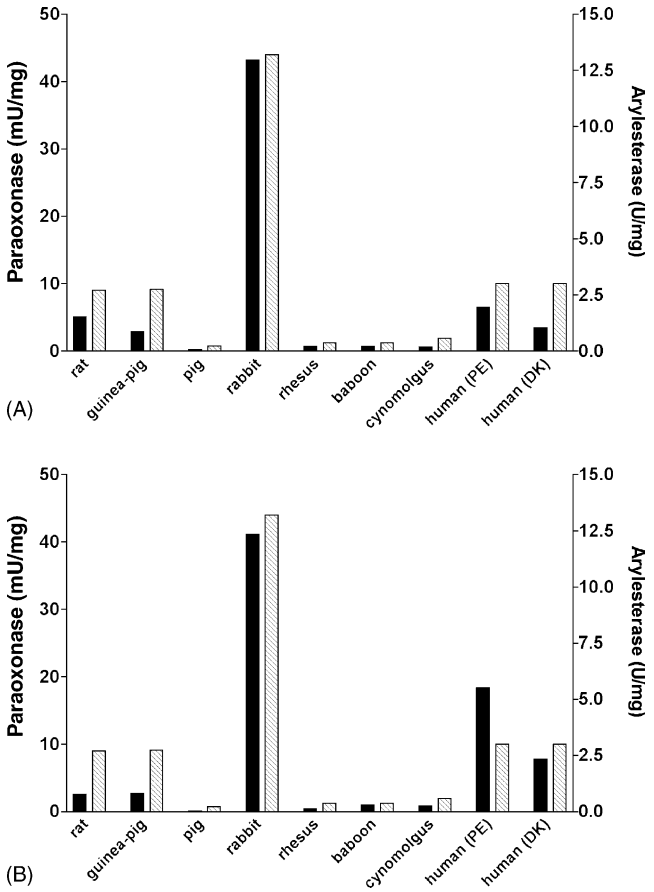


Fig. 7. Distribution of paraoxonase and arylesterase activities in soman-pretreated plasma of various species. The plasma samples were dialyzed against 25 mM Tris–HCl/1 mM Ca^{2+}/0.15 M NaCl/0.1% Triton X-100, pH 8.0. Paraoxonase activities were measured in the absence (**A**) and presence (**B**) of 1 M NaCl. *Black column*: paraoxonase, *hatched column*: arylesterase. Activities refer to the protein-content and are presented in International Units.

the other hand the analogous compound, diethylphosphoryl-4-PAM, has been isolated and characterized by ^{1}H, ^{13}C and ^{31}P-NMR and additionally by SIMS (liquid single ion mass spectroscopy). This compound was

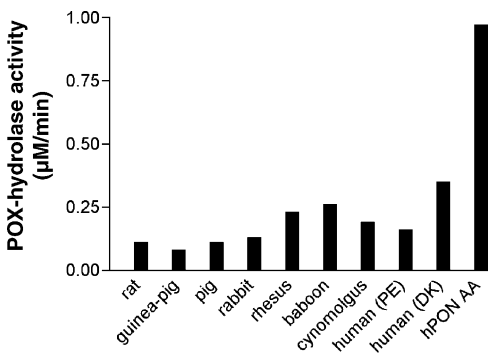


Fig. 8. Distribution of POX-hydrolase activity in soman-pretreated plasma of various species. The plasma samples were dialyzed against 25 mM Tris–HCl/1 mM Ca^{2+}/0.15 M NaCl/0.1% Triton X-100, pH 8.0. For quantification of POX-hydrolase activity, the decrease of DEP-obidoxime (6.4 μM) was followed upon incubation with equal activities of arylesterase (13.9 U/mL) at pH 7.4 and 37 °C for 3 min. (Data are corrected for non-enzymatic degradation of DEP-obidoxime).

relatively stable in H_2O at pH 4.5, but decomposed quickly to the nitrile in 10 mM Tris–buffer, pH 7.8. This POX-derivative of 4-PAM decomposed in alkaline medium into the pyridone and the isonicotinamide derivative [7].

In addition, ethoxymethylphosphonyl-obidoxime was generated in substance, purified by HPLC and characterized with ^{31}P-NMR [8]. Upon phosphylation the absorbance maximum of the compound was shifted hypsochromically from 286 nm to 276 nm, in accordance with the observation of Waser et al. [34] who noticed a hypsochromic shift of the obidoxime absorbance maximum (284–278 nm) after reaction of obidoxime with sarin. Luo et al. [8] did not succeed in generating the DEP-obidoxime and supposed that the compound was not stable enough to be isolated.

That we finally succeeded in isolating DEP-obidoxime and its mononitrile-derivative was due to the unexpected high stability of these compounds in the cold and the high activation energy necessary for degradation (85 kJ/mol). The results of mass spectrometry and the characteristic oximate absorbance at alkaline pH pointed to mono(-diethylphosphoryl) obidoxime and not the bisphosphorylated obidoxime derivative.

The spontaneous decomposition of DEP-obidoxime at neutral pH resulted predominantly in formation of the mononitrile as the primary degradation product. The corresponding half-lives for the decrease of DEP-obidoxime and the simultaneous increase of the mononitrile support this assumption. This reaction proceeds probably by a synchronous cyclic elimination mechanism as suggested by Hagedorn et al. [25]. The nitrile hydrolyzes to the pyridone with cyanide liberation or produces the carboxamido-derivative [23,35]. The degradation of phosphyloximes was reportedly enhanced by the presence of oximes [36]. We did not observe such an effect. Neither obidoxime nor TMB-4 accelerated DEP-obidoxime decomposition. Because attack of obidoxime on the phosphorus atom would regenerate DEP-obidoxime, TMB-4 was tested which should give DEP-TMB-4. Formation of such a compound was not observed.

The enzyme-catalyzed decomposition of DEP-obidoxime by POX-hydrolase in human plasma [13], however, supposedly occurs by a nucleophilic attack at the phosphorus atom followed by hydrophilic decomposition resulting in the parent obidoxime. This behavior is comparable with the hydrolysis of *O*-(*O*,*O*-diethylphosphoryl)-1-methyl-4-pyridiniumaldoxime by the phosphotriesterase of *Pseudomonas* sp. [7]. In the presence of sufficient enzyme activity obidoxime is generated quantitatively. Hence, the existence of POX-hydrolase in human plasma may contribute to optimal reactivation of diethylphosphoryl-AChE by obidoxime; otherwise DEP-obidoxime results in rapid reinhibition of the reactivated enzyme thus retarding net reactivation.

4.1.1. Inhibitory activity of DEP-obidoxime

The bimolecular inhibition rate constant of human Ery-AChE by DEP-obidoxime was determined with

$1.2 \times 10^8\ M^{-1}\ min^{-1}$ at pH 7.4 and 10 °C and was found to be by 350 times a stronger inhibitor than paraoxon. At 37 °C, the inhibition constant is calculated as $7.5 \times 10^8\ M^{-1}\ min^{-1}$, assuming the influence of temperature to be comparable for DEP-obidoxime and paraoxon.

This value approaches the constant for the inhibition of recombinant mouse-AChE by 7-(*O*,*O*-diethylphosphinyloxy)-1-methylquinolinium-methylsulfate (29 °C, 10 mM Tris, pH 7.8 [37]) and the inhibition of human Ery-AChE by cyclosarin (37 °C, pH 7.4 [38]) and exceeds the inhibitory activity of soman [39]. As DEP-obidoxime and reactivated AChE are produced at equimolar concentrations, the half-life of reinhibition can be calculated, which is 1.3 min for 1×10^{-9} M AChE. With the therapeutic reactivation half time of 4 min at therapeutic obidoxime concentration (10 μM), only one-third of AChE could be reactivated after reaching equilibrium conditions, which was also confirmed experimentally [13]. However, in the presence of enough POX-hydrolase the reactivation potency of obidoxime was fully exploited.

4.1.2. Reactivating potency of the degradation products

Interestingly, neither the nitrile nor the pyridone structure fundamentally influenced the reactivation potential of the remaining pyridinium-4-aldoxime-function, as the potency was only little less than expected for the remaining oxime function. Oldiges and Schoene reported on a similar behavior of HI 2, the 4-carboxamido analogue of obidoxime: Its potency to reactivate AChE in paraoxon-poisoned mice was 2.5–2.8 times lower than that of obidoxime [40].

4.2. Characterization of POX-hydrolase

The plasma compound which was able to inactivate DEP-obidoxime, was considered to be an enzyme with a molecular weight of >100 kD and was reminiscent of human paraoxonase (hPON1) [13]. The enzyme activity, which was tentatively termed POX-hydrolase, was not inactivated by soman or paraoxon, thus belonging to the family of A-esterases [41,42].

Upon purification of POX-hydrolase by chromatography on Cibacron Blue 3 GA, co-chromatography with paraoxonase/arylesterase was observed. The most interesting question was the structural similarity of the active center of POX-hydrolase and PON1.

In the active center of PON1 at least one tryptophan should be involved, because Tb^{3+} (0.1 mM) completely inhibited both the Ca^{2+}-dependent PON1 and POX-hydrolase. Modification of tryptophan by N–bromosuccinimide did also result in comparable sensitivity of paraoxonase/arylesterase and POX-hydrolase. Experiments with carbodiimides as reagents for specific modification of carboxyl groups of peptidic aspartate and glutamate and also with PMA and *p*-OHMB as SH group blockers revealed that both enzyme activities were influenced in the same manner. The similar behavior of POX-hydrolase and paraoxonase/arylesterase towards these reagents strongly suggested that tryptophan, aspartate/glutamate and cysteine residues are in or close to the active site and to Ca^{2+}-binding sites of hPON1. Meanwhile the crystal structure of a variant of PON1 obtained by directed evolution has been resolved at 2.2 Å [43]. The six-bladed propeller structure contains two calcium ions in the central tunnel, one responsible for catalytic action and one for structural integrity. Further efforts with site-directed mutagenesis will shed more light on this multifunctional protein [44].

4.2.1. Substrate specificity of POX-hydrolase

As already described [13], DEP-TMB-4, dimethylphosphoryl-obidoxime and diisopropylphosphoryl-obidoxime are also substrates of POX-hydrolase though with lower affinity, while the sarin conjugate of obidoxime is probably not hydrolyzed by the POX-hydrolase [45].

Preliminary experiments with HLö7, a bis-pyridinium-2,4-dialdoxime, allowed the conclusion that DEP-HLö7 may also be generated in situ, and was cleaved by POX-hydrolase [26].

4.3. Identity of POX-hydrolase and human PON1?

The above results strongly pointed to the identity of POX-hydrolase and hPON1. Serious doubts arose, however, when considering the different distribution of these two enzyme activities in human individuals and animal species. The known polymorphisms of human PON1 with arginine or glutamine at position 192 and, to a lesser degree the leucine/methionine polymorphism at position 55, are probably responsible for the different activities of these two forms when hydrolyzing paraoxon. Conceivably, arginine in hPON1 type BB is protonated at physiological pH thereby impeding the access of positively charged substrates such as DEP-obidoxime. In case of hPON1 type AA, however, the glutamine residue is uncharged under physiological conditions and may render better accessibility for charged molecules. This consideration could explain why the individuals PE and GH with PON1possessing higher paraoxonase activity that is also more stimulated by NaCl did not exhibit significant POX-hydrolase activity and may therefore represent the homozygous $_{192}$arginine hPON1-type BB (still awaiting sequence analysis or genotyping). The other individuals, however, should represent the AB-mixed forms of hPON1. This assumption is plausible since there is a difference at human polymorphic PON1-types concerning the hydrolysis of the various organophosphates (for review, see [46]).

Comparison of the expression of paraoxonase, arylesterase and POX-hydrolase in the plasma of various species elucidated fundamental differences. Referring to arylesterase-activity, rodents, pig and rabbit did only exhibit very poor POX-hydrolase activity, whereas in primates this activity seems to be comparable with the putative human PON1-type AB (DK).

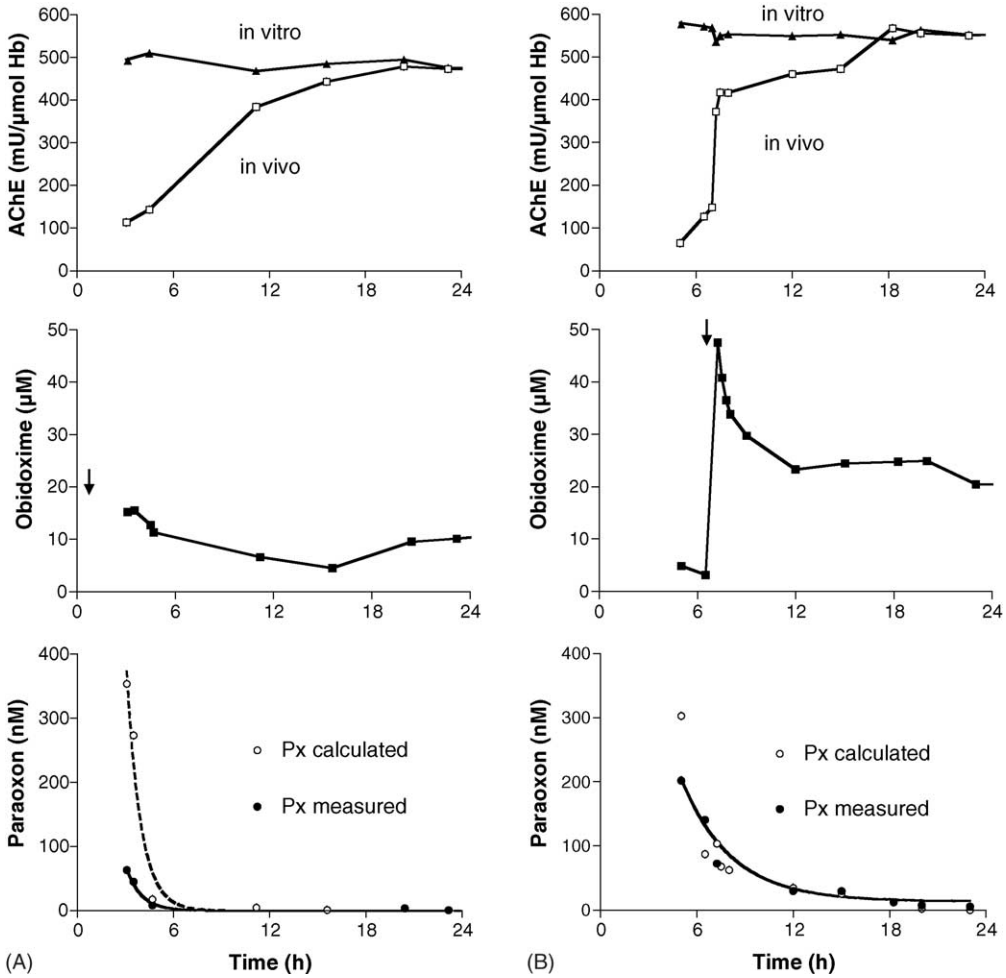


Fig. 9. Influence of the PON 1 status on the efficacy of obidoxime to reactivate inhibited AChE in two patients poisoned with parathion. *Upper panel*: Ery-AChE activity in patient's blood (□) and after reactivation of diluted blood in vitro (▲), to assess reactivatability (100 μM obidoxime, 30 min). Activities refer to the hemoglobin content of the samples [47]. *Mid panel*: Plasma obidoxime concentration as determined by HPLC [20]; arrows indicate beginning of the obidoxime scheme, i.e. 250 mg bolus i.v., followed by continuous infusion at 750 mg/24 h. *Lower panel*: Plasma paraoxon concentration (●) as determined by HPLC [51]. The depicted data refer to free paraoxon (Px) in plasma water. The measured amounts in organic plasma extracts were corrected on the assumption of 55% protein binding [51]. The calculated Px values (○) were derived from the kinetic constants of inhibition and reactivation and the ratio of active vs. inhibited AChE. (For calculation see [2].) Time scale refers to the period elapsed after poison ingestion. (A) Patient SG 25 (left column) exhibited low POX-hydrolase activity (37%, method with AChE reactivation), correlating with poor reactivating efficacy of obidoxime in vivo and comparably higher calculated Px concentrations. (B) Patient LR 31 (right column) exhibited high POX-hydrolase activity (89%, method with AChE reactivation), correlating with high reactivation efficacy of obidoxime in vivo. Measured and calculated Px concentrations were virtually identical. (The residual obidoxime found in the plasma before beginning with our scheme stem from a bolus dose of 250 mg i.v. given by the emergency physician.)

4.4. Influence of POX-hydrolase activity on the effectiveness of obidoxime in organophosphate poisoning

If one transfers these aspects to the therapeutic effectiveness of obidoxime in patients, poisoned with diethyl or dimethylphosphorylating agents, one would expect that patients with high POX-hydrolase activity will benefit more from the therapy with obidoxime. Some hints for this assumption came from a study with parathion-poisoned patients. The therapy of these patients consisted of atropine and obidoxime as continuous infusion. The important parameters of Ery-AChE-activity in vivo, maximal reactivatability in vitro [47], obidoxime concentration [20,48] and plasma paraoxon concentration [49] were determined. With the kinetic parameters K_D, k_r and k_i [50] and the measured concentrations of active AChE, inhibited AChE and obidoxime at hand, one could also calculate the expected poison-concentration [2]. Fig. 9 represents the course of these values in two parathion-poisoned patients. Case A, SG25, exhibited poor POX-hydrolase activity (37%, method with AChE-reactivation), and the calculated paraoxon concentration in plasma water was higher than the measured one. This may be some hint for the existence of a more potent poison such as some accumulation of DEP-obidoxime. Somewhat different was the progress in another parathion-poisoned patient, case B, LR31, (Fig. 9). Here, calculated paraoxon concentration in plasma water corresponded to the measured one with POX-hydrolase activity being particularly high (89%, method

with AChE-reactivation). These two examples may be taken as a first hint, pointing to the relevance of POX-hydrolase activity for optimal effectiveness of obidoxime therapy. Thus, the PON1-polymorphism seems to be a further factor, which may influence the therapeutic success in organophosphate poisoning.

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