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PHOSPHONYLATION OF PURIFIED HUMAN, CANINE AND PORCINE CHOLINESTERASE BY SOMAN
STEREOSELECTIVE ASPECTS

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Abstract—Cholinesterases (EC 3.1.1.8, acylcholine acylhydrolase) from the sera of man, dog and pig were purified 400–600-fold using a combination of ion-exchange and affinity chromatography. In a first approach, phosphonylation by soman was studied by using the half-resolved epimers $C(+)P(\pm)$-soman and $C(-)P(\pm)$-soman. The degradation of soman at the nanomolar level was followed in time by determining the remaining soman by capillary gas chromatography with NP detection. In the three sera investigated the P-(−)-epimer phosphonylates at a higher rate than its corresponding $P(+)$-counterpart and the stereoselectivity is greater for the $C(+)$-epimers than for the $C(-)$-epimers. Individual soman isomers were isolated from $C(+)$- and $C(-)$-epimers and quantified by gas chromatography. Second-order rate constants were determined for the phosphonylation of purified cholinesterase by isolated soman isomers. The $C(+)P(-)$-isomer has the highest phosphonylation rate for the three species; the other toxic isomer, $C(-)P(-)$, has a five to ten-fold lower rate. The overall stereoselectivity is more marked in human cholinesterase than in canine. Porcine serum cholinesterase is phosphonylated by the $P(-)$-isomers at a slightly higher rate than the human enzyme.

Phosphonylation of B-esterases is defined as a reaction between an organophosphonate compound and the protein, which results in the formation of a covalent bond between the serine-hydroxyl in the active site and the phosphorus [1]. Acetylcholinesterase (EC 3.1.1.7, acetylcholine acetylhydrolase) is irreversibly inhibited by this phosphonylation process, which is the underlying biochemical mechanism of the toxicity of anticholinesterase agents in general and soman (1,2,2-trimethylpropyl methylphosphonofluoridate) in particular. Other serine-hydrolases, such as cholinesterase** (EC 3.1.1.8, acylcholine acylhydrolase) and carboxylesterase (EC 3.1.1.1, carboxylic-ester hydrolase) are also inhibited [2]. Since these latter enzymes have no known function, their inhibition can be considered also as an endogenous scavenging mechanism, whereby the agent is irreversibly bound; the amount of such enzymes present in the various species explains in part the interspecies difference in the toxicity of soman [3]. More recently, a role as candidate exogenous scavengers has been suggested for these enzymes [4].

Soman, however, consists of four stereoisomers and some stereoselective aspects of the toxicity of this compound have been reviewed [5]. The phosphonylation as scavenging reaction should aim primarily at the elimination of the most toxic isomers and therefore it is important to study the stereoselectivity of this reaction. We reported previously on these aspects for the phosphonylation of cholinesterase in native serum, under the hypothesis that the hydrolysis of the $P(-)$-isomers is negligible as compared with the phosphonylation of human serum ChE by these isomers and by using a selective blocking of enzymatic hydrolysis [6]. This approach was not valuable in native dog serum [7]. Since we presumed that this was due to interferences with other mechanisms, it was decided to purify the enzyme prior to the phosphonylation studies.

We now report on the phosphonylation of ChE purified from man, dog and pig with the aim of: (1) comparing these results with our previously obtained rate constants in native human serum; (2) contributing to the study of the possibilities of ChE as a potential exogenous scavenger agent in soman poisoning. The relative ranking for phosphonylation, $C(+)P(-) \gg C(-)P(-) > C(-)P(+) > C(+)P(+)$, is confirmed for human and canine ChE, with the human enzyme showing a more marked overall stereoselectivity than canine ChE. For porcine ChE, only partial results are available indicating a similar behaviour as the human enzyme.

MATERIALS AND METHODS

The half-resolved soman epimeric pairs $C(+)P(\pm)$ and $C(-)P(\pm)$-soman (10.0 mg in 1.0 mL 2-propanol) were obtained from the Prins Maurits Laboratory, TNO, The Netherlands (Dr H. P. Benschop). Individual isomers were isolated in 2-propranol using a slightly modified approach as described by Benschop *et al.* [8]. For the isolation of the $P(-)$-isomers about 2 μg $C(+)P(\pm)$ or

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** Abbreviated in this text as ChE.

$C(-)P(\pm)$-soman in 10 μL 2-propranol are added to 1 mL porcine serum; after 20 min soman is extracted from the sample with a solid phase adsorbent (C-18) and eluted with diethylether; the ether is dehydrated in solid CO_2 and evaporated under nitrogen for about 80%; 400 μL 2-propranol are added and evaporation is continued until practically no ether remains in the solvent. An analogous procedure is used for the isolation of $P(+)$-isomers but porcine serum is replaced by a solution of chymotrypsine (2 mg/mL) in Tris (10 mM, pH 7.4). The solutions of individual isomers in 2-propranol are assayed by capillary chromatography with NP-detection [6] against a series of calibrated (by weight) standards of $C(\pm)P(\pm)$-soman.

*Preparation of affinity gel.** (a) The compound 6-(benzyloxycarbonyl)aminocaproaic acid (1) was synthesized according to Ref. 9.

(b) Synthesis of *N*-[6-benzyloxycarbonyl)amino-caproyl]-*N'*,*N'*-dimethylethylenediamine (2): To a cold (−20°) solution of (1) (5 g, 18.8 mmol) and *N'*,*N'*-dimethylethylenediamine (1.66 g, 2.06 mL, 18.8 mmol) in 60 mL of dichloromethane is added dropwise a solution of dicyclohexylcarbodiimide (DCC, 4.2 g, 20 mL, 20.7 mmol, 10% molar excess) during 30 min. After 1 hr at −20° the reaction mixture is allowed to warm up to room temperature and is left overnight. The reaction mixture is extracted with 5% citric acid (3 × 50 mL). The desired product is precipitated from the acid water layer by addition of 1 M NaOH up to pH 13. The crystalline product is collected by filtration and is resolved by addition of 300 mL of chloroform and isolated by evaporation of the chloroform under reduced pressure. Yield: 1.33 g, 21%.

(c) Synthesis of *N*-[6-aminocaproyl]-*N'*,*N'*-dimethyl-ethylenediamine (3): 100 mg of Pd/C 10% was suspended in a solution of (2) (1 g, 2.98 mmol) in methanol. The hydrogenation was carried out for 16 hr (left overnight) at 1 atm. TLC analysis (eluent EtOAc:H_2O:BuOH:HOAc, 1:1:1:1 vol) showed complete conversion. Compound (3) was obtained after evaporation of the solvent under reduced pressure. Yield: 0.588 g, 98%.

(d) Coupling of ligand (3) to activated CH-sepharose® 4B is done as commercially prescribed†. The concentration of ligand in the gel was determined by conductometric titration of the counter chloride ion by 1 mM $AgNO_3$ at pH < 10 and was found to be 7 μmol/mL gel.

Cholinesterase purification. Pooled serum (about 30 humans, six pigs, four male beagle dogs) was used in portions of 200–400 mL. The sera are dialysed against a 20 mM NaOAc-buffer (pH 5.0), prior to the transfer to a DEAE Sepharose® CL-6B, ion-exchange column. Elution is done with a NaOAc buffer (pH 4.0)/NaCl salt gradient (0–0.5 M NaCl). The fractions containing ChE-activity are pooled and dialysed against 20 mM Tris buffer, pH 7.5. The dialysate is further purified with affinity chromatography on a *N*,*N*-dimethyl ethylenediamine hydrochloride ligand, bound to activated CH-sepharose® 4B. Elution is done with a salt gradient and the fractions containing ChE activity are again dialysed against 20 mM Tris pH 7.5 and concentrated with an Amicon ultrafiltration unit (M_r 30,000) to obtain a ChE-activity between 1 (porcine) and 20 units/mL. Cholinesterase activity is assayed colorimetrically using butyrylthiocholine iodide (BTCI) as a substrate and dithio(bis)nitrobenzoic acid as a chromogenic reagent [10] (Unit definition: 1 unit ChE hydrolyses 1 μM BTCI per minute at 30° and pH 7.4). Purification by this procedure is typically 350–650-fold, which is suitable for our needs. The purified ChE-solution is stored at −18° until further use. For our purposes, ChE concentration is expressed as soman-binding sites, obtained from the difference between a known amount of $C(+)P(-)$-soman added to the ChE-solution and the amount of free $C(+)P(-)$-isomer remaining after 20–30 min [6].

Phosphonylation. Soman, $C(+)P(\pm)$ or $C(-)P(\pm)$ or isolated isomer, is added to a solution of purified ChE in Tris (10 mM) at pH 7.4 and 25°. Final concentrations vary, both for soman and for ChE, between 10 and 100 μM. After various times internal standard is added together with an excess of 2-methylcyclohexyl methylphosphonofluoridate (competitor). The organophosphorus compounds are immediately extracted with solid phase (C-18) cartridges, and analysed by capillary gas chromatography with NP detection; the epimers are separated on an achiral stationary phase [6].

Calculations. The ratios of rate constants are calculated according to the theory of competing reactions [11]:

$$k_1/k_2 = \frac{\log(s_1/s_1^0)}{\log(s_2/s_2^0)}$$

with s_1 and s_2 the concentrations of epimers at t min, and s_1^0 and s_2^0 the concentrations of epimers at zero min; k_1 and k_2 are the phosphonylation rate constants for epimers 1 and 2, respectively.

Second-order rate constants are calculated by using the integrated equation for second-order kinetics [11].

$$k = \frac{1}{(p_0 - s_0)t} \ln \frac{s_0(p_0 - x)}{p_0(s_0 - x)}$$

s_0 is the initial concentration of soman isomer, p_0 is the initial concentration of ChE and x is the concentration of ChE that is phosphonylated at time t.

RESULTS

The stereoselectivity of the phosphonylation of purified serum ChE by soman is, in a first approach, qualitatively investigated by using the $C(+)P(\pm)$- and $C(-)P(\pm)$-epimers. For the three species it is found that the ranking of the phosphonylation rates

* All synthesized compounds were checked for their purity and their identity by ^{1}H-NMR, 360 MHz.

† Chromatographie d'Affinité, Principes et Méthodes, Pharmacia, 1979.

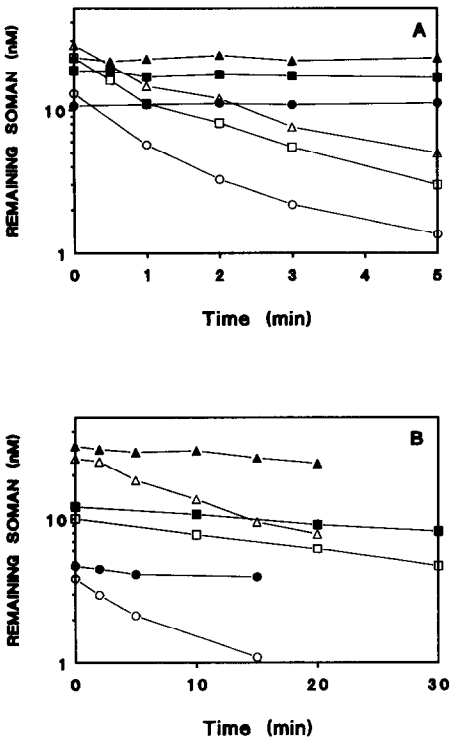

Fig. 1. Degradation of soman epimers by phosphonylation of cholinesterase purified from human, canine and porcine serum at 25° and pH 7.4. Each point is the mean of at least two determinations. (A) $C(+)P(\pm)$-soman; filled symbols $C(+)P(+)$, empty symbols $C(+)P(-)$ (○, ●): human; (□, ■): canine; (△, ▲): porcine. (B) $C(-)P(\pm)$-soman; filled symbols $C(-)P(+)$, empty symbols $C(-)P(-)$ (○, ●): human; (□, ■): canine; (△, ▲): porcine.

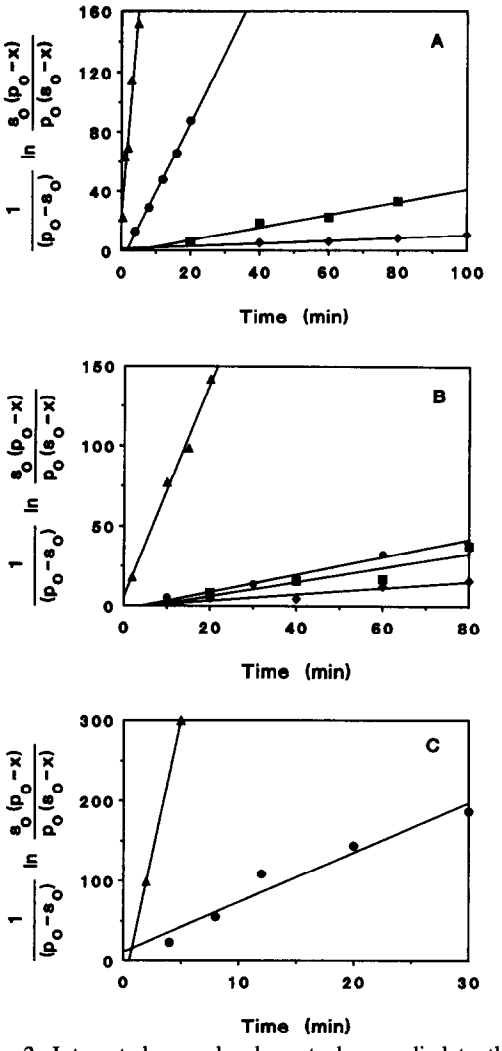

Fig. 2. Integrated second-order rate law applied to the phosphonylation of serum cholinesterase by isolated soman isomers: ▲, $C(+)P(-)$; ●, $C(-)P(-)$; ■, $C(-)P(+)$; ◆, $C(+)P(+)$ (A) human, (B) canine; (C) porcine.

Table 1. Ratio of rate constants (mean value of all the time points ± 90% confidence interval limits) for the phosphonylation of serum cholinesterase by the $C(-)P(\pm)$-isomers of soman (pH 7.4 and 25°)

Species	Man	Dog	Pig
$\frac{k_{C(-)P(+)}}{K_{C(-)P(-)}}$	0.14 ± 0.05 N = 8	0.50 ± 0.06 N = 9	0.16 ± 0.05 N = 10

N is the number of time points.

is, as shown on Fig. 1: $C(+)P(-) \gg C(+)P(+)$ and $C(-)P(-) > C(-)P(+)$. The $C(-)P(\pm)$-epimers both phosphonylate appreciably in time; consequently it is possible for this pair to estimate the ratio of phosphonylation rate constants by the kinetic theory of parallel reactions [11] (Table 1). For the $C(+)P(\pm)$-isomers the ratio cannot be determined experimentally since the ratio (s_1/s_1^0) is nearly 1.

For the isolated isomers similar phosphonylation experiments are done. Rate constants are calculated according to a second-order bimolecular model. Results are shown on Fig. 2. The linear fitting shows the compatibility of the results with the used model. The rate constants are calculated from the slopes of the fitted lines (Table 2).

DISCUSSION

The relative ranking of epimers for the phosphonylation of purified serum ChE is consistent with previous reports [12, 13, 6] and is the same as found for acetylcholinesterase [8]. The absolute values of the individual $P(-)$isomers correspond fairly well with our previously reported values in native human serum [6]. However, the $P(+)$-values and, more in particular the $C(+)P(+)$-value, now obtained from purified human ChE, are much lower than the previously reported rate constants. Since these P(+)-values were measured after blocking the enzymatic hydrolysis, which is highly selective

Table 2. Second-order phosphonylation rate constants* ($\mu M^{-1}\,min^{-1} \pm 90\%$ confidence interval limits) of serum cholinesterase and isolated soman isomers at pH 7.4 and 25°

Serum	$C(+)P(-)$	$C(-)P(-)$	$C(-)P(+)$	$C(+)P(+)$
Human	39 ± 5	3.6 ± 0.4	0.33 ± 0.08	0.09 ± 0.05
Canine	7.0 ± 1.6	0.67 ± 0.4	0.40 ± 0.10	0.27 ± 0.11
Porcine	53 ± 12	7.2 ± 1.0†	1.2‡	

* Mean value of two to five runs.
† Mean value of one run (nine time points); confidence limits calculated on the slope.
‡ Estimated from the ratio of epimer rate constants in Table 1.

towards $P(+)$, this difference may be due either to an insufficient blocking, or to an alternative $P(+)$-selective soman degradation in human serum. Our previously calculated values of the first-order hydrolysis rate constant of $C(+)P(+)$ ($4.2\,min^{-1}$) and of the ChE concentration in human serum (~70 nM) allow us to estimate that about 10% of phosphorylphosphatase activity should have remained for the effect to be due to insufficient blocking of hydrolysis; this is rather unlikely in view of our previous experiences. For an alternative degradation mechanism, however, we have as yet no further evidence.

The respective $P(-)$-isomers phosphonylate canine serum ChE more slowly than human ChE, while the rate of each of the $P(+)$-isomers is about the same for both enzymes. Consequently the overall stereoselectivity is more marked in human serum. This also holds if one compares with equine serum ChE: the non-toxic $C(-)P(+)$-isomer was found to phosphonylate at the same rate as $C(-)P(-)$-soman [12].

The results now obtained for purified canine ChE show that the problems we reported with the determination of the phosphonylation rate constants of the $P(-)$-isomers in native dog serum were due to interference with enzymatic hydrolysis. Indeed, for $C(-)P(-)$-soman, the phosphonylation rate constant of $0.67\,\mu M^{-1}\,min^{-1}$ combined with a concentration of canine ChE of about 40 nmol of phosphonylation sites per litre serum results in an initial rate of $0.027\,min^{-1}$; this is in the same order as the enzymatic hydrolysis rate constant ($0.034\,min^{-1}$), which, in our previous approach, was assumed to be negligible.

The ratios of phosphonylation rate constants obtained from experiments with $C(-)P(\pm)$-epimers (Table 1) are in fairly good agreement with those calculated from values of isolated isomers.

The phosphonylation rate constants of purified porcine serum ChE and the isolated $P(-)$-isomers are found to be slightly higher, but still in the same order of magnitude as human ChE. The results in Table 1 allow the estimation of a phosphonylation rate constant of $C(-)P(+)$-soman (Table 2).

The possibility of using exogenous scavengers in organophosphate poisoning has been suggested earlier [14] and some experimental results have been reported in the case of soman poisoning [15] [16] [4]. A good candidate-scavenger should have a high reaction rate with the organophosphate and present a good stereoselectivity towards the toxic isomers. The rate constants for the reaction of ChE with the toxic soman isomers are about one order in magnitude less and stereoselectivity is less pronounced as compared with acetylcholinesterase [8]. On the basis of these results, the latter enzyme remains the prime candidate for a soman scavenger; ChE can be considered as a reasonable substitute.

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