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IN VITRO DEGRADATION OF THE STEREOISOMERS OF SOMAN IN GUINEA-PIG, MOUSE AND HUMAN SKIN

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Abstract—The fate of the four stereoisomers of soman [C(−)P(+), C(+)P(+), C(+)P(−) and C(−)P(−)] was studied by incubating 10 μM C(±)P(±)-soman at pH 7.4 and 37° for various periods in the presence or absence of homogenates (1:10 and 1:20 w/v) of guinea-pig, mouse or human skin. The remaining concentrations of the soman isomers were determined gas chromatographically. Similar rates of spontaneous (non-enzymatic) hydrolysis ($K = 0.005$ min^{-1}) were found for the four isomers of soman. Hydrolysis of the toxic (C(±)P(−)-isomers is not accelerated in the presence of the skin homogenates. In contrast, the non-toxic C(±)P(+)-isomers are enzymatically hydrolysed. As the amount of proteins present in the homogenates varied the rate constants for enzyme hydrolysis per protein concentration were calculated. Except for the high hydrolysis rate constant of >0.127/min.g.l for C(+)P(+) in human skin, these values were almost similar (0.031–0.045/min.g.l) for the skin homogenates tested. Irreversible binding sites for the four soman-stereoisomers are only found in homogenates of mouse skin; 122–195 pmol soman-isomer are bound per mg protein. After preincubation of mouse homogenate with 10 μM soman during 18 hr at 0–4° no further binding of the isomers was detected. It is concluded that skin of the three species tested does not contain enzymes that degrade the toxic C(±)P(−)-isomers of soman, whereas phosphorylphosphatase activity for the C(±)P(+)-isomers is present in the skin of all three species. Binding sites for all four soman isomers are only present in mouse skin.

Soman is a highly toxic organophosphorus compound by virtue of its extremely strong potency as inhibitor of acetylcholinesterase. Soman consists of a mixture of four stereoisomers, i.e. C(−)P(+), C(+)P(+), C(+)P(−) and C(−)P(−), respectively, due to the presence of two asymmetric centers: one at the pinacolyl moiety and the other at the phorphorus atom.

The concentration of free soman *in vivo* can be reduced by two processes: (1) hydrolysis, either spontaneous (non-enzymatic) hydrolysis or enzymatic hydrolysis catalysed by A-esterases (e.g. phosphorylphosphatases; [1]) or (2) irreversible binding to B-esterases (e.g. carboxylesterases and cholinesterases) or to other proteins [2].

The rates of degradation vary widely for the four stereoisomers of soman by either hydrolysis or irreversible binding [3]. The C(±)P(−)-isomers of soman appear to be the toxic ones in mice due to a relatively slow enzymatic hydrolysis and their potent anticholinesterase properties [4]. Although intoxication by soman may occur after contamination of the skin, only a few studies have been performed on the fate of soman penetrating the skin. Fredriksson [5] showed that homogenates of guinea-pig skin contain an enzyme which is particularly active in splitting soman. Van Hooidonk *et al.* [6] concluded that soman is substantially degraded in the skin. They measured the penetration of ^{32}P-soman through excised skin from humans and from guinea-pigs, using a combination of a radiometric method and an enzymatic method. Recently, in our laboratory the penetration of soman through the skin from nude mice was investigated *in vivo* in comparison with the penetration of this agent through human skin grafted onto nude mice [7]. It was found that human skin is less permeable to soman than mouse skin and that soman is degraded in both types of skin.

From the above-mentioned studies it can be concluded that substantial amounts of soman can be degraded in skin. However, these studies did not discriminate between the degradation of the four stereoisomers of soman separately. Insight into the mechanisms whereby detoxification of the separate stereoisomers occurs in various species may be very important for the development of countermeasures. In the present study hydrolysis and irreversible binding of the four soman isomers were investigated employing guinea-pig, mouse and human skin.

MATERIALS AND METHODS

Materials. C(±)P(±)-soman (1,2,2-trimethylpropyl methylphosphonofluoridate) and C(−)P(+)-isomer of (U-^{2}H)-1,2,2,-trimethylpropyl methylphosphonofluoridate were synthesized at the Prins Maurits Laboratory TNO (Rijswijk, The Netherlands).

Dye reagent concentrate was obtained from Bio-Rad Laboratories (Munchen, West Germany).

All other chemicals were commercial products of analytical grade.

Homogenization of skin. Outbred, male guinea-pigs (Dunkin' Hartley, 6–7-week-old, weight 500–600 g) were purchased from Charles River (West Germany). The abdominal skin from the guinea-pigs was clipped and shaved 24 hr prior to excising.

Congenitally athymic (nude) male mice (C57 B1/KA-nu/nu, 10–12-week-old, body weight approximately 20 g) were obtained from REP-Institutes

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TNO (Rijswijk, The Netherlands). Before being skinned, guinea-pigs and mice were killed by carbogen-air. Abdominal and back skin of mice and abdominal skin of guinea-pigs (epidermis and dermis, thickness approximately 0.5 mm) were taken.

Mammary skin of humans was obtained fresh from patients undergoing cosmetic operations. Varying amounts of the dermis were trimmed so that the thickness of the human skin approximated 0.5 mm (see also [8]).

The skins were weighed and the areas were measured before homogenization. The homogenization procedure was performed at 0–4°. Skin (approximately 1.5–2 g) was cut up fine with a pair of scissors and was homogenized with a glass–glass homogenisator in 0.154 M NaCl/NaOH, pH 7.4 (1:10 or 1:20, w/v). For homogenization of human skin, it was necessary to treat the skin/0.154 M NaCl, pH 7.4 mixture with an ultra-turrax three times during 15 sec before treating it with the glass–glass potter.

Preincubation. In some experiments homogenates were preincubated with soman before studying the degradation of soman. Preincubation was performed by adding 10 μl C(±)P(±)-soman (given a concentration of 10 μM) to 10 ml skin homogenate (1:10, w/v) in 0.154 M NaCl, pH 7.4. The mixture was kept at 0–4° for 18 hr. The remaining soman was determined gas chromatographically (see below).

Degradation experiments. Homogenate, preincubate homogenate or 0.154 M NaCl (10 ml) was thermostatically kept at 37°. Incubation was started by addition of 10 μl C(±)P(±)-soman (given a concentration of 10 μM). During incubation the solution was stirred magnetically and was kept at pH 7.4 by means of a pH-stat equipment (Radiometer). Two samples of 200 μl were taken every 5 min during 30 min of incubation. The first two samples ($t = 0$) were taken within 15 sec after the addition of soman. Each sample was added to 1 ml hexane containing 40 ng C(−)P(+)-isomer of (U-^{2}H)-1,2,2-trimethylpropyl methylphosphonofluoridate as internal standard. The mixture was stirred on a Vortex for 30 sec. Subsequently, the organic layer was taken off and analysed.

Analysis of soman-isomers. Determination of the concentration of the four soman-isomers was performed by using a Carlo Erba gas chromatograph equipped with a wide bore (i.d. = 0.50 mm) Chirasil-Val (Type II) column (length = 50 m) which was kindly obtained from the Prins Maurits Laboratory TNO (Rijswijk, The Netherlands). The conditions of the gas chromatographic procedure were identical to those described by Benschop *et al.* [3]. In short, after cold on-column injection of 2 μl of the organic layer chromatography was performed at 110°. The temperature of the nitrogen–phosphorus detector was 275°. Helium was used as carrier gas at a flow of 2.5 ml/min.

Amounts of the isomers in the 200 μl samples were calculated from the ratios of peak heights of the isomers and the internal standard using calibration curves constructed with various concentrations of racemic soman with addition of known amounts of the internal standard. The concentrations of remaining soman-isomers in the incubation media were calculated from the amounts of the isomers found in the duplicate 200 μl samples.

Table 1. Weights of 1 cm^2 skin (thickness of 0.5 mm) and concentrations of protein in homogenates of skin

Type of skin	Weight of skin (mg)	Protein concentration (g/l)
Guinea-pig	110 ± 4 (8)	2.4 ± 0.1 (6)
Mouse	49 ± 6 (5)	4.7 ± 0.2 (6)
Human	96 ± 6 (3)	3.1 ± 0.3 (7)

Abdominal skin from guinea-pigs, abdominal and back skin from mice and mammary skin from humans were taken. Homogenates were prepared by homogenizing skin in 0.154 M NaCl, pH 7.4 (1:10, w/v). Results are given as mean ± SEM, the number of animals in parentheses.

Calculation of hydrolysis rate constants. First-order rate constants of the hydrolysis were determined from the plot of the logarithm of the percentage of remaining soman versus time, using linear-least-squares curve fitting. The enzymatic hydrolysis rate constants were calculated as the differences between the total hydrolysis rate constants obtained from incubation experiments with the homogenate and the rate constant for spontaneous hydrolysis (no homogenate added).

Calculation of amount of binding sites. The concentration of binding sites was evaluated from the difference between the starting soman-isomer concentration and the isomer concentration calculated from the *Y*-intercept in the linear plot of the logarithm of the percentage of remaining soman versus time.

Determination of protein concentration. Protein concentration was determined in triplicate by a dye binding method [9] as modified by Bio-Rad using bovine serum albumin as a standard.

RESULTS

Characteristics of the skin tissues used, namely weight per cm^2 and protein content per g tissue are collected in Table 1. The weight per cm^2 is the lowest for the mouse tissue, whereas after homogenization in 0.154 M NaCl (1:10, w/v) the protein concentration of tissue homogenate was highest for this tissue and lowest for the tissue of guinea-pigs. The variations in weight and protein concentration may be due to differences in the ratio of epidermis and dermis. It is not likely that the variations in protein concentrations may be due to variations in the presence of insoluble material in the homogenates, since the standard errors of the protein concentrations estimated in triplicate were very small.

Little spontaneous hydrolysis of the four soman-isomers occurred in 0.154 M NaCl at 37° and pH 7.4 for 30 min (Fig. 1). No significant differences were found in rate constants (C(±)P(±): $k = 0.005 \pm 0.003$/min/mean ± SEM, N = 16).

The results of the degradation of C(±)P(±)-soman in the homogenates of guinea-pig, mouse and human

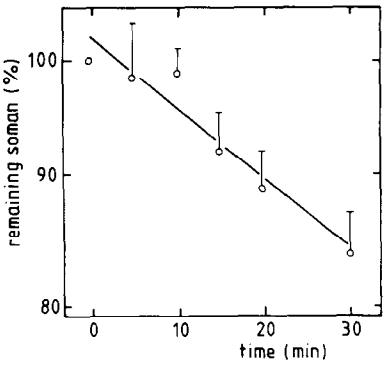

Fig. 1. Spontaneous degradation of C(+)P(+)-soman (○). C(±)P(±)-soman (10 μM) was incubated in 0.154 M NaCl at 37° and pH 7.4. After different times the remaining concentrations of the four soman isomers were determined. The degradation of the three other isomers proceeded similarly. SEM (N = 16) is indicated by the bars.

skin are shown in Fig. 2. The concentrations of the C(±)P(−)-isomers remaining in the incubation medium after various periods were consistently larger than those of the C(±)P(+)-isomers. No difference was found between the lines of the C(+)P(−)- and C(−)P(−)-isomers, except for the intercepts of the lines obtained from the experiments with the homogenate of mouse skin (1:10, w/v, Fig. 2e); the slopes of both curves, however, are similar. The degradation of the C(+)P(+)- and C(−)P(+)-isomers proceeds similarly in the homogenates of guinea-pig and mouse skin. In homogenates of human skin the C(+)P(+)-isomer is very rapidly degraded and could not be detected any more after 5 min of incubation.

The total hydrolysis rate constants were calculated using all the data points for the C(±)P(−)-isomers and the first 3–5 points of the curves from Fig. 2 for the C(±)P(+)-isomers (Table 2). As the amounts of proteins present in the homogenates varied, the rate constants for enzymatic hydrolysis per protein concentration were calculated. Apparently, these values are independent of the protein concentration used (1:20 and 1:10 w/v homogenate). The enzymatic hydrolysis rate constants of the C(±)P(+)-isomers are much higher than those of the C(±)P(−)-isomers. Hypdrolysis of the C(±)P(−)-isomers is not accelerated by the homogenates.

The concentration of irreversible binding sites was calculated from the *Y*-intercepts of the semi-logarithmic plots for soman decay (Fig. 2). In the 1:20 (w/v) homogenates and in 1:10 (w/v) homogenates of guinea-pig and human skin (not shown) hardly any binding sites were detectable. All soman-isomers were almost equally bound in 1:10 (w/v) homogenates of mouse skin. In order to ascertain that the

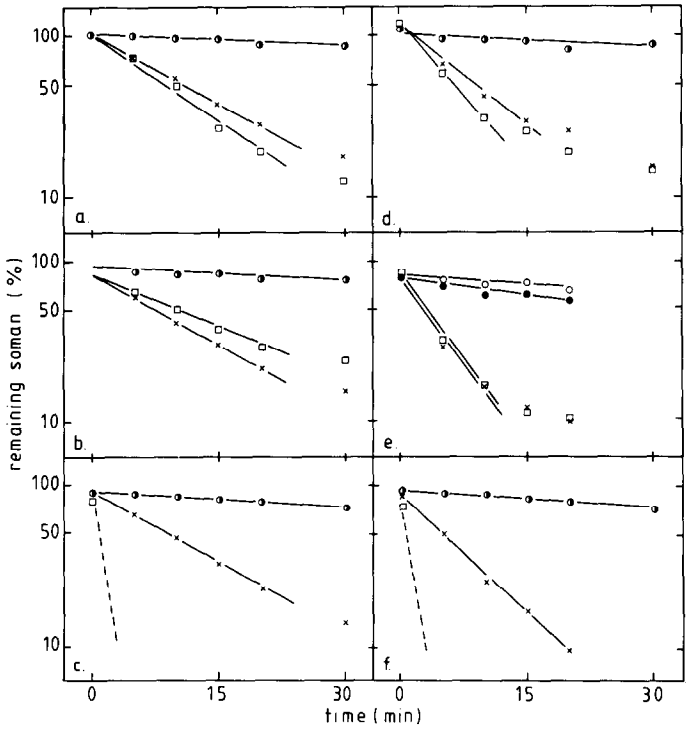

Fig. 2. Degradation of C(−)P(+)—(×), C(+)P(+)—(□), C(+)P(−)—(●) and C(−)P(−)—(○) soman by 1:20 (w/v; a, b, c) and 1:10 (w/v; d, e, f) skin homogenate. Homogenates of guinea-pigs (a, d), mice (b, e) and humans (c, f) were incubated with C(±)P(±)-soman (10 μM) in 0.154 M NaCl at 37° and pH 7.4 and after different time periods the remaining concentrations of the four soman-isomers were determined. The SEM values (N = 5–6) are not shown, but are smaller than 10%. The degradation of C(+)P(+)-isomers in human skin is drawn with dotted lines (c+f), because no C(+)P(+)-isomers were detectable after 5 min.

Table 2. Enzymatic hydrolysis rate constants of the four soman isomers in homogenates of guinea-pig, mouse and human skin

Type of skin	Rate constant (10^{-3}/min.g.l)				N
	C(−)P(+)	C(+)P(+)	C(+)P(−)	C(−)P(−)	
Guinea-pig (1:20)	45 ± 3**	55 ± 3**	1 ± 1	2 ± 1	6
Guinea-pig (1:10)	37 ± 5**	41 ± 5**	6 ± 1	6 ± 5	6
Mouse (1:20)	31 ± 4**	25 ± 3**	1 ± 1	0 ± 1	5
Mouse (1:10)	33 ± 2**	32 ± 3**	4 ± 1	3 ± 1	4
Human (1:20)	41 ± 5**	>127**	2 ± 1	2 ± 1	7
Human (1:10)	39 ± 5**	>63**	2 ± 1	2 ± 1	7

The enzymatic hydrolysis rate constants were calculated by subtracting the spontaneous hydrolysis rate constants* from the total hydrolysis rate constants obtained from the results shown in Fig. 2 and dividing by the concentration of protein (Table 1). The results are given as means ± SEM.

* The spontaneous hydrolysis rate constants of the C(±)P(±)-isomers were $(5 \pm 3) \times 10^{-3}$/min.

** The total hydrolysis rate constant (Fig. 1) was significantly different ($P < 0.01$; Student's *t*-test) from the spontaneous hydrolysis rate constant.

binding in mouse skin homogenate is caused by irreversible binding the effect of preincubation of mouse skin with 10 μM soman for 18 hr at 0–4° was studied. After preincubation no C(±)P(+)-soman isomers, but 40% of the C(+)P(−)-isomer and 55% of the C(−)P(−)-isomer were left. Rate constants for enzymatic hydrolysis of 10 μM soman in preincubated homogenate were similar to those found in non-preincubated homogenate; no binding of the isomers was detected any more.

In homogenates from human skin binding of C(+)P(+)-isomer could not be analysed due to rapid degradation (Fig. 2c,f). Lowering the incubation temperature to 0–4° reduced considerably the hydrolysis rate constants [C(−)P(+): 0.008, C(+)P(+): 0.055, C(+)(P−): 0.003 and C(−)P(−): 0.003/min.g.l]. The hydrolysis of C(+)P(+)-isomer was still too rapid for estimation of binding.

DISCUSSION

Intoxication with organophosphates may occur after exposure of skin with these agents. In the present study the degradation of soman-isomers was studied in homogenates of guinea-pig, mouse and human skin. Those homogenates were incubated with 10 μM soman, a concentration which was close to the amount applied on 1 cm^2 skin in a previous study on skin penetration in our laboratory [7].

Spontaneous hydrolysis of soman-isomers was determined in order to be able to differentiate between spontaneous and enzymatic catalysed hydrolysis. It appeared that the four stereoisomers underwent hydrolysis at a similarly slow rate. Previously, the same phenomenon was observed by Broomfield *et al.* ([10], $k = 0.0003$/min). However, De Bisschop *et al.* [11] have found different rates of spontaneous hydrolysis for the soman-diastereoisomers C(−)P(+) and C(+)P(−): $k = 0.014\ min^{-1}$; C(−)P(−) and C(+)P(+): $k = 0.022\ min^{-1}$). Since the incubation conditions varied considerably, differences in absolute values of initial rate constants may be due to them.

First-order kinetics were observed for the hydrolysis process of C(±)P(±)-soman in the skin homogenate as should be expected at the low initial concentrations of soman used (Fig. 2). A deviation from linearity was found for the C(±)P(+)-isomers during the incubation when >80% of the isomers were degraded. This non-linearity may be due to the inaccuracy of the determination of the remaining soman concentration which approaches the detection limit of the gas chromatographic analysis (approximately 0.2 pmol).

In the present study it was found that in skin homogenates the non-toxic C(±)P(+)-isomers of soman are hydrolysed more rapidly than the toxic C(±)P(−)-isomers (Fig. 2) which agrees with similar results in blood [3, 11–14]. As it is very unlikely that enzymes from blood might have contaminated our skin homogenates, the hydrolysis of the C(±)P(+)-isomers must be attributed to skin enzymes. A difference in rate of hydrolysis between C(+)P(+)- and C(−)P(+)-isomers we only found in human skin. A difference in the rate of hydrolysis between the two P(+)-isomers was also found in human serum [11, 15], in mouse plasma and by phosphorylphosphatase activity isolated from porcine kidney [14].

Since total hydrolysis rate constants of C(±)P(−)-isomers in skin did not differ from spontaneous hydrolysis rate constants, it can be concluded that the hydrolysis process of C(±)P(−)-isomers is not catalysed by skin enzymes. Previously, De Bisschop *et al.* [11] also concluded that in human blood these isomers are also not enzymatically hydrolysed.

Irreversible binding sites for soman were only found in mouse skin homogenates. They contain about 0.7 nmol irreversible binding sites per mg protein (Table 3), a concentration that seems high when compared with that found in other tissues by Reynolds *et al.* [16] after injection of ^{3}H-soman intravenously to mice. This difference may be due to a difference in accessibility to binding sites *in vivo* and *in vitro*.

Table 3. Concentrations of irreversible binding sites of soman isomers in homogenates (1:10, w/v) of mouse skin

Type of skin	Concentration of binding sites (pmol/mg protein)			
	C(−)P(+)	C(+)P(+)	C(+)P(−)	C(−)P(−)
Mouse	195 ± 38*	186 ± 30*	185 ± 48*	122 ± 36*

The concentrations were calculated by subtracting the concentrations found using the *Y*-intercepts of the degradation curves (Fig. 2) from the initial concentrations of the isomers and dividing by the amount of protein (Table 1). The results are given as mean ± SEM (N = 4).

Y-intercept was significantly different from 100%. $P < 0.01$; Student's *t*-test.

Up to now, the degradation of soman in skin has been investigated in a very limited number of studies [5–7]. Fredriksson [5] has found with the aid of a titrator that the homogenate of guinea-pig skin contains enzymes which are very active in hydrolysing soman. On account of the present results, it can be concluded that he probably determined only the hydrolysis of the C(±)P(+)-isomers. Van Hooidonk *et al.* [6], using a combination of radiometric and enzymatic measurements, found that 24 hr after application of radioactive soman of guinea-pig and human skin, approximately 50 and 80% or more, respectively, of the amount of soman which penetrated, was degraded during passage. Again, on the basis of our results it could be concluded that most of the 50% degradation in guinea-pig skin may be attributed to the hydrolysis of the C(+)P(+)-isomer. However, for human skin they concluded that there was at least 80% degradation. At the end of their experiment, half of the total amount of radioactivity had penetrated; 80% of the penetrated amount of radioactivity had no potency any more to inhibit cholinesterase. If also in this case all C(±)P(+)isomers were hydrolysed, it might be that the (labelled) products of this hydrolysis penetrated more rapidly than intact C(±)P(−)-isomers, which could offer an alternative explanation for the above-mentioned 80% reduction in activity.

In another study, the rate of penetration of soman through mouse skin was examined *in vivo* by determination of inhibition of mouse blood cholinesterase [7]. By this method only the penetration of the C(±)P(−)-isomers was determined. The inactivation of soman in the skin was calculated by comparing the degree of inhibition of blood cholinesterase obtained 2.5 hr after topical administration of soman with that obtained after intravenous administration. After topical application to 60 μg/kg soman onto 1 cm^2 of the mouse skin (i.e. 750 ng C(±)P(−)-isomers per cm^2 for a 25 g mouse = 1791 pmol C(±)P(−)-isomers per skin mg protein; Table 1), it was found that 85% of the C(±)P(−)-isomers were inactivated before they reached the blood stream. On the basis of our present results we have calculated that 70% of the amount of applied C(±)P(−)-isomers can be degraded in mouse skin in 2.5 hr: 17% of the applied C(±)P(−) isomers can be degraded in the dermis and epidermis by irreversible binding and 53% can be degraded by spontaneous hydrolysis ($k = 0.005$/min; Fig. 1). The difference between 70 and 85% may be due to the fact that a part of the C(±)P(−)-isomers did not penetrate through the mouse skin in 2.5 hr. An alternative explanation therefore could be that the soman-isomers reach the blood stream via subcutaneous tissue.

In conclusion, in this study it was found that in guinea-pig, mouse and human skin C(±)P(+)-isomers are enzymatically hydrolysed, whereas C(±)P(−)-isomers are only spontaneously hydrolysed. Binding sites for all four soman-isomers were only found in mouse skin.

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