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HYDROLYSIS OF THE FOUR STEREOISOMERS OF SOMAN CATALYZED BY LIVER HOMOGENATE AND PLASMA FROM RAT, GUINEA PIG AND MARMOSET, AND BY HUMAN PLASMA*

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Abstract—Stereoselective hydrolysis at pH 7.5 and 37° of C(±)P(±)-soman by liver homogenate and plasma from rat, guinea pig and marmoset, and by human plasma is studied by using the four single stereoisomers. The fast hydrolysis of the C(±)P(+)-isomers is monitored titrimetrically, whereas the decay of the much slower reacting C(±)P(−)-isomers is followed by gas chromatographic determination of the residual concentration. Values of K_m and V_{max} are evaluated for the enzymatic hydrolysis of the two relatively nontoxic C(±)P(+)-isomers. The plasma enzymes have a high affinity for these isomers (K_m: 0.01–0.04 mM); the K_m values of the liver enzymes vary between 0.04 and 0.7 mM. Except for rat liver homogenate, only first-order rate constants can be obtained for catalyzed hydrolysis (k_c) of the highly toxic C(±)P(−)-isomers: most measurements with C(±)P(−)-isomer concentrations >0.3 mM are complicated by epimerization to C(±)P(+)-isomers, which may conceal enzyme saturation with the C(±)P(−)-isomers. The first-order rate constants of catalyzed hydrolysis (V_{max}/K_m or k_c) by all liver homogenates and plasmata decrease in the order: C(+)P(+)- > C(−)P(+)- ≫ C(−)P(−)- > C(+)P(−)-soman. The highest P(+)-/P(−)-stereoselectivity is found for rat plasma. Rat liver homogenate is more potent than the other liver homogenates in catalyzing the hydrolysis of both the C(±)P(+)- and the C(±)P(−)-isomers. Rat plasma shows the highest activity for degradation of the C(±)P(+)-isomers, but is approximately as active as marmoset and human plasma for degradation of the C(±)P(−)-isomers.

The nerve agent soman (1,2,2-trimethylpropyl methylphosphonofluoridate) is highly toxic, mainly due to its ability to phosphonylate the active centre of acetylcholinesterase. The anticholinesterase activity is eliminated by hydrolytic cleavage of the phosphorus–fluorine bond leading to nontoxic 1,2,2-trimethylpropyl hydrogen methylphosphonic acid. This hydrolytic detoxification of soman and of other anticholinesterase organophosphates can be catalyzed by so-called phosphorylphosphatases or A-esterases, such as DFP-ase and paraoxonase. Enzymatic activity for organophosphate hydrolysis, first described by Mazur [1], has been identified in plasma and various tissues of mammals, as reviewed by Mounter [2].

Qualitatively, we have demonstrated [3] that catalysis of the hydrolysis of chiral C(±)P(±)-soman‡ is stereoselective. De Bisschop *et al.* [4–6] investigated the stereoselective hydrolysis of the agent by human plasma in a quantitative way. By using a low substrate concentration (50 nM) of C(+)P(±)- and C(−)P(±)-soman, these investigators determined first-order rate constants for catalyzed hydrolysis of the four stereoisomers. In the present work hydrolysis of higher concentrations (up to 0.5 mM) of the single stereoisomers was investigated, attempting to determine the kinetic characteristics, V_{max} and K_m, for the catalytic activity of some phosphorylphosphatases towards the various stereoisomers. Catalysis by rat, guinea-pig and marmoset liver homogenate and plasma and by human plasma was studied, in order to obtain insight into one of the major pathways that play a role in toxicokinetics of soman stereoisomers in these species [7].

MATERIALS AND METHODS

Materials

The stereoisomers of soman were isolated according to Benschop *et al.* [8]. Before use, rabbit serum was passed over an XAD-2 column to remove unknown components which are otherwise isolated together with the C(±)P(−)-isomers of soman and which generate acid in the presence of liver homogenate and plasma. The stereoisomers are obtained as a solution in ethyl acetate. Since this solvent is a substrate for enzyme(s) present in liver homogenate and plasma, it was replaced by acetonitrile as follows. The stereoisomer solution was diluted with 40 volumes of acetonitrile and concentrated at reduced pressure (7.3 kPa) to the original volume. This pro-

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‡ Due to chirality at phosphorus and in the pinacolyl moiety, soman consists of four stereoisomers denoted as C(+)P(+), C(+)P(−), C(−)P(+), C(−)P(−). In this notation P stands for phosphorus and C for pinacolyl, whereas C(±)P(±)-soman stands for a mixture of the four stereoisomers in any ratio.

cedure was repeated once. Three preparations of each stereoisomer were made. The preparations containing 10–50 mg stereoisomer/ml (total volume 0.2–0.9 ml) were contaminated with *ca.* 5% of the other epimer with respect to asymmetry at phosphorus, as analyzed by gas chromatography. C(+)P(±)- and C(−)P(±)-soman and D_{13}-C(±)P(±)-soman were prepared as described previously [8 and 9, respectively]. All other chemicals were commercial products.

Blood of rats (male Wistar (WAG/Rij), bred in the Medical Biological Laboratory TNO) and guinea pigs (male outbred of the Hartley type, obtained from Charles River, F.R.G.) was obtained by heart puncture under nembutal and ketamine hydrochloride anaesthesia, respectively. Blood of marmosets (*Callithrix jacchius*, obtained from the Primate Center TNO) was taken via a carotid cannula after ketamine hydrochloride anaesthesia. Human blood was obtained from volunteers in our laboratory by venapuncture. The blood was collected in a syringe containing 0.2 ml heparin solution (5000 U/ml) or in heparinized vessels. Blood from two marmosets or at least three rats, guinea pigs or individuals, was pooled. Homogenates of the liver of the animals were made 25% in 0.9% NaCl. The supernatant obtained after centrifugation of the homogenate at 1000 *g* for 4 min was used. The supernatant of the marmoset liver homogenate was diluted 1.5-fold.

Hydrolysis experiments

The hydrolysis runs were carried out at pH 7.5 and 37° in the thermostated vessel of a Radiometer pH-stat apparatus consisting of a TTA31 titration assembly, a PHM64 pH meter, a TTT60 titrator, an ABU80 autoburette equipped with a 0.25 ml syringe and a REC80 servograph. The reactions proceeded in a nitrogen atmosphere; nitrogen was first washed by passing (10 ml/min) through soda lime and water. The titrant was 0.01 N NaOH.

(i) *Enzymatic hydrolysis of C(±)P(+)-isomers.* A solution (10–50 μl) of a single C(±)P(+)-isomer or of C(+)P(±)-soman was added to 4 ml (final C(±)P(+)-isomer concentrations ≥0.3 mM) or to 6 or 8 ml (final C(±)P(+)-isomer concentrations <0.3 mM) of a 0.9% NaCl solution that has been equilibrated at pH 7.5 and 37° in the pH-stat equipment. Rat plasma was diluted fourfold (C(−)P(+)-isomer) or tenfold (C(+)P(+)-isomer), whereas human and marmoset plasma were diluted twofold (C(−)P(+)-isomer) or fivefold (C(+)P(+)-isomer); rat liver homogenate was diluted tenfold. The pH of diluted plasma and homogenate was adjusted to 7.5 immediately before use. The enzyme-catalyzed hydrolysis was started by addition of 50 μl (rat) or 62.5 μl (other species) of plasma or liver homogenate per ml reaction mixture, after monitoring spontaneous hydrolysis for at least 5 min. Hydrolysis was followed titrimetrically. The initial soman concentration was determined by gas chromatography in a sample taken 1 min before the start of the enzymatic hydrolysis.

(ii) *Catalyzed hydrolysis of C(±)P(−)-isomers.* Soman isomer solution (10–25 μl) was added to 4 ml (final isomer concentration ≥0.3 mM) or to 6 or 8 ml (final isomer concentration <0.3 mM) of a 0.9% NaCl solution that had been equilibrated at pH 7.5 and 37° in the pH-stat equipment. After monitoring spontaneous hydrolysis for at least 5 min, the catalyzed hydrolysis was started by addition of plasma or liver homogenate, 62.5 μl/ml reaction mixture, immediately followed by 10–20 μl 0.1 N HCl (plasma) or 0.2 N NaOH (liver homogenate) to adjust pH to 7.5. Human and marmoset plasma were diluted with an equal volume of 0.9% NaCl. The pH was kept constant by addition of titrant as controlled by the pH-stat equipment and by manual addition of small volumes of 0.01 N HCl. Samples of 25, 40 or 50 μl were taken from 4, 6 or 8 ml reaction mixtures, respectively, 1 min before the start of the catalyzed hydrolysis and at various times during the reaction. Residual isomer concentrations were determined by extracting the samples with 2 ml hexane containing 0.8 μg D_{13}-C(±)P(±)-soman and gas chromatographic analysis of the extract.

(iii) *Spontaneous hydrolysis.* Hydrolysis was started by addition of 10–50 μl solution of a single isomer or of C(+)P(±)-soman to 4 ml (final isomer concentration ≥0.3 mM) or 8 ml (final isomer concentration <0.3 mM) of 0.9% NaCl that had been equilibrated at pH 7.5 and 37°. The pH was kept constant in the pH-stat equipment. The decrease of the soman concentration was followed for 1.5 hr as described for the catalyzed hydrolysis experiments with C(±)P(−)-isomers. Similarly, hydrolysis of C(+)P(±)- and C(−)P(±)-soman was studied in 0.9% NaCl containing 0.1 M EDTA without or with human plasma added, and in 0.9% NaCl to which a 6% bovine serum albumin solution in Krebs Ringer buffer was added.

Gas chromatographic analysis

A Carlo Erba HRGC 5160 gas chromatograph was equipped with an alkali flame ionization detector and an autosampler AS 550 for cold on-column injection. The CPSil 8CB fused silica column (length, 51 m; i.d., 0.32 mm; film thickness, 1.3 μm) was obtained from Chrompack, The Netherlands. The column was fitted with a retention gap (Carlo Erba) consisting of a piece of uncoated and deactivated fused silica (length, 2 m; i.d., 0.50 mm). The injected volume was *ca.* 1 μl (10 sec secondary cooling time). For each chromatographic run, the column was heated from 87 to 140° at 6.2°/min or from 87 to 130° at 5°/min. The detector block was held at 250°. Carrier gas helium was used at a flow of 1.5 ml/min; flows of air and hydrogen through the detector were 370 and 35 ml/min, respectively. Make-up gas for the detector was helium, at a flow of 24.6 ml/min. Under these conditions, the retention time for the soman stereoisomers is 14–15 min. Peak heights were measured with a CI-10 integrator (LDC/Milton Roy).

Calibration curves for calculation of the concentrations of the individual stereoisomers of soman were constructed by measuring the sum of peak heights for the two epimers of C(+)P(±)-soman at six concentrations (0.1–1.6 μg/ml), relative to the sum of peak heights of the enantiomeric pairs of D_{13}-C(±)P(±)-soman (0.4 μg/ml).

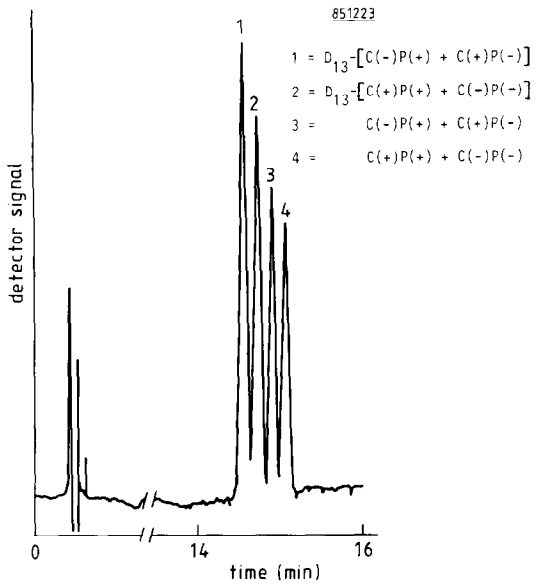

Fig. 1. Gas chromatographic separation of the enantiomeric pairs of D_{13}-C(±)(P±)-soman (peaks 1 and 2) and of C(±)P(±)-soman (peaks 3 and 4) on a CPSil 8CB column. See Materials and Methods for further details.

RESULTS

Gas chromatographic analysis

A gas chromatographic analysis was applied to determine the initial concentrations of the isomers and to follow the concentration decrease of the C(±)P(−)-isomers upon hydrolysis. The capillary column (CPSil 8CB) almost completely separates the two enantiomeric pairs of C(±)P(±)-soman and of the internal standard D_{13}-C(±)P(±)-soman, as shown in Fig. 1. Consequently, this gas chromatographic method allows the separate analysis of a soman stereoisomer and its epimer that may be present as a contaminant due to epimerization at phosphorus [8]. Moreover, the hydrolysis of the two epimers of C(+)P(±)- or C(−)P(±)-soman can be studied simultaneously by using this analytical technique.

Spontaneous hydrolysis of soman stereoisomers

Hydrolysis runs were carried out in the absence of plasma or liver homogenate in order to estimate the contribution of spontaneous hydrolysis at pH 7.5 and 37°. The rates of hydrolysis of two enantiomeric pairs should be equal, whereas the rates of hydrolysis of the epimeric pairs may differ. C(+)P(±)-soman was used at five different concentrations* (0.15–0.88 mM) to determine the rate constants of two epimers simultaneously. The first-order rate constants are approximately independent of the initial concentration of the soman isomer, as expected for spontaneous hydrolysis at constant pH. The averaged rate constants found are $(3.6 \pm 0.4) \times 10^{-3}$ and $(3.4 \pm 0.4) \times 10^{-3}$ min^{-1} for C(+)P(+)- and C(+)P(−)-soman, respectively. In addition, hydrolysis of the single C(+)P(−)-isomer was studied at five concentrations (0.08–0.42 mM): the averaged rate constant was $(3.5 \pm 0.7) \times 10^{-3}$ min^{-1}.

The results obtained with C(+)P(±)-soman and with the single isomer are similar, indicating that the two isomers in C(+)P(±)-soman react independently. The rate constants obtained for C(+)P(+)- and C(+)P(−)-soman are approximately equal. Therefore, our results are in contrast with those of De Bisschop *et al.* [4, 5], who found a 1.6-fold faster hydrolysis of C(+)P(+)- and C(−)P(−)-soman than of C(+)P(−)- and C(−)P(+)-soman in partly deproteinated human serum at pH 8.8 and 25°.

Enzymatic hydrolysis of C(±)P(+)-isomers of soman

Initial rates of enzymatic hydrolysis of the single C(±)P(+)-isomers of soman were estimated from the recordings of alkali consumption at pH-stat conditions, according to the method reported by Goldenberg [10] and Boeker [11]. Goldenberg derived the following equation, which is a very close approximation of the integrated Michaelis–Menten equation

$$(S_0 - S_t)/t = V_{max}S_0/(K_m + S_0) - (S_0 - S_t)V_{max}K_m/2(K_m + S_0)^2 \quad (1)$$

where S_0, S_t, K_m and V_{max} are the substrate concentration at time zero and time t, the Michaelis–Menten constant and the maximum velocity, respectively. This is the equation of a straight line if $(S_0 - S_t)/t$, the average rate over t, is plotted as a function of $S_0 - S_t$. The intercept equals the initial rate of the enzymatic reaction, v_0, according to the Michaelis–Menten equation

$$v_0 = \frac{V_{max}S_0}{K_m + S_0}. \quad (2)$$

Since the recording of alkali consumption is inaccurate at the beginning of the reaction, the first reading from the titration curve was generally taken after 10 sec of reaction. This time was set to t_0 and values of $S_0 - S_t$, expressed as nmole soman hydrolyzed at time t per ml reaction mixture, were calculated as 0.5 (nmole alkali consumed at t − nmole alkali consumed at t_0)/(volume of reaction mixture at t_0). Since the C(±)P(+)-isomers are already hydrolyzed to a considerable extent within a few minutes, the contribution of spontaneous hydrolysis can be neglected. The substrate concentrations at t_0 determined by gas chromatography were corrected for the slight concentration change occurring between the time of sampling for the gas chromatographic determination and the time chosen as t_0. Values of K_m and V_{max} calculated from sets of v_0, S_0 data by using the nonlinear regression method of Wilkinson [12] are collected in Table 1. Illustrations of the dependence of v_0 on the concentration is given in Fig. 2 for the hydrolysis of C(+)P(+)-soman by liver homogenate and plasma from guinea pigs. Similar plots were obtained for the other hydrolysis experiments with the C(±)P(+)-isomers.

* The ratio of C(+)P(+)- to C(+)P(−)-soman in synthetic C(+)P(±)-soman was previously established as 45 : 55 [8]. This ratio was confirmed by analysis (GLC) of the initial concentrations of the epimers found in the reaction mixtures.

Table 1. Kinetic parameters and their standard deviations for the enzymatic hydrolysis of C(+)P(+)- and C(−)P(+)-soman by liver homogenate and plasma from rat, guinea pig and marmoset, diluted in 0.9% NaCl solution (pH 7.5, 37°). Data for diluted human plasma are also given

Source*	Soman isomer	Range of initial concn‡ (μM)	K_m (μM)	V_{max}† (nmole/min/ml)	V_{max}/K_m† (min^{-1})
Plasma: man (1.2)	C(+)P(+)	50–450 (10)	45 ± 15	39 ± 2	0.9 ± 0.3
(3)	C(−)P(+)	20–400 (10)	23 ± 8	11 ± 1	0.5 ± 0.2
marmoset (1.2)	C(+)P(+)	70–480 (10)	36 ± 14	44 ± 3	1.2 ± 0.5
(3)	C(−)P(+)	60–440 (10)	40 ± 9	12 ± 1	0.31 ± 0.07
guinea pig (6)	C(+)P(+)	60–380 (9)	14 ± 4	3.1 ± 0.1	0.22 ± 0.07
(6)	C(−)P(+)	40–340 (10)	29 ± 17	2.1 ± 0.2	0.08 ± 0.05
rat (0.5)	C(+)P(+)§	30–320 (12)	25 ± 9	85 ± 6	3.4 ± 1.3
(1.2)	C(−)P(+)	40–520 (9)	24 ± 10	28 ± 2	1.2 ± 0.5
Liver: marmoset (1)	C(+)P(+)	70–430 (10)	99 ± 25	8.6 ± 0.7	0.09 ± 0.02
(1)	C(−)P(+)	70–400 (10)	171 ± 38	7.1 ± 0.7	0.04 ± 0.01
guinea pig (1.5)	C(+)P(+)	30–370 (11)	40 ± 16	20 ± 2	0.5 ± 0.2
(1.5)	C(−)P(+)	40–330 (10)	40 ± 10	11 ± 1	0.27 ± 0.02
rat (0.12)	C(+)P(+)§	20–450 (14)	51 ± 13	130 ± 11	2.5 ± 0.7
(0.12)	C(−)P(+)	90–610 (9)	710 ± 170	300 ± 50	0.4 ± 0.1

* The figure in parentheses denotes the final concentration (%) of liver homogenate or plasma used in the hydrolysis experiments.

† For comparison, all values of V_{max} are calculated as nmole/min per ml 0.6% plasma or per ml 0.15% liver homogenate; it is assumed that the rate of enzymatic hydrolysis is proportional to the enzyme concentration.

‡ The figure in parentheses denotes the number of hydrolysis runs.

§ Most hydrolysis runs were performed with C(+)P(±)-soman; see also Fig. 3.

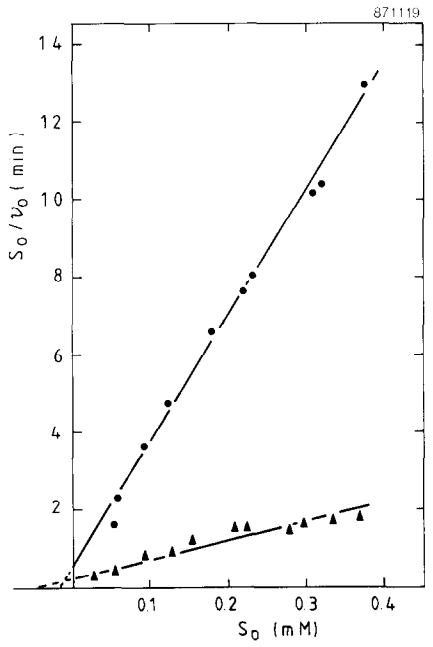

Fig. 2. Eadie plot of the dependence of initial rate (v_0) of the enzymatic hydrolysis (pH 7.5, 37°) of C(+)P(+)-soman catalyzed by guinea-pig plasma (●——●; final concentration 6%) and guinca-pig liver homogenate (▲——▲; final concentration 1.5%) on the initial substrate concentration (S_0). Solid lines were calculated from the data points according to eqn (2) by using the nonlinear regression method of Wilkinson [12].

The hydrolysis measurements of C(+)P(+)-soman with the rat enzymes were performed with C(+)P(±)-soman. It had been found from preliminary experiments that the C(+)P(+)-soman is enzymatically hydrolyzed much faster than the C(+)P(−)-isomer, especially by the rat enzymes. Hence, the acid initially formed during enzymatic hydrolysis of C(+)P(±)-soman will be liberated almost exclusively by C(+)P(+)-soman and initial rates derived from the course of acid release can be assumed to represent rates of C(+)P(+)-soman hydrolysis. Accordingly, the results obtained in control experiments with the single C(+)P(+)-isomer are in agreement with those obtained from C(+)P(±)-soman hydrolysis (see Fig. 3).

Phosphorylphosphatases from plasma show a high affinity for the C(±)P(+)-isomers, the K_m values being only slightly different for the enzymes from the various species. The affinities of the enzymes from liver towards C(+)P(+)-soman are somewhat less than those of the plasma enzymes, whereas the affinities towards C(−)P(+)-soman are even lower, at least for the rat and marmoset enzymes. Both rat plasma and rat liver enzyme show the highest rates of catalytic reaction (V_{max}) of the three species, whereas the guinea-pig plasma is the least active as far as plasma enzymes are concerned. Since the K_m values differ only slightly in the various species, the first-order constants, V_{max}/K_m, of the plasma enzymes for the C(±)P(+)-isomers have a ranking similar to that of V_{max}. On the basis of this first-order rate constant, the rat has the highest enzyme activity in the liver also. In contrast with the results for plasma enzymes, the value of V_{max}/K_m for guinea-pig liver is higher than that for marmoset liver by virtue of a somewhat faster rate of the catalytic reaction as well as a greater

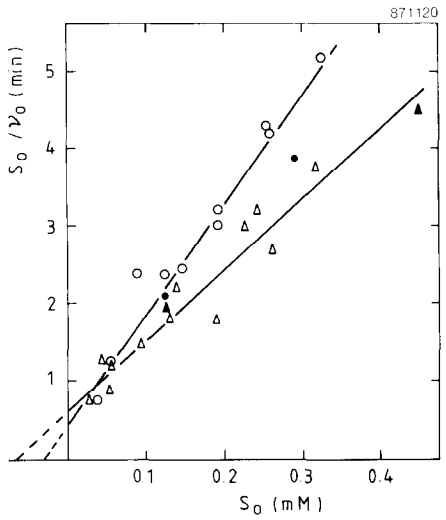

Fig. 3. Eadie plot of the dependence of initial rate (v_0) of the enzymatic hydrolysis (pH 7.5, 37°) of C(+)P(+)-soman catalyzed by rat plasma (○——○; final concentration 0.5%) and rat liver homogenate (△——△; final concentration 0.12%) on the initial substrate concentration (S_0). Experiments were carried out with C(+)P(±)-soman (open symbols) and C(+)P(+)-soman (filled symbols). Solid lines were calculated from the data points according to eqn (2) by using the nonlinear regression method of Wilkinson [12].

affinity for the C(±)P(+)-isomers. The somewhat faster hydrolysis of C(+)P(+)-soman than of C(−)P(+)-soman as found for all enzymes is mostly due to a higher catalytic reaction rate, but is caused mainly by a higher affinity in case of the rat and marmoset liver enzyme.

Catalyzed hydrolysis of C(±)P(−)-isomers of soman

The relatively slow hydrolysis reactions of the C(±)P(−)-isomers were followed by GLC analysis of residual soman concentrations at various reaction times. Plots of the logarithm of the residual C(±)P(−)-isomer concentration against time were made for each hydrolysis run to evaluate kinetic parameters of the reaction.

Invariant rate constants at increased substrate concentrations. Reasonably straight lines were obtained in the semilogarithmic plots for the hydrolysis reactions catalyzed by rat plasma. The slopes of the lines, k_{obs}, are not significantly different when the initial C(±)P(−)-isomer concentration is varied. These results are consistent with enzymatic hydrolysis if $K_m \gg S_0$ (see eqn 2), with a general catalysis of the hydrolysis by rat plasma constituents or with simultaneous catalysis according to these two mechanisms, in addition to spontaneous hydrolysis. Values for the rate constants of catalyzed hydrolysis, k_c, are obtained as the average value of k_{obs} corrected for spontaneous hydrolysis according to

$$\ln S_t = \ln S_0 - (k_c + k_w)t \qquad (3)$$

where k_w is the rate constant for spontaneous hydrolysis.

Increased rate constants at increased substrate concentrations. The results obtained for most hydrolysis runs with the guinea-pig, marmoset and human enzymes also show a linear relationship in the semilogarithmic plots. An apparent increase in hydrolysis rate occurring after some reaction time, however, is observed for runs with higher initial C(+)P(−)-soman concentrations and, in a less prominent way, for some runs with the highest initial C(−)P(−)-soman concentrations. Examples of the dependence of the residual isomer concentration on time are given in Figs 4 and 5 for the hydrolysis of C(+)P(−)-soman by guinea-pig plasma and of C(−)P(−)-soman by marmoset plasma, respectively. Rate constants for these hydrolysis runs were evaluated as the slopes of the initial linear part of the semilogarithmic plots (six to nine data points). The means of the rate constants were calculated for three ranges of initial C(±)P(−)-isomer concentrations, i.e. <0.15 mM, 0.15–0.25 mM and >0.25 mM, and are collected in Table 2. In general, rate constants are somewhat greater at higher C(±)P(−)-isomer concentrations, especially in case of C(+)P(−)-soman.

Both deviations from first-order kinetics observed

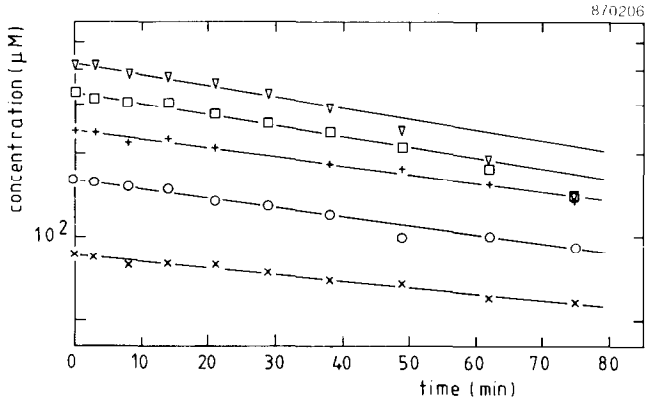

Fig. 4. Plots of the residual concentration of C(+)P(−)-soman vs time of hydrolysis catalyzed by guinea-pig plasma (final concentration 6%) in 0.9% NaCl solution (pH 7.5, 37°). Different symbols refer to different runs performed with various initial C(+)P(−)-soman concentrations.

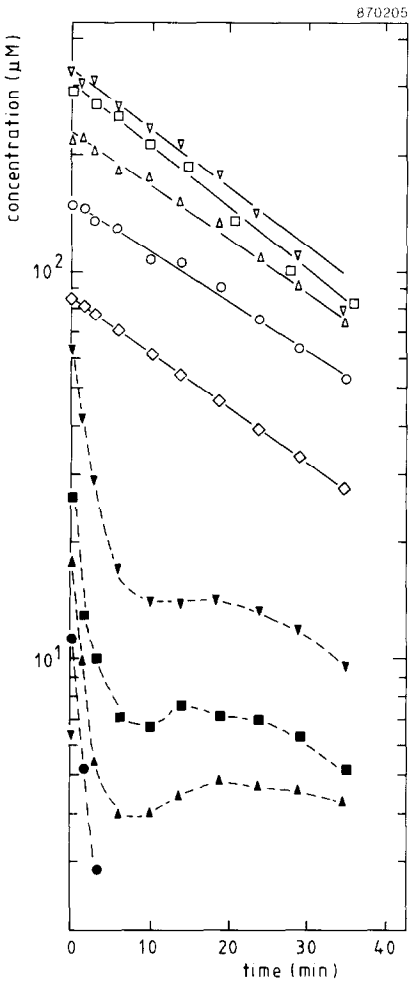

Fig. 5. Plots of the residual concentration of C(−)P(−)-soman (open symbols) and of C(−)P(+)-soman (filled symbols) vs time of hydrolysis for a C(−)P(−)-soman preparation by marmoset plasma (final concentration 3%) in 0.9% NaCl solution (pH 7.5, 37°). Different symbols refer to different runs performed with various initial C(−)P(−)-soman concentrations.

for hydrolysis runs with higher initial C(±)P(−)-isomer concentrations, i.e. increase in hydrolysis rate at the end of a run and enhanced rate constants, may be attributed to simultaneous epimerization at phosphorus. A considerably higher concentration of fluoride ions is generated in the reaction mixture with the highest initial soman concentrations. Fluoride ions induce epimerization at phosphorus [8] leading to the corresponding P(+)-epimer, which is enzymatically hydrolyzed at a high rate. Since the fluoride concentration increases during hydrolysis, this additional pathway becomes increasingly important, resulting in deviations from first-order kinetics. In this connection, the enhanced rate constants found for higher C(±)P(−)-isomer concentrations is assumed to be due to epimerization occurring at a rate sufficiently high to contribute to the overall rate of decay but apparently too slow to cause noticeable deviations from first-order kinetics. The smaller contribution of epimerization in case of C(−)P(−)-soman hydrolysis should be expected in view of its faster rate of hydrolysis by all enzyme preparations. Similarly, no indications were obtained for the involvement of this alternative pathway for C(±)P(−)-soman degradation by rat plasma, due to the relatively high rates at which these reactions proceed.

Values of $k_c + k_w$ (see eqn 3) are evaluated as the means of rate constants that are approximately independent of the initial C(±)P(−)-isomer concentrations, i.e. the rate constants obtained for hydrolysis of C(+)P(−)-soman at initial concentrations <0.15 mM, or ≤0.25 mM in case of marmoset liver homogenate, and those obtained for hydrolysis of C(−)P(−)-soman at initial concentrations <0.25 mM or from all runs in case of marmoset liver homogenate (see Table 2). Values of k_c are obtained after correction for spontaneous hydrolysis (Table 2).

The steady state concentrations of the P(+)-epimer formed by epimerization relative to the C(±)P(−)-isomer concentration depend on the ratio of the rate constants for formation and for hydrolysis of the C(±)P(+)-isomer. The rate of epimerization of the two C(±)P(−)-isomers should be approximately the same. Hence, a higher steady state concentration should be expected for C(−)P(+)- than for C(+)P(+)-soman when studying the hydrolysis of the C(±)P(−)-isomers at similar initial concentrations, due to the faster enzymatic hydrolysis of C(+)P(+)-soman. Indeed, residual C(−)P(+)-soman was found in the reaction mixtures of higher concentrations of C(−)P(−)-soman with all enzymes during the whole run (see for instance Fig. 5). C(+)P(+)-soman was only observed in runs with higher concentrations of C(+)P(−)-soman hydrolyzed by marmoset liver homogenate, which is the least active enzyme preparation for hydrolysis of C(+)P(+)-soman (Table 1).

Decreased rate constants at increased substrate concentrations. Deviations from first-order kinetics were also observed for hydrolysis of the C(±)P(−)-isomers with rat liver homogenate. In contrast with the above-mentioned results, however, the C(±)P(−)-isomer decay slightly increases at the end of each hydrolysis run, independent of the initial C(±)P(−)-isomer concentration used, and smaller k_{obs} values are obtained from the initial linear part of the semilogarithmic plots when using higher initial C(±)P(−)-isomer concentrations (Table 2). These observations are consistent with hydrolysis according to Michaelis–Menten kinetics (eqn 2). In order to evaluate values of K_m and V_{max}, initial rates, v_0, of the enzymatic hydrolysis were estimated from k_{obs} values after correction for spontaneous hydrolysis according to

$$v_0 = (k_{obs} - k_w)S_0 \quad (4)$$

K_m and V_{max} values are estimated from sets of v_0, S_0 data by using the nonlinear regression method of Wilkinson [12]. The K_m values are approximately one order of magnitude higher than most values found for the C(±)P(+)-isomers (see Table 2).

For comparison, the values of the first-order rate constants for catalyzed hydrolysis, k_c or V_{max}/K_m, are calculated for hydrolysis with 1 ml 0.6% plasma

Table 2. Means and standard deviations of first-order rate constants (k_{obs}) obtained for hydrolysis of C(+)P(−)- and C(−)P(−)-soman in 0.9% NaCl solution in the presence of rat, guinea-pig and marmoset liver homogenate and plasma, and of human plasma (pH 7.5, 37°), for three ranges of initial C(±)P(−)-isomer concentrations (The average values of the rate constant of catalysis by liver homogenate and plasma (k_c) derived from these data are included)

Source*	Isomer	Average k_{obs}† (10^{-2} min^{-1}) for initial soman concentrations (mM) <0.15	0.15–0.25	>0.25	k_c‡ (10^{-2} min^{-1})
Plasma: man (3)	C(+)P(−)	0.68 ± 0.07 (2)	0.95 ± 0.09 (2)	1.4 ± 0.1 (6; 3)	0.3 (2; 0.09–0.12)
(3)	C(−)P(−)	2.0 ± 0.1 (4)	2.0 ± 0.1 (3)	2.5 ± 0.3 (4; 1)	1.6 (7; 0.06–0.23)
marmoset (3)	C(+)P(−)	0.95 ± 0.01 (2)	1.3 ± 0.2 (2)	1.7 ± 0.3 (6; 5)	0.6 (2; 0.10–0.11)
(3)	C(−)P(−)	3.0 ± 0.1 (3)	3.2 ± 0.1 (3)	3.6 ± 0.2 (4; 1)	2.8 (6; 0.09–0.23)
guinea pig (6)	C(+)P(−)	0.60 ± 0.10 (4)	0.85 ± 0.08 (5)	0.99 ± 0.11 (4; 4)	0.24 (4; 0.07–0.12)
(6)	C(−)P(−)	1.1 ± 0.1 (4)	1.3 ± 0.2 (4)	1.6 ± 0.1 (4)	0.8 (8; 0.07–0.25)
rat (6)	C(+)P(−)	1.7 ± 0.2 (2)	1.7 ± 0.1 (3)	1.9 ± 0.1 (5)	1.5 (10; 0.08–0.41)
(6)	C(−)P(−)	3.9 ± 0.1 (2)	3.7 ± 0.3 (5)	3.5 ± 0.3 (3)	3.2 (10; 0.07–0.33)
Liver: marmoset (1)	C(+)P(−)	0.96 ± 0.01 (2)	1.05 ± 0.07 (3)	1.4 ± 0.1 (5; 3)	0.7 (5; 0.09–0.25)
(1)	C(−)P(−)	1.7 ± 0.2 (2)	1.5 ± 0.1 (3)	1.7 ± 0.1 (4; 1)	1.3 (10; 0.08–0.37)
guinea pig (1.5)	C(+)P(−)	1.3 ± 0.1 (2)	1.5 ± 0.1 (4)	1.7 ± 0.1 (4; 4)	0.9 (2; 0.07–0.08)
(1.5)	C(−)P(−)	1.9 ± 0.1 (4)	1.9 ± 0.1 (2)	2.3 ± 0.1 (4; 2)	1.6 (6; 0.07–0.21)
rat (1.5)	C(+)P(−)	3.0 ± 0.9 (3; 3)	2.1 ± 0.3 (3; 3)	2.0 ± 0.1 (4; 4)	2.8§ (10; 0.06–0.41)
(1.5)	C(−)P(−)	4.1 ± 0.5 (4; 4)	3.2 ± 0.1 (2; 2)	3.0 ± 0.3 (4; 4)	4.4§ (10; 0.07–0.36)

* The figure in parentheses denotes the final concentration (%) of liver homogenate or plasma used in the hydrolysis experiments.

† The figure in parentheses denotes the number of k_{obs} values from which the mean value is calculated; a second figure denotes the number of runs in which deviations from first-order kinetics were observed at the end of the run.

‡ The value of k_c at the final concentration used of plasma or liver homogenate is the mean of k_{obs} values corrected for spontaneous hydrolysis. The number of k_{obs} values used and the range of initial C(±)P(−)-isomer concentrations (mM) at which these values were obtained are given in parentheses.

§ The value of k_c is calculated as the ratio of the V_{max} and K_m values estimated by using all k_{obs} values after correction for spontaneous hydrolysis; K_m is 0.50 ± 0.12 and 0.46 ± 0.20 mM and V_{max} is 14 ± 3 and 20 ± 5 nmole/min per ml 1.5% rat liver homogenate, for C(+)P(−)- and C(−)P(−)-soman, respectively.

or 0.15% liver homogenate; it is assumed that the rate of catalyzed hydrolysis is proportional to the plasma or liver homogenate concentration. These values together with those calculated for the C(±)P(+)-isomers in a similar way are given in Table 3. As for the C(±)P(−)-isomers, the guinea-pig plasma is less active than the plasma enzymes of the other species. The liver homogenates of guinea pig and marmoset show about the same activity; the highest activity is found for the rat liver.

Table 3. First-order rate constants, k_c, for hydrolysis of the four stereoisomers of soman, catalyzed by diluted liver homogenate and plasma from rat, guinea pig and marmoset, and by diluted human plasma (pH 7.5, 37°)

Source	k_c* (10^{-3} min^{-1}) C(+)P(−)	C(−)P(−)	C(−)P(+)	C(+)P(+)
Plasma: man	0.7	3.2	480	860
	(1)	(4.7)	(700)	(1300)
marmoset	1.2	5.5	310	1200
	(1)	(4.7)	(260)	(1000)
guinea pig	0.2	0.8	80	220
	(1)	(3.5)	(350)	(900)
rat	1.5	3.2	1200	3400
	(1)	(2.2)	(800)	(2300)
Liver: marmoset	1.0	1.9	40	90
	(1)	(2.0)	(40)	(90)
guinea pig	0.9	1.6	270	510
	(1)	(1.7)	(300)	(600)
rat	2.8	4.4	420	2500
	(1)	(1.5)	(150)	(900)

* Calculated for hydrolysis by 1 ml 0.6% plasma or 0.15% liver homogenate.

For the C(±)P(+)-isomers k_c equals V_{max}/K_m.

Figures in parentheses give the relative values of k_c for hydrolysis of the four stereoisomers catalyzed by the same plasma or liver homogenate. The value for C(+)P(−)-soman is arbitrarily set at 1.

Table 4. Nonenzymatic catalysis of the hydrolysis of C(+)P(±)- and C(−)P(±)-soman in 0.9% NaCl solution upon addition of human plasma in which the phosphorylphosphatase activity is inhibited by 0.1 M EDTA, and of bovine serum albumin (60 mg/ml) in Krebs Ringer buffer (pH 7.5, 37°) (For comparison, rate constants for hydrolysis of the C(±)P(−)-isomers by noninhibited human plasma are included)

Reaction medium*	Reactants	Rate constants† (10^{-3} min^{-1}) for P(+)-isomer	P(−)-isomer
0.9% NaCl/EDTA/plasma (3)	0.4 mM C(+)P(±)-soman	0.6	0.7
	0.4 mM C(−)P(±)-soman	1.0	1.1
0.9% NaCl/EDTA/plasma (6)	0.4 mM C(+)P(±)-soman	1.7	1.7
	1.1 mM C(−)P(±)-soman	1.8	1.7
0.9% NaCl/albumin in Krebs Ringer buffer (6)	0.6 mM C(−)P(±)-soman	1.4	1.4
0.9% NaCl/plasma (3)	C(+)P(−)-soman		3‡
	C(−)P(−)-soman		16‡

* The figure in parentheses denotes the final concentration (%) of human plasma or of albumin solution in Krebs Ringer buffer, in the reaction medium.

† Corrected for spontaneous hydrolysis in 0.9% NaCl or in 0.9% NaCl containing 0.1 M EDTA.

‡ Data from Table 2.

Nonenzymatic catalysis of the hydrolysis of soman stereoisomers

In order to obtain some information on the contribution of nonenzymatic catalysis of the C(±)P(−)-isomers by diluted human plasma, hydrolysis reactions were performed with C(+)P(±)- and C(−)P(±)-soman in the presence of 0.1 M EDTA. Under these conditions, the C(±)P(+)- and C(±)P(−)-isomers are hydrolyzed at a similarly slow rate (Table 4), confirming the finding of De Bisschop [6] that EDTA inhibits the phosphorylphosphatase activity. The inhibited rate of hydrolysis is slightly higher than in aqueous solution. This nonenzymatic catalysis affords only a small contribution (<7%) to the noninhibited hydrolysis of C(−)P(−)-soman by human plasma, whereas the rate constant of the noninhibited hydrolysis of C(+)P(−)-soman exceeds more than three times that of the nonenzymatic hydrolysis by the plasma. The acceleration of soman hydrolysis brought about by addition of a 6% bovine serum albumin solution in Krebs Ringer buffer is approximately equal to that found for the nonenzymatic acceleration by diluted human plasma (Table 4).

DISCUSSION

Values of K_m and V_{max} were evaluated for the hydrolysis of the C(±)P(+)-isomers of soman catalyzed by phosphorylphosphatases from rat, guinea-pig and marmoset plasma and liver, and from human plasma. These isomers, which are very weak acetylcholinesterase inhibitors and relatively nontoxic [8], are rapidly degraded by the enzymes. Especially the plasma enzymes show a high affinity for these substrates.

K_m and V_{max} could not be determined for the toxic C(±)P(−)-isomers, except in case of the rat liver enzyme. At first glance it might be concluded that the values of K_m of the other enzymes towards the C(±)P(−)-isomers are evidently greater than the highest substrate concentrations that could be used (0.4–0.5 mM). Rapid epimerization will hamper a proper investigation of the catalyzed hydrolysis of the C(±)P(−)-isomers at substrate concentrations >0.5 mM. Actually, this reaction already prevents the determination of reliable rate constants at the highest concentrations used. It should be taken into account that saturation of the enzyme and increasing formation of the corresponding C(±)P(+)-isomer via epimerization at higher concentrations of the C(±)P(−)-isomers will have opposite effects on the rate of hydrolysis. Therefore, a decrease of the rate constant caused by some saturation might go unnoticed due to the compensating effect of epimerization. Consequently, it cannot be ruled out that K_m values for the C(±)P(−)-isomers are of the order of magnitude of the highest substrate concentration used.

Alternatively, the observation of first-order kinetics for the hydrolysis of the C(±)P(−)-isomers might indicate that the acceleration of hydrolysis is not caused by an enzymatic reaction, but is due to a general catalysis by plasma or liver constituents. Acceleration of the spontaneous hydrolysis of the analogous organophosphate sarin (isopropyl methylphosphonofluoridate) by anions, such as hydrogen phosphates and hydrogen carbonates (Krebs Ringer buffer), was demonstrated by Christen [13]. Albumin also accelerated the hydrolysis, possibly due to esterase-like activity [14]. Similar results were recently obtained by De Bisschop [6] for the hydrolysis of the soman stereoisomers. Moreover, this investigator presented results suggesting that hydrolysis of the C(±)P(−)-isomers in human plasma occurs mainly according to this alternative mechanism. In the present study evidence for enzymatic hydrolysis of the C(±)P(−)-isomers by diluted human plasma was obtained from the considerable retardation of the hydrolysis of the isomers after inhibition of phosphorylphosphatase activity with EDTA. Since the low nonenzymatic catalysis of the hydrolysis of the C(±)P(−)- or C(±)P(+)-isomers induced by diluted human plasma is similar to the enhancement brought about by an albumin solution in Krebs Ringer buffer, it is assumed that the nonenzymatic

contribution to soman hydrolysis by human plasma is representative for that of other species. Consequently, nonenzymatic catalysis probably affords a minor contribution (<30%) to the observed hydrolysis rates of the C(±)P(−)-isomers in diluted rat, guinea-pig and marmoset plasma, except for the hydrolysis of C(+)P(−)-soman by guinea-pig plasma. In the latter case, nonenzymatic catalysis is probably the major pathway (70%; see Tables 2 and 3).

Upon addition of soman to plasma or liver homogenate, the soman concentration decreases not only because of hydrolysis but also due to irreversible reactions with binding sites. Christen and Cohen [15] found a relatively high concentration of irreversible binding sites in rat plasma, the concentrations in guinea-pig and human plasma being threefold to fivefold lower. In rat plasma, carboxylesterases are the major binding sites [16]; the concentration of active sites is *ca.* 3 μM [15, 17]. During enzymatic hydrolysis of the C(±)P(−)-isomers the carboxylesterase concentration in diluted rat plasma is *ca.* 0.18 μM, and even lower during hydrolysis of the C(±)P(+)-isomers. Hence, the contribution of irreversible binding to the decrease of soman concentration can be neglected even in rat plasma ($S_0 \geq 0.07$ mM). Consistent with this conclusion, an initially rapid decrease of the concentration of the C(±)P(−)-isomers due to a contribution of irreversible binding was not observed.

The first-order rate constant, V_{max}/K_m, controls the rate of enzymatic hydrolysis at low substrate concentrations ($S_0 \ll K_m$). This parameter probably determines the enzymatic hydrolysis rate *in vivo* after administration of C(±)P(±)-soman. Comparison of the first-order rate constants for catalyzed hydrolysis, V_{max}/K_m or k_c, reveals that all enzyme preparations studied hydrolyze the soman stereoisomers in the order C(+)P(+)- > C(−)P(+)- ≫ C(−)P(−)- > C(+)P(−)-soman (Table 3).

Qualitatively, a similar ranking was found by De Bisschop *et al.* [5] for catalyzed hydrolysis in twofold diluted human plasma, i.e. 235:29:1.7:1. The most outspoken difference in activity towards the C(±)P(+)- and C(±)P(−)-isomers is found for rat plasma, whereas the results for marmoset liver homogenate show the smallest ratio. The data for rat plasma and liver are fully in accordance with observations made in studies on toxicokinetics of C(±)P(±)-soman in the rat: a much faster elimination of the C(±)P(+)-isomers, C(+)P(+)-soman being most labile [7, 18, 19].

The rates of degradation of the C(±)P(+)-isomers and, more interesting, also of the highly toxic C(±)P(−)-isomers, by liver homogenate and plasma are species-dependent. The guinea-pig plasma is less potent than the other plasmata studied in acceleration of the degradation of the C(±)P(−)- and C(±)P(+)-isomers, whereas both the guinea-pig and the marmoset liver homogenates show a lower activity than rat liver. In contrast with these results, Christen [13] found approximately equal activities for rat, guinea-pig and human plasma towards the (toxic) P(−)-isomer of the analogous phosphonofluoridate sarin. Dependence of the relative activities of phosphorylphosphatases from various species on the structure of the substrate was reported by Becker and Barbaro [20]. These investigators found that the relative activities of guinea-pig, human and rabbit plasma towards a series of ethyl *p*-nitrophenyl phosphonates vary with the length of the carbon chain linked to the phosphorus atom.

The large P(+)-/P(−)-stereoselectivity offers the possibility to study the enzymatic hydrolysis of C(+)P(+)-soman by using C(+)P(±)-soman, as shown in this work. Possibly, the hydrolysis of C(−)P(+)-soman can be studied analogously by using C(−)P(±)-soman. When determining the initial rate of catalyzed hydrolysis of C(±)P(±)-soman, the rate of degradation of the C(±)P(+)-isomers will be obtained, especially that of C(+)P(+)-soman. The degradation of the C(±)P(−)-isomers will mainly be measured in the second phase of hydrolysis. Consistently, somanase activities (pH 7.3, 25°) determined by Harris *et al.* [21] in rat plasma (9.3 μmole/min/ml plasma) and human plasma (2.7 μmole/min/ml plasma) at rather high (1 mM) soman concentration are in the same order of magnitude as our extrapolated data for V_{max} of C(+)P(+)-soman (14 and 6.5 μmole/min/ml plasma, respectively; see Table 1).

The large differences in anticholinesterase activities between the C(±)P(−)- and C(±)P(+)-isomers of soman are reflected in differences in toxicity [8]. These differences in toxicity are even enhanced by the preferential degradation of C(±)P(+)-isomers. So far, stereoselectivity of phosphorylphosphatase-catalyzed hydrolysis has been demonstrated for only a small number of other chiral organophosphates [22–30] (for a recent review see [31]). As for soman, the less toxic enantiomers of these compounds are more rapidly degraded.* In view of these results, it should be kept in mind that initial rate of enzymatic hydrolysis for C(±)P(±)-soman and for the racemic mixtures of other chiral organophosphates, as usually determined to characterize enzymatic activity, are generally not relevant for the rate of detoxification of the compound, i.e. for the rate of degradation of the toxic isomer(s).

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* The, generally low, stereoselectivity for hydrolysis of the nerve agent tabun (ethyl dimethylphosphoramidocyanidate) appears to be species-dependent [29, 30].

REFERENCES

1. Mazur A, An enzyme in animal tissue capable of hydrolyzing the phosphorus-fluorine bond of alkyl fluorophosphates. *J Biol Chem* **146**: 271–289, 1946.
2. Mounter LA, Metabolism of organophosphorus anticholinesterase agents. In: *Handbuch der Experimentellen Pharmakologie*, Vol. XV *Cholinesterases and Anticholinesterase Agents* (Ed. Koelle GB), pp. 486–504. Springer, Berlin, 1963.
3. Benschop HP, Konings CAG and De Jong LPA, Gas chromatographic separation and identification of the four stereoisomers of 1,2,2-trimethylpropyl methylphosphonofluoridate (soman). Stereospecificity of in

vitro "detoxification" reactions. *J Am Chem Soc* **102**: 4260–4262, 1981.
4. De Bisschop HC, Mainil JG and Willems JL, *In vitro* degradation of the four stereoisomers of soman in human serum. *Biochem Pharmacol* **34**: 1895–1900, 1985.
5. De Bisschop HC, Van Driessche EE, Alberty MLM and Willems JL, *In vitro* detoxification of soman in human plasma. *Fundam Appl Toxicol* **5**: S175–S179, 1985.
6. De Bisschop HCJV, Biodegradation of 1,2,2-trimethylpropyl methylphosphonofluoridate (soman). Studies in human and canine serum and plasma. Ph.D. thesis, Catholic University of Nijmegen, The Netherlands, 1986.
7. Benschop HP, Bijleveld EC, De Jong LPA, Van der Wiel HJ and Van Helden HPM, Toxicokinetics of the four stereoisomers of the nerve agent soman in atropinized rats—Influence of a soman simulator. *Toxicol Appl Pharmacol* **90**: 490–500, 1987.
8. Benschop HP, Konings CAG, Van Genderen J and De Jong LPA, Isolation, anticholinesterase properties, and acute toxicity in mice of the four stereoisomers of soman. *Toxicol Appl Pharmacol* **72**: 61–64, 1984.
9. Benschop HP, Bijleveld EC, Otto MF, Degenhardt CEAM, Van Helden HPM and De Jong LPA, Stabilization and gas chromatographic analysis of the four stereoisomers of 1,2,2-trimethylpropyl methylphosphonofluoridate (soman) in rat blood. *Anal Biochem* **151**: 242–253, 1985.
10. Goldenberg H, Rectification of nonlinear enzyme activity curves—I. Preliminary. *Arch Biochem Biophys* **52**: 288–291, 1954.
11. Boeker EA, Initial rates. A new plot. *Biochem J* **203**: 117–123, 1982.
12. Wilkinson GN, Statistical estimations in enzyme kinetics. *Biochem J* **80**: 324–332, 1961.
13. Christen PJ, De stereospecifieke enzymatische hydrolyse van sarin in plasma. Ph.D. thesis, State University of Leiden, The Netherlands, 1967.
14. Yoshida K, Kurono Y, Mori Y and Ikeda K, Esterase-like activity of human serum albumin—V. Reaction with 2,4-dinitrophenyl diethyl phosphate. *Chem Pharm Bull* **33**: 4995–5001, 1985.
15. Christen PJ and Cohen EM, Binding of [^{32}P]-sarin to esterases and other proteins in plasma from rat, man and guinea pig. *Acta Physiol Pharmacol Neerl* **15**: 36–37, 1969.
16. Polak RL and Cohen EM, The influence of triorthocresyl phosphate on the distribution of [^{32}P] in the body of the rat after the injection of [^{32}P]-sarin. *Biochem Pharmacol* **18**: 813–820, 1969.
17. De Jong LPA and Van Dijk C, Formation of soman (1,2,2-trimethylpropyl methylphosphonofluoridate) via fluoride-induced reactivation of soman-inhibited aliesterase in rat plasma. *Biochem Pharmacol* **33**: 663–669, 1984.
18. Benschop HP, Berends F and De Jong LPA, GLC-analysis and pharmacokinetics of the four stereoisomers of soman. *Fundam Appl Toxicol* **1**: 177–182, 1981.
19. Benschop HP, Konings CAG, Van Genderen J and De Jong LPA, Isolation, *in vitro* activity, and acute toxicity in mice of the four stereoisomers of soman. *Fundam Appl Toxicol* **4**: S84–S95, 1984.
20. Becker EL and Barbaro JF, The enzymatic hydrolysis of *p*-nitrophenyl ethylphosphonates by mammalian plasma. *Biochem Pharmacol* **13**: 1219–1227, 1964.
21. Harris L, Broomfield C, Adams N and Stitcher D, Detoxification of soman and *O*-cyclopentyl *S*-diethylaminoethyl methylphosphonothioate *in vivo*. *Proc West Pharmacol Soc* **27**: 315–318, 1984.
22. Christen PJ and Van den Muysenberg JACM, The enzymatic isolation and fluoride catalysed racemization of optically active sarin. *Biochim Biophys Acta* **110**: 217–220, 1965.
23. Lee PW, Allahyari R and Fukuto TR, Studies on the chiral isomers of fonofos and fonofos oxon—II. *In vitro* metabolism. *Pestic Biochem Physiol* **8**: 158–169, 1978.
24. Ohkawa H, Mikami N and Miyamoto J, Stereoselectivity in metabolism of the optical isomers of cyanofenphos (*O*-*p*-cyanophenyl *O*-ethyl phenylphosphonothioate) in rats and liver microsomes. *Agric Biol Chem* **41**: 369–376, 1977.
25. Nomeir AA and Dauterman WC, Stereospecific hydrolysis of the optical isomers of *O*-ethyl *O*-*p*-nitrophenyl phenylphosphonate by liver microsomes. *Biochem Pharmacol* **28**: 2407–2408, 1979.
26. Dudman NPB and Zerner B, Enzymatic preparation of an optically active phosphotriester, asymmetric only at phosphorus. *J Am Chem Soc* **95**: 3019–3021, 1973.
27. Grothusen JR and Brown TM, Stereoselectivity of acetylcholinesterase, arylester hydrolase, and chymotrypsin toward 4-nitrophenyl alkyl(phenyl) phosphinates. *Pestic Biochem Physiol* **26**: 100–106, 1986.
28. Ooms AJJ and Boter HL, Stereospecificity of hydrolytic enzymes in their reaction with optically active organophosphorus compounds—I. The reaction of cholinesterases and paraoxonase with *S*-alkyl *p*-nitrophenyl methylphosphonothiolates. *Biochem Pharmacol* **14**: 1839–1846, 1965.
29. Augustinsson K-B, Enzymatic hydrolysis of organophosphorus compounds—VII. The stereospecificity of phosphorylphosphatases. *Acta Chem Scand* **11**: 1371–1377, 1957.
30. Degenhardt CEAM, Van den Berg GR, De Jong LPA, Benschop HP, Van Genderen J and Van de Meent D, Enantiospecific complexation gas chromatography of nerve agents. Isolation and properties of the enantiomers of ethyl *N*,*N*-dimethylphosphoramidocyanidate (tabun). *J Am Chem Soc* **108**: 8290–8291, 1986.
31. De Jong LPA and Benschop HP, Biochemical and toxicological implications of chirality in anticholinesterase organophosphates. In: *Stereoselectivity of Pesticides. Biological and Chemical Problems* (Eds. Ariëns EJ, Van Rensen JJS and Welling W), pp. 109–149. Elsevier, Amsterdam, 1988.