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SHORT COMMUNICATIONS

The mechanism of soman detoxification in perfused rat liver

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It is well established that highly toxic organophosphorus compounds are detoxificated through hydrolysis catalyzed by phosphorylphosphatase, an enzyme widely distributed in the body. The irreversible organophosphorus anticholinesterase soman consists of two pairs of stereoisomers. Stereospecificity has been reported for the hydrolysis of soman by phosphorylphosphatase [1], as well as for the binding of soman to cholinesterase [2]. The pair of stereoisomers which are preferentially hydrolyzed by phosphorylphosphatase have a low anticholinesterase activity [1]. The inhibition of acetylcholinesterase in brain and diaphragm is the fatality in organophosphate poisoning, whereas the inhibition of other serine esterases, such as butyrylcholinesterase and carboxylesterase, has no known toxic effect. Rather, the binding of soman to these enzymes in rodent plasma has been shown to contribute substantially to the detoxification of low doses of soman [3, 4]. However, in rat liver, which possesses a high capacity of soman detoxification [5], the cholinesterase and carboxylesterase activities were inhibited to a lesser extent than in other intoxication in other organs following soman intoxication [3, 6]. This has been taken into consideration in the present work, where we have studied the rate and mechanism for the detoxification of soman during perfusion of the liver.

Materials and methods

Animals. Male Wistar rats (200–300 g) were purchased from the National Institute of Public Health, Oslo, Norway.

Soman (1,2,2'-trimethylpropyl methylphosphonofluoridate). Soman, assessed to be more than 99% pure by nuclear magnetic resonance spectroscopy, was synthesized in this laboratory.

Vein catheterization. Animals were anaesthetized with Hypnorm vet (Mekos) (0.1–0.15 ml s.c.) and Valium (Roche) (0.1–0.15 mg i.p.), and 1000 IE Heparin (A/S Apothekernes Laboratorium) was injected into the vena cava inferior to avoid coagulation of blood in the liver. Thereafter the vena porta was exposed, and a polyethylene tubing (Intramedic, Clay-Adams 318) was inserted and fastened 5–10 mm from the vein entrance of liver. The animals were killed, and the liver with tubing was carefully removed, washed in physiological saline, and placed on a coarse-meshed filter in a funnel. Then the vein cathether was connected to a perfusion tubing (Silicon 'Versilic tube', inner diameter = 1 mm).

Perfusion procedure. The perfusion buffer consisted of 114 mM NaCl, 4.8 mM KCl, 1.9 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 26 mM $NaHCO_3$, 40 mM glucose, 30 mg/ml bovine serum albumin (BSA) and one of several different concentrations (0–64 μM) of soman. Before the addition of glucose, BSA and soman the buffer was oxygenated for 5–10 min. The perfusion buffer was delivered by a peristaltic pump (12000 Varioperpex, LKB) (6–7 ml/min) and passed through a heat exchanger at 37° before it reached the liver.

Blood was removed from the liver by perfusion with buffer (no soman) until the perfusate became clear (10–15 ml). The perfusion was continued with 50 ml of buffer containing soman, followed by 15–20 ml of buffer (no soman) to wash out soman in the liver and tubings. The perfusate (67–70 ml) was collected and analyzed immediately. A sample of the soman in buffer was stored at 37° and analyzed at the same time.

Determination of soman in perfusate. The amount of soman in the perfusate was determined either from its inhibition of commercial acetylcholinesterase or by the combination of gas chromatography and mass spectrometry (GC/MS).

(a) Enzymatic method. The activity of bovine erythrocyte acetylcholinesterase (Sigma Type I) (EC 3.1.1.7, acetylcholine hydrolase) incubated with perfusate of unknown soman concentration was compared with activities in presence of the corresponding perfusion buffer diluted to contain a series of known adequate soman concentrations: 10 μl of enzyme (0.8 mg/ml) and 10 μl of soman solution was incubated at 30° for 5 min. Then 180 μl of substrate was added to a final concentration of 1.3 mM [^{14}C]acetylcholine (0.05 Ci/mole) and 15 mM sodium phosphate buffer, pH 7.4. The mixture was then incubated and assayed as previously described [7, 8].

(b) GC/MS method. Perfusate was extracted with an equal volume of dichlormethane for 5 min and centrifuged (350 *g*, 5 min). The organic phase containing the extracted soman was shaken vigorously for 5–10 sec with dehydrated Na_2SO_4 to exclude traces of water and proteins. After centrifugation (350 *g*, 5 min), the clear solution was mixed with an adequate amount of *n*-decane (internal standard), and 1 μl of the mixture was analyzed in the LKB 2091 gas chromatograph–mass spectrometer. A glass column (5 feet) with 10% SP–1200/1% H_3PO_4 on 80/100 mesh Chromosorb W-AW DMCS (Suppelco Inc., PA) was used, and the temperature was 95° isotherm for 1 min, then raised 8°/min to 130°. The analysis was performed by mass fragmentography of the m/e = 126 ion. Parallel analysis was made on standard soman solution treated similar to the perfusate.

Determination of soman in liver. The liver was homogenized with a Potter–Elvehjem homogenizer to give 10% homogenate in 0.3 M formic acid. Soman was extracted with dichlormethane and determined by GC/MS as above.

Determination of enzyme activities in liver. The liver was homogenized with a Potter–Elvehjem homogenizer to give 20% homogenate in 25 mM $NaHCO_3$.

Cholinesterase. The liver homogenate, which contains mainly butyrylcholinesterase (EC 3.1.1.8, acylcholine acylhydrolase), was diluted with 3 volumes of 20 mM sodium phosphate buffer, pH 7.4, and the rate of hydrolysis of acetylcholine was measured by the radiochemical method of Sterri and Fonnum [7] as previously described [8].

Carboxylesterase (E.C. 3.1.1.1, *carboxylic-ester hydrolase*). The liver homogenate was diluted with 9 volumes of 25 mM $NaHCO_3$, and the rate of hydrolysis of tributyrin was measured by the Warburg technique as described by Du Bois *et al.* [9] with minor modifications [3].

Results and discussion

The difference in the amount of soman between the original perfusion buffer and final corresponding perfusate, i.e. the soman lost during perfusion, was a linear function of the soman concentration in the perfusion buffer almost up to 64 μM or a total of 576 μg soman (Fig. 1). In addition, almost all the soman was hydrolyzed. Thus, when the perfusion was performed with 360 μg soman or lower in

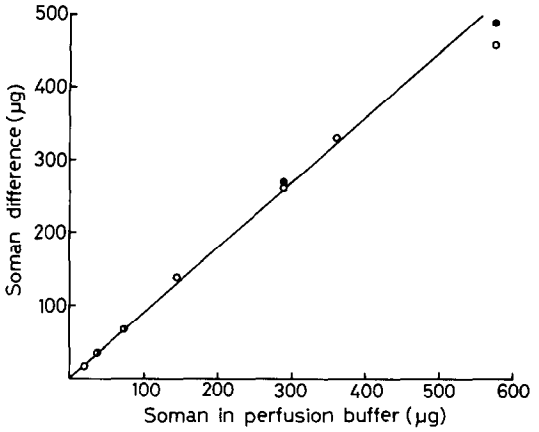

Fig. 1. The amount of soman lost during perfusion of rat liver. Liver was perfused with 50 ml of buffer (6–7 ml/min) containing different amounts (18–576 μg) of soman. The amount of soman recovered in the final perfusate was determined enzymatically (○) or by GC/MS (●). The calculated differences between soman in perfusion buffer and corresponding perfusate are mean values of 2–6 experiments. S.D. is within 5% of the values.

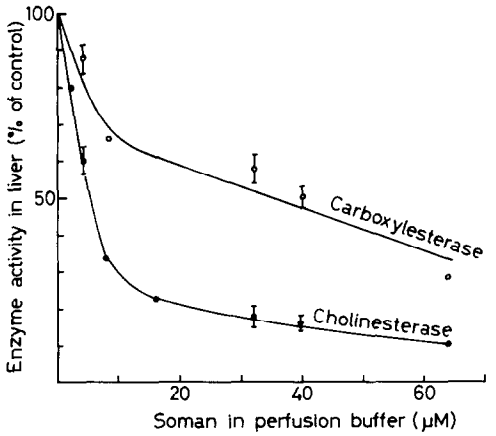

Fig. 2. Carboxylesterase (aliesterase) and cholinesterase activities in rat liver after perfusion with soman buffer. Liver was perfused with 50 ml of buffer (6–7 ml/min) containing one of several different concentrations (2–64 μM) of soman. Results are mean value of 2–6 experiments ± S.E.M.

the buffer (40 μM), less than 15% of the initial amount of soman was recovered in the perfusate (Fig. 1, Table 1). This proportion was increased only to about 20% in the case of perfusion with 64 μM soman buffer (Fig. 1). The soman content in the liver itself after perfusion with 32 μM soman buffer was less than 10% of the initial amount in the buffer (Table 1). This means that during perfusion with 32 μM soman buffer, 80–90% of the soman became detoxificated (Table 1). This concentration corresponds to eight times the intraperitoneal LD_{50} dose for the animal [5]. At the same time, both carboxylesterase and cholinesterase activities were not maximally inhibited (Fig. 2), indicating that the main part of soman was hydrolyzed before it reached these enzymes. Thus, the results show that soman is extensively detoxificated, probably by phosphorylphosphatase during perfusion of liver. This may be explained by the subcellular localization of these enzymes in liver. The phosphorylphosphatase is located predominantly in the supernatant fraction [10, 11], the esterases in the microsomal fraction [11–13]. It also accords with the very high activity of phosphorylphosphatase in liver compared to other organs [14]. The linearity between hydrolysis of soman and initial soman concentration (Fig. 1) indicates that the soman-hydrolyzing capacity has not yet been saturated. The observation is consistent with the reported K_m values of about 7 mM for hydrolysis of sarin and tabun in rat plasma [15].

Table 1. Soman in rat liver and perfusate after perfusion with soman buffer*

	Amount of soman			
	Liver		Perfusate	
Rat No.	(μg)	(%)	(μg)	(%)
1	17	6	40	14
2	16	6	26	9
3	23	8	4	1
4	20	7	9	3
5	—	—	23	8
6	—	—	17	6

* Liver was perfused with 50 ml of 32 μM soman buffer (6–7 ml/min) containing 288 μg soman (=100%). The amount of soman in liver and perfusate was determined by GC/MS.

Two different methods were used for determination of soman in the perfusate to allow us to detect any enrichment of soman stereoisomers during the perfusion. The GC/MS method should give the total amount of soman regardless of stereoisomers. The enzymatic method should measure only one of the pair of stereoisomers (see Materials and Methods), due to the stereospecificity of cholinesterase inhibition by soman [2]. The two methods led to similar results (Fig. 1) indicating little or no enrichment of soman stereoisomers during the perfusion. Taken together with the very high proportion of soman detoxificated (Fig. 1, table 1), and the fact that the hydrolysis of soman by phosphorylphosphatase is stereospecific [1], this indicates that some form of racemization of soman has taken place during the perfusion. The result was unexpected since incubation of soman with liver homogenate gives a soman highly enriched in the pair of stereoisomers showing high anticholinesterase activity [1]. In conclusion, 80–90% of the amount of soman became hydrolyzed during a single pass through rat liver. The detoxification may mainly be accounted for by the action of phosphorylphosphatase and only to a lesser extent by soman binding to carboxylesterase and cholinesterase. Analysis of the remaining soman by GC/MS and by enzyme inhibition showed no enrichment of any stereoisomer indicating rapid racemization of soman during the perfusion.

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The influence of filtrability on β-adrenergic ligand binding to membrane fragments from human erythrocytes and mononuclear leucocytes

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β-Adrenergic receptors have been identified on nucleated erythrocytes from frog [1] and turkey [2]. Also non-nucleated rat erythrocytes contain β-adrenergic receptors [3]. Saturable binding sites with affinity similar to that of β-adrenergic receptors have been detected on intact human erythrocytes by centrifugation [4], but not on broken cells by ultrafiltration [5–7]. Before accepting the absence of β-adrenergic receptor binding sites on human erythrocytes, we evaluated the application of ultrafiltration for membrane fragments from these cells in order to substantiate the hypothesis that human erythrocyte membrane fragments may deform and pass through the glass fibre filters during the ultrafiltration. Human mononuclear leucocytes were applied as the reference system since the existence of β-adrenergic receptors has been established for these cells [5, 8]. The radioactivity of [^{3}H]-(−)-dihydroalprenolol on the glass fibre filter was used to evaluate binding, and the presence of proteins in the filtrate to evaluate filter retention efficacy.

Materials and methods

[^{3}H]-(−)-Dihydroalprenolol ([^{3}H]DHA) with a specific activity of 49.1 Ci/mmole was obtained from New England Nuclear (Dreieich, F.R.G.); (±)-propranolol hydrochloride was obtained from Imperial Chemical Industries Ltd., Pharmaceutical Division (Cheshire, U.K.). Other chemicals were of analytical grade.

Erythrocytes and mononuclear leucocytes were obtained from heparinized human blood (10 IU/ml). The erythrocytes were prepared according to [9]. After three washes with 110 mM NaCl/10 mM Tris–HCl, pH 7.4 (hematrocrit = 0.40) and removal of buffy coat, the cells were lysed in distilled water (1 ml packed cells/10 ml ice-cold water). Membrane fragments of human erythrocytes (HEM) were obtained by homogenization of the lysate in a Potter–Elvehjem glass homogenisator with three gentle strokes by the motor driven Teflon pestle. The homogenate was adjusted to 5 mM Tris–HCl/2 mM $MgCl_2$, pH 7.4 and centrifuged at 30,000 *g* for 15 min. The pellet from 1 ml packed cells was washed twice in this buffer and finally resuspended in 75 mM Tris–HCl/25 mM $MgCl_2$, pH 7.4 Packed cells (1 ml) yielded 1 ml of HEM suspension. Total protein concentrations were determined according to Lowry [10] and residual haemoglobin concentrations according to [11]. Membrane protein concentration was 5 mg/ml, defined as the difference in concentration between total protein and residual haemoglobin. All procedures were performed at 0–4°. Mononuclear leucocytes were obtained according to [12] and prepared according to [5]. The cells (0.5–1.0 × 10^8) were washed twice in 20 ml 50 mM Tris–HCl/10 mM $MgCl_2$, pH 8.1, and homogenized like the erythrocytes. The homogenate was centrifuged at 30,000 *g* for 15 min and the pellet was washed twice in 15 ml 50 mM Tris–HCl/10 mM $MgCl_2$, pH 8.1. Finally, membrane fragments from human mononuclear leucocytes (HLM) were resuspended in 3 ml 50 mM Tris–HCl/10 mM $MgCl_2$, pH 7.4, with a protein concentration of 2 mg/ml determined by the method of Lowry [10]. All procedures were performed at 0–4°.

Results

When HEM were incubated, diluted, filtered and the filters washed at 22°, approximately 90% of the applied membrane proteins were recovered in the filtrate (Table 1). The use of ice-cold dilution buffer with increasing equilibration time at 0–4° before filtration and washing the filters with ice-cold buffer reduced the filtrability of HEM. When HEM were incubated, diluted, filtered and the filters washed at 0–4°, approximately 25% of the applied membrane proteins were present in the filtrate (Table 1).

In parallel incubations with 10 nM [^{3}H]DHA in the absence or presence of 10 μM (±)-propranolol, a significant negative correlation between filter radioactivity and the fraction of applied membrane protein recovered in the filtrate was found (Fig. 1). Specific binding became similar for the different incubation and filtration conditions when filter radioactivity was correlated to filter protein content and not to the incubation mixture protein content (Table 1).

In order to identify saturable β-adrenergic ligand binding to HEM, different concentrations of [^{3}H]DHA were incubated in the absence or presence of 10 μM (±)-propranolol. For incubations at 22°, with dilution and immediate filtration/washing of filters at 0–4°, without any correction for the amount filtered protein small differences were found between radioligand binding in the absence and presence of propranolol (data not shown). When all procedures were performed at 0–4°, saturable [^{3}H]DHA binding was found (Fig. 2). When plotted according to Scatchard [14], a dissociation constant of 1.7 ± 0.6 nM (mean ± S.D., $n = 3$) and a maximum binding capacity (B_{max}) of 21 ± 5 fmole/mg (mean ± S.D., $n = 3$) were found (Fig. 2, inset).

In contrast, saturable radioligand binding to HLM was obtained after incubation at 22° with dilution, filtration and washing of the filters at 0–4° (Fig. 3) with no detectable protein in the filtrate [12]. When plotted according to Scatchard [14], a dissociation constant of 3.5 ± 0.5 nM