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FORMATION OF SOMAN (1,2,2-TRIMETHYLPROPYL METHYLPHOSPHONOFLUORIDATE) VIA FLUORIDE-INDUCED REACTIVATION OF SOMAN-INHIBITED ALIESTERASE IN RAT PLASMA

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Abstract—After incubation (37°) of rat blood or plasma with the nerve agent soman, $(CH_3)_3C(CH_3)C(H)O(CH_3)P(O)F$ (7.7 μM), for 10 min, only a small amount of this organophosphate (7 or 1%, respectively) is left, as determined enzymatically (acetylcholinesterase) and gas chromatographically. Comparison of the results obtained with both analyses shows that this residual soman consists only of its P(−)-isomers. Incubation (25°) at pH 4.8–6.1 of such soman-treated rat blood or plasma with sodium fluoride (2.5 mM) for 0.5 min leads to (i) a substantial increase of the P(−)-soman concentration, and (ii) a (partial) reactivation of the soman-inhibited aliesterase, proportional to the amount of generated P(−)-soman. These results indicate strongly that added fluoride ions regenerate soman by a reversal of the inhibition reaction. From the relationship between percentage of reactivation and increase of soman concentration the aliesterase concentration in rat plasma is calculated as 2.6 μM.

Sodium fluoride has a similar effect in blood taken from rats to which soman was administered intravenously.

The increase of the P(−)-soman concentration is higher with higher sodium fluoride concentrations and at lower pH values.

In accordance with the absence of aliesterase, addition of sodium fluoride does not induce an increase of the P(−)-soman concentration in soman-treated human plasma.

Recently, Benschop *et al.* [1] developed a method to separate gas chromatographically the nerve agent soman into its four stereoisomers (see Fig. 1 for chemical structures). Application of this method to study the fate of soman in rat blood showed that only P(−)-isomers of soman remained even after a relatively short period of incubation [1]. This observation has been attributed to a preferential hydrolysis of the P(+)-isomers of soman by phosphoryl phosphatase(s) present in the blood.

In the course of our studies on the stereospecific degradation of soman in rat blood we investigated the induction of racemization of the residual P(−)-soman by sodium fluoride as a control experiment. Fluoride ions will react with the phosphonofluoridate leading to a molecule with the inverse configuration around the phosphorus atom [2]. All four stereoisomers of soman were detected after reaction of the residual P(−)-soman with sodium fluoride at a pH of approximately 5.5, at which the activity of phosphoryl phosphatase is strongly inhibited. From quantitative determinations of soman, however, it turned out unexpectedly that the treatment with sodium fluoride increases considerably the total soman concentration.

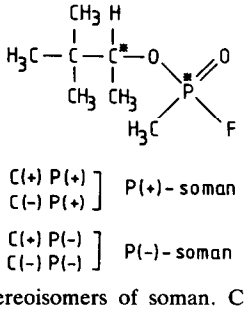


Fig. 1. Four stereoisomers of soman. C stands for the asymmetric α-carbon atom in the pinacolyl moiety and P for the asymmetric phosphorus atom.

In this paper a study on the effect of sodium fluoride on the residual concentration of soman in soman-treated rat blood and rat and human plasma is described.

MATERIALS AND METHODS

Materials. Rat blood was obtained from male Wistar (WAG) rats, bred in the Medical Biological Laboratory TNO under SPF conditions, by heart puncture under nembutal anaesthesia. Blood was collected in a syringe containing 0.2 ml of a heparin solution (5000 IE/ml). Human blood was obtained from volunteers of our laboratory by venapuncture. Blood was collected in heparinized vessels. Blood pooled from at least three animals or individuals was used. Plasma was prepared by centrifugation for 20 min at 1200 *g*.

Electric eel acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) was preparation type VI-S from Sigma Chemical Co., St. Louis, U.S.A.

Physostigmine sulphate (eserine sulphate) was purchased from Nutritional Biochemicals Corp., Cleveland, U.S.A.

Soman, at least 99% pure (GLC), 1,2-dimethylpropyl methylphosphonofluoridate and *o*-nitrophenyl acetate, m.p. 40–41°, were synthesized in this laboratory and had satisfactory elemental analyses. XAD-2 was purchased from Serva, Heidelberg, FRG. Fine particles were removed by repeated slurrying with methanol and decanting. The resin was cleaned by soxhletting for 8 hr with ethyl acetate. Ethyl acetate was freshly distilled over calcium oxide. All other chemicals were commercial products of an analytical grade.

Determination of residual soman concentrations in blood or plasma. Blood or plasma was incubated with soman by addition of a few μl of a stock solution of soman in isopropanol to at least 0.5 ml blood or plasma. The incubation was stopped by adding 3, 1.5 or 1.8 vol of a cold (0°) 25 mM sodium formate solution titrated with 25 mM formic acid to pH 3.75, to one volume of rat blood, rat plasma or human plasma, respectively. In case of blood samples the solution contained 0.84 g saponin/l. The final pH of the mixtures was approximately 5.5. At this pH the activity of phosphoryl phosphatases is highly reduced [3]. Next, the mixture was centrifuged at 20,000 *g* for 10 min (4°). The pH of the resulting supernatant was adjusted to the desired value, if necessary. Soman assays were carried out in the supernatant directly or after incubation of 50 vol of supernatant with one volume of a sodium fluoride solution in water.

Gas chromatographic determination of soman (achiral GLC). For gas chromatographic determinations, soman was adsorbed from the supernatant to XAD-2 by passing the solution through a column (7 × 0.8 cm) of 1 g of the resin at a flow of 0.5–1 ml/min. Before use XAD-2 was washed with methanol, water, 0.1 N NaOH, water, 0.1 N HCl, water and with 25 mM sodium formate/formic acid, pH 3.75, titrated with 0.07 N NaOH to pH 5.5 (formate solution, pH 5.5). The XAD-2 column to which soman was adsorbed, was subsequently washed three times with 5 ml formate solution, pH 5.5. Next, soman was extracted by percolating 8 ml ethyl acetate through the column at a flow of 0.5–1 ml/min. One milliliter of a solution of the soman analogue 1,2-dimethylpropyl methylphosphonofluoridate (100–200 ng) in ethyl acetate was added to the eluate as an internal standard. The residual aqueous phase was removed by dipping the ethyl acetate eluate into solid CO_2/ethanol mixture until water was frozen. The organic layer was decanted and the ice was washed with 1 ml ethyl acetate. The combined ethyl acetate fractions were concentrated to approximately 0.1 ml in a vessel according to Junk *et al.* [4], consisting of a 25 ml pearshaped flask equipped with a calibrated taper of about 0.5 ml, at room temperature and reduced pressure (7.3–8.0 kPa, i.e. 55–60 mm Hg) by using a rotatory evaporator. In control experiments it was found that this treatment does not affect the ratio of the concentrations of soman and internal standard. Soman determinations in the ethyl acetate concentrates were performed by using a Perkin–Elmer Sigma 3 gas chromatograph equipped with a capillary column (35 m × 0.7 mm pyrex glass) coated with SE-30 (film thickness 1 μm) and with an alkaline-bead or flame photometric detector operating in the P-mode configuration. The injector and the first part of the detector up to 1 cm from the detector-tip were glasslined and the glass surfaces of injector, column and detector were deactivated with carbowax 1540 M via the gas phase [5, 6]. The temperatures of the column oven, injection port and detector block were 130, 210 and 210°, respectively, while the flow rates of helium carrier gas, detector hydrogen, helium make-up gas to the detector and detector air were 5, 2.5, 10 and 100 ml/min, respectively. Samples of 0.2–0.5 μl were injected. The quantity of soman in a sample was evaluated from the average of 3–5 values of the ratio of the peak heights of soman and of the internal standard on the basis of a calibration curve. Calibration curves were determined by using 5 mixtures of various concentrations of soman and of a fixed concentration of the internal standard in ethyl acetate.

Determination of relative concentrations of the stereoisomers of soman (chiral GLC). The determination of the relative concentrations of the stereoisomers of soman was performed gas chromatographically in the ethyl acetate concentrate (see Gas Chromatographic Determination of Soman), as described by Benschop *et al.* [1].

Enzymatic determination of soman. Supernatant (20 μl), supernatant treated with sodium fluoride (5, 10 or 20 μl) or, a sufficient dilution of the sample in formate solution, pH 5.5, was added to a solution of 2 μg acetylcholinesterase/ml 0.01 M phosphate buffer, pH 7.5, containing 0.2 mg bovine serum albumin/ml. The mixture, final volume being 1.2 ml, was incubated for 5 min at 25°. Next, 1 ml of the incubation mixture was assayed titrimetrically for the residual enzyme activity by using a Radiometer pH-meter PHM64 equipped with a titrator TTT60 and an autoburette ABU80. The assay was performed with 20 ml of a 3 mM acetylcholine perchlorate solution in 0.1 M potassium chloride at pH 7.5 and 25° under a stream of nitrogen. The titrant was 0.01 N NaOH. Concentrations of P(−)-soman were evaluated from the obtained percentages of residual enzyme activity with the aid of a calibration curve. Calibration data were obtained from quadruplicate determinations carried out with 20 μl of at least 7 standard soman solutions or with 10 μl of 7 standard soman solutions containing 2.5 mM sodium fluoride. Standard soman solutions were 100- or 200-fold dilutions of stock solutions in isopropanol with formate solution, pH 5.5. Calibration curves were calculated as a two or three degree polynomial fit to sets of data for percentage of residual enzyme activity, P(−)-soman concentration (being half the concentration of racemic soman in the standard solution). The calibration curves for soman standard solution without and with added 2.5 mM sodium fluoride differ only very slightly. Therefore, the calibration curve obtained with standard soman solutions without added sodium fluoride was used also for the evaluation of the soman concentration in samples treated with sodium fluoride at a concentration lower than 2.5 mM.

Enzyme assay with o-nitrophenyl acetate as a substrate. An aliquot of 20 μl rat plasma, of 70 μl human plasma, or of a sample containing a similar amount of plasma, was added to a mixture of 2.9 ml of 50 mM veronal buffer, pH 7.0, and 0.1 ml of 20 mM *o*-nitrophenyl acetate in 25% ethanol. After incubation for 5 (rat plasma) or 10 min (human plasma) at 25° the enzymatic hydrolysis of *o*-nitrophenyl acetate was stopped by addition of 0.1 ml of a sodium dodecyl sulphate solution (130 mg/ml). Subsequently, the extinction of the solution was measured at 368 nm after approximately 4 min.

Enzyme assay with methyl butyrate as a substrate. An aliquot of 60 μl rat plasma, of 200 μl human plasma or of a sample containing a similar amount of plasma, was added to a mixture of 20 ml (rat plasma) or 40 ml (human plasma) of a solution of 0.06 M methyl buryrate in water and of 0.1 ml 0.2 M phosphate buffer, pH 7.5. The hydrolysis of methyl butyrate was followed titrimetrically at pH 7.5 and 25° by using a Radiometer pH-stat equipment. The titration was carried out under a stream of nitrogen with 0.01 N NaOH as a titrant.

Enzyme assay with acetylthiocholine as a substrate. An aliquot of 30 μl plasma or of a sample containing a similar amount of plasma was added to 4 ml of a solution of 0.5 mM acetylthiocholine iodide in 0.05 M phosphate buffer, pH 7.0, containing 0.5 mM 5,5′-dithiobis(2-nitrobenzoic acid). After incubation for 5 min (human plasma) or 20 min (rat plasma) at 25° the substrate hydrolysis was stopped by addition of 0.1 ml of a sodium docecyl sulphate solution (130 mg/ml). Subsequently, the extinction of the solution was measured at 412 nm after approximately 4 min.

Evaluation of percentage of reactivation. The percentages of reactivation at time t (% react$_t$) were calculated according to

$$\%\ \text{react}_t = \frac{\text{AIR}_t(\text{A}/\text{AR}) - \text{AI}_0}{\text{A} - \text{AI}_0} 100 \qquad (1)$$

where AIR_t is the activity of inhibited enzyme after incubation with sodium fluoride for time t and A, AR and AI_0 are blanks for the enzyme activity, the enzyme activity in the presence of sodium fluoride and the inhibited enzyme activity at the start of the reactivation experiment, respectively.

RESULTS

Analysis of soman. Residual concentrations of soman in blood or plasma were determined from its inhibitory effect on electric eel acetylcholinesterase. Residual endogenous cholinesterase activity was not detected in soman-treated blood or plasma samples and, hence, does not interfere with the enzymatical assay. Because of the high reaction rate of P(−)-isomers of soman with the enzyme in comparison with the P(+)-isomers [7], the concentration of P(−)-soman is obtained in this assay. Soman concentrations were determined in supernatant obtained from blood or plasma after adjusting the pH to approximately 5.5 and the temperature to 0°. The P(−)-soman concentration decrease about 5% upon storage of a sample for 60 min at these conditions, wheras upon storage at 25° the P(−)-soman concentration decreased about 20% within 30 min.

In a limited number of experiments additional information on residual soman was obtained from two gas chromatographic analyses. In the quantitative achiral GLC assay the diastereoisomers are not separated and the total soman concentration is determined. In the chiral GLC analysis the relative amounts of the four stereoisomers of soman are determined separately but in a qualitative manner.

Assay of aliesterase activity. Initially, aliesterase (carboxylic ester hydrolase, EC 3.1.1.1) activities were measured titrimetrically with the specific substrate methyl butyrate [8]. This assay, however, is time-consuming and rather inaccurate in our hands. Therefore, the major part of the activity determinations were carried out according to a more simple spectrophotometric assay with *o*-nitrophenyl acetate as a substrate [9], although both arylesterases and cholinesterase exhibit some catalytic activity towards the hydrolysis of this substrate. The activity due to arylesterases, which are not inhibited by organophosphates [10], does not interfere with the determination of the restoration of organophosphate-inhibited enzyme activity. The cholinesterase activity will only slightly contribute in comparison with the relatively high aliesterase activity in rat plasma. In fact, only $6 \pm 2\%$ ($N = 4$) of the enzyme activity assayed with *o*-nitrophenyl acetate was inhibited after incubation of rat plasma for 10 min at 37° with 10 μM physostigmine which is a specific cholinesterase inhibitor at this concentration [10].

Effect of sodium fluoride on the residual soman concentration in rat blood and plasma. Residual concentrations of soman in rat blood and plasma after incubation for 10 min at 37° are given in Table 1. The correspondence between the data obtained in the two assays used shows that only P(−)-isomers are present in blood or plasma even after the short period of incubation. This conclusion based on quantitative data confirms the previous finding made by using the qualitative chiral GLC assay [1]. Analyses of the present samples with this assay led to the same answer (Table 1). Incubation of the residual soman with 2.5 mM sodium fluoride for 30 min at 25° and pH 5.5 causes complete racemization: the P(−)-soman concentration determined enzymatically is about half the total soman concentration as determined by achiral GLC and by chiral GLC four peaks having approximately the same height, are observed (Table 1). However, this treatment with sodium fluoride highly increases the concentration of soman. This increase is a fast process as appears from the considerable effect within 0.5 min of treatment. To examine which stereoisomers are formed, a sample taken after treatment for 0.5 min was rapidly extracted with an equal volume of ethyl acetate. Only P(−)-isomers were found in the ethyl acetate extract upon chiral GLC analysis. In these experiments plasma was used which had been incubated with 7.7 μM soman for 10 min at 37°. The P(−)-soman concentration before and after treatment with sodium fluoride were 0.07 or 0.09 and 0.57 or 0.54 μM, respectively, as a result of two experiments. So, at least 80% of soman in the analyzed samples were formed upon treatment with sodium fluoride. Evidently, the soman formed consists of P(−)-soman.

Table 1. Residual soman concentration in (A) rat blood incubated with 7.7 μM soman and in (B) rat plasma incubated with 12.6 μM soman for 10 min at 37°, subsequently mixed with 25 mM sodium formate/formic acid, pH 3.75 (final pH 5.5) and centrifuged

Incubate	Treatment with NaF	P(−)-soman concentration enzyme assay (μM)	Soman concentration GLC assay (μM)	Peak ratio of soman stereoisomers chiral GLC assay C(−)P(−)	C(+)P(−)	C(−)P(+)	C(+)P(+)
A	none	0.52 ± 0.05	0.45 ± 0.09	1.3	1*	N.D.†	N.D.
	0.5 min	1.09 ± 0.07	—‡	—	—	—	—
	30 min	1.16 ± 0.08	2.30 ± 0.18	0.9	1	0.8	0.8
B	none	1.07 ± 0.08	0.97 ± 0.23	1.3	1	N.D.	N.D.
	0.5 min	2.11 ± 0.14	—	—	—	—	—
	30 min	1.72 ± 0.07	3.67 ± 0.69	0.9	1	0.9	0.9

* The peak height of the C(+)P(−)-isomer is arbitrarily set to 1.
† Not detected.
‡ Not determined.
Mean values and their standard errors of enzymatic and gas chromatographic determinations performed directly and after treatment with 2.5 mM sodium fluoride at pH 5.5 and 25° in supernatants of three separate incubates are calculated as molar concentrations in blood or plasma. The mean values of the ratios of the peak heights of the soman stereoisomers analyzed by chiral GLC are also given.

Recently, we published preliminary experiments in which the course of the soman concentration in blood was followed as a function of time after intravenous injection of 6 LD50 soman (495 μg/kg) to anaesthetized and atropinized rats which were kept alive with artificial respiration [11]. In these experiments it was found from comparison of the soman concentrations determined by GLC and enzymatically that only P(−)-soman is circulating in the blood even within two minutes after injection. In addition to the above-mentioned experiments, enzymatic analyses were carried out after treatment of the blood samples with 2.5 mM sodium fluoride for 30 min at pH 5.5 and 25°. Also in these samples treatment with sodium fluoride increased the P(−)-soman concentration. This increase, 0.40 ± 0.03 μM (N = 28), was independent of the time interval (2–90 min) between administration of soman and sampling of the blood.

Dependence of sodium fluoride-induced increase of soman concentration on sodium fluoride concentration and on pH. The dependence of the sodium fluoride-generated increase of the P(−)-soman concentration on the sodium fluoride concentration and on pH was studied with the supernatant obtained in the usual manner from rat plasma which was incubated with 7.7 μM soman for 10 min at 37°. Sodium fluoride treatment was carried out for a short period of time (0.5 min) only. Treatment for a longer time leads to complicated results due to additional reactions taking place simultaneously, like racemization and degradation of soman. The results obtained for treatment with various sodium fluoride concentrations at pH 5.5 and 25° are given in Table 2. Obviously, the generation of the P(−)-soman increases with increasing sodium fluoride concentration.

Data on the dependence of the increase of the P(−)-soman concentration on the pH at which the treatment with 2.5 mM sodium fluoride was carried out, are given in Table 3. The amount of generated soman decreases gradually when the pH is increased from 4.8 to 6.1. In addition to the data given in Table 3, it should be mentioned that generation of soman with 2.5 mM sodium fluoride was not observed at pH 8 in the absence or in the presence of added formate/formic acid buffer.

Reactivation with sodium fluoride of soman-inhibited aliesterase in rat plasma. As mentioned before, endogenous cholinesterase activity was completely inhibited in the supernatant obtained from rat plasma incubated with 7.7 μM soman for 10 min at 37°. Incubation with 2.5 mM sodium fluoride for 30 min at 25° and pH 5.5 did not lead to any restoration of cholinesterase activity.

Incubation of the plasma with 7.7 μM soman for 10 min at 37° led to 87 ± 8% (N = 23) inhibition of the activity towards methyl butyrate and to 90 ± 5% (N = 48) inhibition of the activity measured with *o*-nitrophenyl acetate. No spontaneous reactivation

Table 2. The effect of treatment with various sodium fluoride concentrations for 0.5 min at pH 5.5 and 25° on the P(−)-soman concentration in rat plasma

Sodium fluoride concentration (mM)	Increase* of P(−)-soman concentration (μM)
0.01	−0.03; 0.005
0.03	0.02 ± 0.01 (4)
0.1	0.03 ± 0.01 (7)
0.3	0.07 ± 0.01 (3)
1.0	0.26 ± 0.05 (4)
2.5	0.47 ± 0.12 (15)

* The concentration of P(−)-soman present before addition of sodium fluoride was 0.08 ± 0.05 μM (N = 36).
Rat plasma had been incubated for 10 min at 37° with 7.7 μM soman, mixed with 25 mM sodium formate/formic acid, pH 3.75, and centrifuged. The mean values with their standard errors obtained from enzymatic assays in the supernatant are calculated as molar concentrations in rat plasma. Values in parentheses denote the number of determinations.

Table 3. The effect of treatment with 2.5 mM sodium fluoride for 0.5 min at 25° and various pH values on the P(−)-soman concentration in rat plasma

pH	Initial* P(−)-soman concentration (μM)	Increase of P(−)-soman concentration (μM)
4.8	0.10 ± 0.05 (6)	1.05 ± 0.26 (6)
5.2	0.08 ± 0.04 (3)	0.76 ± 0.18 (3)
5.5	0.08 ± 0.05 (36)	0.46 ± 0.12 (15)
5.8	0.10 ± 0.03 (5)	0.38 ± 0.13 (5)
6.1	0.03 ± 0.02 (8)	0.30 ± 0.03 (8)

* Concentration of P(−)-soman present in the plasma after incubation with 7.7 μM soman but before treatment with sodium fluoride.

Rat plasma had been incubated for 10 min at 37° with 7.7 μM soman, mixed with 25 mM sodium formate/formic acid, pH 3.75, and centrifuged. The mean values with their standard errors obtained from enzymatic assays in the supernatant are calculated as molar concentrations in rat plasma. Values in parentheses denote the number of determinations.

Table 4. Restoration of aliesterase activity in rat plasma after treatment with sodium fluoride for 0.5 min

Conditions of treatment NaF concn (mM)	pH	Reactivation (%) Substrate I	Substrate II
0.03	5.5		0 ± 1 (5)
0.1	5.5		0.7 ± 0.4 (5)
2.5	6.1	7 ± 3 (8)	9 ± 1 (14)
2.5	5.5	22 ± 5 (9)	20 ± 2 (10)
2.5	4.8		40 ± 1 (3)

Rat plasma had been incubated for 10 min at 37° with 7.7 μM soman, mixed with 25 mM sodium formate/formic acid, pH 3.75, and centrifuged. Aliesterase activities were assayed with methyl butyrate (I) or *o*-nitrophenyl acetate (II) as a substrate. Mean values with their standard errors are given. Values within parentheses denote the number of determinations.

of the aliesterase activity was found after incubation of the supernatant for 0.5 min at 25° in the pH range 4.8–6.1.

Results on reactivation by sodium fluoride of soman-inhibited aliesterase are given in Table 4. No reactivation was observed when treating plasma incubated with soman, directly with 2.5 mM sodium fluoride at 25° and physiological pH for 0.5 min. The percentages of reactivation at lower pH values obtained when assaying the activity with the two substrates, are in close correspondence. Percentages of reactivation are reasonably proportional to the concentration of the P(−)-soman formed at corresponding conditions as shown by the plot of Fig. 2.

As one aliesterase active site binds one soman molecule, the total concentration of aliesterase active sites was calculated as 2.6 μM by extrapolation of the plot.

Effect of sodium fluoride on the residual soman concentration in human plasma. The residual P(−)-soman concentration found in the supernatant of soman-treated human plasma is given in Table 5. The soman concentration decreased only 1% or less upon incubation of the supernatant for 30 min at pH 4.8 and 25°. P(−)-soman concentrations obtained after treatment of the supernatant with sodium fluoride are also given in the table. The data correspond to a complete racemization within 30 min as the only process to which the P(−)-soman which will only be left in the soman-treated plasma [12] is amenable.

Incubation of the plasma with 7.7 μM soman for 10 min at 37° led to 95% inhibition of the cholinesterase activity. No reactivation was observed after incubation with 2.5 mM sodium fluoride at pH 4.8 and 25°.

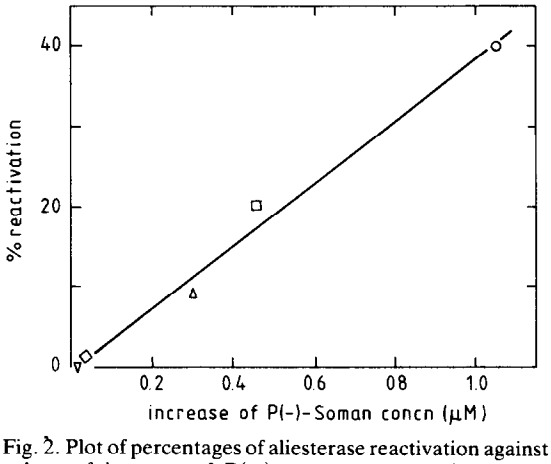


Fig. 2. Plot of percentages of aliesterase reactivation against values of increase of P(−)-soman concentration in rat plasma upon treatment for 0.5 min with 2.5 mM sodium fluoride at 25° and pH 4.8 (○), 5.5 (□) and 6.1 (△) and with 0.03 (▽) and 0.1 (◇) mM sodium fluoride at 25° and pH 5.5. Before incubation with sodium fluoride, rat plasma had been incubated for 10 min at 37° with 7.7 μM soman, mixed with 25 mM sodium formate/formic acid, pH 3.75, and centrifuged. Aliesterase activities were assayed with *o*-nitrophenyl acetate as a substrate. Values are taken from Tables 2, 3 and 4.

Table 5. Residual P(−)-soman concentration in human plasma which was incubated with 7.7 μM soman for 10 min at 37° and subsequently mixed with 25 mM sodium formate/formic acid, pH 3.75, and centrifuged

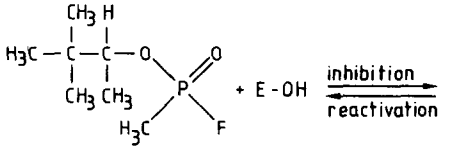
Treatment with NaF	P(−)-soman concentration (μM)
none	2.36 ± 0.04
0.5 min	2.31 ± 0.09
30 min	1.23 ± 0.04

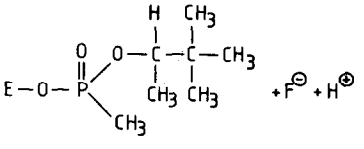
Mean values and their standard errors of enzymatic determinations performed directly and after treatment with 2.5 mM sodium fluoride at pH 4.8 and 25° with the supernatants of 3 separate incubates are calculated as molar concentrations in plasma.

In agreement with the reported absence of aliesterase [10] no activity towards methyl butyrate was measured in untreated human plasma. The plasma showed some activity in the hydrolysis of *o*-nitrophenyl acetate. Incubation of the plasma with 7.7 μM soman or with 10 μM physostigmine for 10 min at 37° caused, however, about the same inhibition of the enzyme activity when assayed with this substrate: 75 ± 1% or 74 ± 1% (N = 3), respectively. Apparently, the relatively high cholinesterase activity present in the plasma is responsible for the catalytic hydrolysis of *o*-nitrophenyl acetate. Neither spontaneous reactivation of the soman-inhibited activity for *o*-nitrophenyl acetate nor sodium fluoride (2.5 mM)-induced reactivation was found (pH 4.8, 25°).

DISCUSSION

It has been shown previously that organophosphates inhibit both cholinesterase and aliesterase by phosphorylation of the hydroxyl group of the serine residue in the active center of the enzymes [13]. In the present work it was found in the pH region 4.8–6.1 that fluoride ions added to soman-treated rat plasma rapidly restore soman-inhibited aliesterase and induce concomitantly a considerable increase of the residual P(−)-soman concentration. The increase of this concentration found after 0.5 min of treatment is reasonably proportional to the percentage of reactivation obtained under the same conditions (Fig. 2). This indicates strongly that upon addition of fluoride ions the reverse of the inhibition reaction will take place, at least in the pH region 4.8–6.1:





(2)

where E–OH represents the aliesterase. As should be expected, this reactivation reaction was also found for soman-inhibited aliesterase formed after intravenous injection of soman into the rat. At higher pH values (7.5–8) neither reactivation nor soman formation by sodium fluoride was found. Under these conditions, reactivation may proceed more slowly, whereas analogously to results reported for aliesterase from pig and horse liver [14, 15], the velocity of the inhibition reaction may be much higher.

It was shown that only P(−)-soman is formed upon sodium fluoride treatment for 0.5 min. It is reasonable to assume that inhibition and reactivation occur with inversion of the configuration around the phosphorus atom of soman by analogy with the racemization reaction with fluoride ions. So probably, only P(−)-soman inhibits aliesterase and this P(−)-soman is regenerated upon reactivation. However, P(+)-soman is very rapidly hydrolyzed in rat plasma. So, it can not be ruled out that P(+)-soman is also a potent inhibitor of aliesterase, but a very short time after addition of soman only P(−)-soman will be available for reaction with the enzyme.

The reactivating properties of fluoride ions towards organophosphate-inhibited cholinesterases were already demonstrated by Heilbronn [16, 17] although soman-inhibited cholinesterase is refractory to this agent. The latter result was confirmed in the present work. Analogously to the pH-dependence found for sodium fluoride-induced reactivation of cholinesterase inhibited by sarin (isopropyl methylphosphonofluoridate), the reactivation with this agent of soman-inhibited aliesterase increases when the pH is lowered.

The relatively high aliesterase concentration found in rat plasma, 2.6 μM, closely corresponds to the values obtained by Christen *et al.* [18, 19], i.e. 3 and 2.8 μM, from the amount of ^{32}P bound to aliesterase in gel electrophoretograms of rat plasma incubated with ^{32}P-sarin or ^{32}P-soman, respectively.

Recently, it was found that more than 1 hr after administration of several LD50-doses there was still intact soman present in the blood of the rat. Evidence has been presented that soman is stored in unidentified "depot" from which it is gradually supplied to the blood stream [11, 20]. Clement [21] suggested soman-inhibited aliesterase as a depot on the basis of his finding that in blood taken from soman-treated mice this inhibited enzyme exhibits spontaneous reactivation and simultaneously cholinesterase is further inhibited. However, as mentioned before, organophosphate is covalently bound to aliesterase from which inhibited enzyme the corresponding hydrogen phosphonate or hydrogen phosphate will be formed upon spontaneous reactivation [19]. We suggest that spontaneous reactivation is not directly related to further inhibition of cholinesterase, a slight residual amount of soman in the blood causing inhibition of cholinesterase. It is now shown that intact soman will be generated from soman-inhibited aliesterase upon incubation with sodium fluoride. It seems highly unlikely, however, that this reaction is the underlying mechanism for a gradual supply of soman from its storage sites, which in that case should be soman-inhibited aliesterase. Sodium fluoride-induced reactivation was not observed to proceed at pH values in the physiological range. Moreover, natural fluoride levels in rat blood or organs are very low (<30 μM) [22, 23], although the occurrence of a local much higher level cannot *a priori* be ruled out.

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