Biochemical Pharmacology, Vol. 36, No. 21, pp. 3587–3591, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00

STEREOSELECTIVE PHOSPHONYLATION OF HUMAN SERUM PROTEINS BY SOMAN

HERBERT C. J. V. DE BISSCHOP,*† WILLEM A. P. DE MEERLEER* and JAN L. WILLEMS‡

*Technical Division of the Army, Department for Nuclear, Biological and Chemical Protection, B-1801 Vilvoorde, and ‡Heymans Institute of Pharmacology, University of Gent Medical School, De Pintelaan, B-9000 Gent, Belgium

(*Received* 3 *February* 1987; *accepted* 27 *April* 1987)

Abstract—Phosphonylation has been reported as part of the degradation of soman in human serum. The concentration of phosphonylation sites can be quantified by comparing the degradation in serum, preincubated with soman (all sites occupied), with the degradation in serum not preincubated. The mean value of 73 nM of phosphonylation sites is in agreement with the concentration of active sites of butyrylcholinesterase (EC 3.1.1.8.), which is known to be phosphonylated by soman. Hence, it is concluded that butyrylcholinesterase accounts for all the phosphonylation sites present in human serum. The stereoselectivity of the reaction was investigated by using epimeric pairs of soman, *in casu* C(+)P(±)- and C(−)P(±)-soman. In a first approach enzymatic hydrolysis was blocked and the ratios of phosphonylation rate constants, C(+)P(+)/C(+)P(−) and C(−)P(+)/C(−)P(−), were determined to be 0.15 and 0.31, respectively. In a second approach, in untreated serum, the bimolecular phosphonylation rate constants of C(+)P(−)- and C(−)P(−)-soman were determined, neglecting their small hydrolysis rate and taking advantage of the fast enzymatically catalysed disappearance of their respective P(+)-epimeric counterparts. Values for C(+)P(−)- and C(−)P(−)-soman are 3.6×10^7 and $0.6 \times 10^7\ M^{-1}.min^{-1}$, respectively. Using a combination of both approaches, a relative ranking of phosphonylation rates of the four isomers was found to be C(+)P(−) ⩾ C(+)P(+) ≃ C(−)P(−) > C(−)P(+).

Certain esterases and possibly other proteins are known to be susceptible to phosphylation. This reaction, which covers phosphorylation, phosphonylation and phosphinylation [1] results in the formation of a covalent bond between the organophosphorus compound and a protein.

The *in vivo* irreversible binding by organophosphorus compounds was studied earlier by other investigators. Jandorf and McNamara [2] reported a high binding capacity in kidney, lung and liver tissue of the rabbit for DF^{32}P (0.2 to 1.2 μg/g of dry tissue). Cohen and Warringa [3] were able to show that a small portion (0.5 to 1 μg/g of plasma nitrogen) of DF^{32}P, injected in humans, was irreversibly bound to plasma proteins. Ramachandran [4] found a binding of ^{32}P by liver esterase or other DFP-susceptible esterases in the liver (>1 μg P/whole organ) of the mice dosed i.p. with DF^{32}P; the amount of bound ^{32}P did not change by pretreatment with atropine or oximes. The biodisposition of [^{3}H]DFP in mice was studied by Martin [5]. He showed that plasma-bound DFP rapidly reached a maximum of 2 ng of DFP-equivalents/g tissue and then steadily decreased in time. For sarin, Christen [6] and Polak and Cohen [7] showed that the phosphonylated rat plasma proteins were identical with aliesterase (EC 3.1.1.1.). Bošković [8] and Clement [9] showed that serum aliesterase was a major phosphonylation site for soman. A study of biodisposition of tritiated soman in mice (25 μg, i.v.) was done recently by Reynolds *et al.* [10]; they showed that the level of irreversibly bound soman in plasma remained fairly constant at *ca.* 100 pg/ml during the first 8 hr, decreasing to a low level (<20 pg/mg tissue) after one day.

Since proteins are involved in phosphylation a stereoselective effect can reasonably be expected. Keijer and Wolring [11] demonstrated a stereoselectivity in inhibition and aging rate of bovine erythrocyte acetylcholinesterase (EC 3.1.1.7.) and horse serum butyrylcholinesterase (EC 3.1.1.8.) by the four isomers of soman. De Jong and Kossen [12] recently reported on the stereoselectivity of the reactivation of human brain and erythrocyte acetylcholinesterase inhibited by soman. The phosphonylation of hog liver aliesterase by C(+)- and C(−)-soman epimers was studied by Nordgren *et al.* [13] and was also shown to be a stereoselective process. The C(−)P(+)-isomer showed a much higher phosphonylation rate constant than C(−)P(−)-soman. When the C(+)-epimers were compared a higher affinity was found for C(+)P(−)- than for C(+)P(+)-soman.

The stereoselectivity of the phosphonylation of horse serum cholinesterase by the four isomers of soman was investigated earlier. Keijer and Wolring [11] found the following order: C(+)P(−) > C(−)P(−) = C(−)P(+) > C(+)P(+). Nordgren *et al.* [13] reported C(+)P(−) > C(+)P(+) and C(−)P(−) > C(−)P(+).

† To whom correspondence should be addressed.

In this paper we report the following aspects of the phosphonylation of human serum by soman: the concentration of phosphonylation sites and the stereoselectivity of the phosphonylation process. The concentration of phosphonylation sites was shown to be equal to the concentration of active sites of butyrylcholinesterase.

MATERIALS AND METHODS

All materials, and the sections on assay, degradation experiments, preincubation and calculation of degradation rate constants were described in refs. 14 and 15.

Calculation of phosponylation sites. The linear parts of the degradation curves in preincubated and not-preincubated serum samples, respectively are extrapolated to zero minutes (Fig. 1).

The values obtained are subtracted and the result, a percentage of initial concentration, is transformed in a concentration of soman used for phosphonylation of serum proteins.

Determination of the ratio of phosphonylation rate constants of soman epimers. When C(+)P(±)- or C(−)P(±)-soman is added to serum treated with EDTA and aluminon, the enzymatic hydrolysis is largely blocked [14] and phosphonylation of serum proteins will be the main process. Assuming that the phosphonylation sites are a homogeneous group and that the reaction is first order with regard to both reactants, i.e. protein and soman, the reaction can be treated according to the classical theory of competing reactions. The ratio of rate constants can be calculated:

$$k_1/k_2 = \frac{\log(s_1/s_1^0)}{\log(s_2/s_2^0)} \quad (1)$$

where s_1 and s_2 are the concentrations of epimers at t min, s_1^0 and s_2^0 are the concentrations of epimers at zero min and k_1 and k_2 are the phosphonylation rate constants of the epimers. In eqn. (1) the fractions of epimers at t min (Fig. 3) are derived from the % remaining soman [15].

Calculation of bimolecular phosphonylation rate constants. Accepting a bimolecular reaction model, the rate equation of the phosphonylation by a soman isomer can be written as

$$ds/dt = -kps \quad (2)$$

where k is the rate constant and p and s are the concentrations of protein and soman isomer at time t, respectively.

Bimolecular rate constants are calculated by using

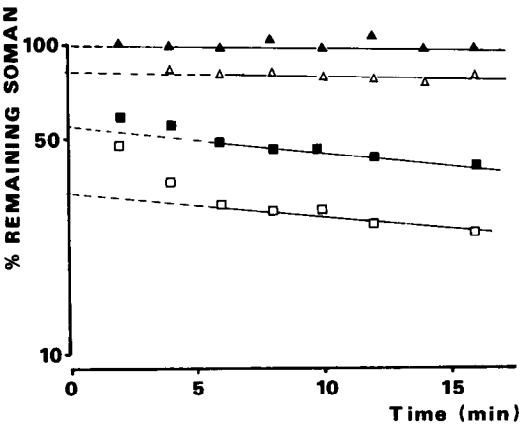

Fig. 1. Determination of phosphonylation site—concentration in human serum at 25°, pH 7.4 and an initial C(±)P(±) concentration of 520 nM. Open symbols: no preincubation; closed symbols: with preincubation. ▲ and △ represent the degradation of C(±)P(±)-soman in human serum, treated with EDTA/aluminon (10 mM).

the integrated equation [16]

$$kt = \frac{1}{p^0 - s^0} \ln \frac{s^0(p^0 - x)}{p^0(s^0 - x)} \quad (3)$$

where s^0 and p^0 are the initial concentrations of soman isomer and phosphonylation site respectively and x is the concentration of soman or protein that has phosphonylated on time t. For various sampling times t, x is calculated from the % remaining soman and the initial isomer concentration.

RESULTS

Quantitative determination of phosphonylation site concentration

Figure 1 shows the results obtained when C(+)P(±)-soman was added to human serum. Some 17% of the initial 520 nM was used for the phosphonylation of serum proteins, regardless of whether the serum has been treated with EDTA or not. The concentration of phosphonylated sites was determined for some individual sera (Table 1). A mean value of 73 nM is found.

Stereoselectivity of the phosphonylation of human serum proteins

In a first approach to the study of the stereoselectivity of the phosphonylation reaction, the serum *somanase* activity was largely inhibited by adding EDTA and the model of competing reactions was applied to the epimers C(+)- and C(−)-soman.

Table 1. Concentration* of phosphonylation sites (±95% confidence limits) in the sera of some individuals

Individual No.	1	2	3	4	5	6
Concentration phosphonylation sites (nM)	56 ± 15	79 ± 16	68 ± 13	93 ± 16	57 ± 16	87 ± 14

* Mean value: 73 nM (standard deviation = 16 nM).

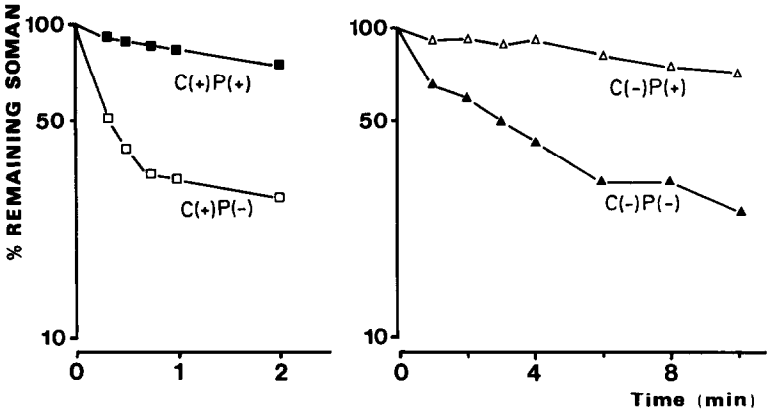

Fig. 2. Degradation of C(−)P(±)- and C(+)P(±)-soman in not-preincubated human serum treated with EDTA/aluminon (10 mM) at 25° and pH 7.4. Initial concentration of C(+)P(±)- and C(−)P(±)-soman is 110 nM. ■-C(+)P(+); □-C(+)P(−); ▲-C(−)P(−); △-C(−)P(+).

Table 2. Mean values* of the ratio of phosphonylation rate constants for C(−)- and C(+)-epimers in not preincubated human serum spiked with EDTA/aluminon (10 mM final concentration) (pH 7.4; 25°)

Initial concentration of C(−)P(±) or C(+)P(±) (nM)	Human serum batch	$\frac{k_{C(-)P(+)}}{k_{C(-)P(-)}}$	$\frac{k_{C(+)P(+)}}{k_{C(+)P(-)}}$
110	A	0.26 ± 0.04 (9)	0.19 ± 0.04 (11)
110	A	0.25 ± 0.01 (13)	0.16 ± 0.04 (11)
55	B	0.26 ± 0.05 (7)	0.12 ± 0.01 (5)
110	C	0.41 ± 0.13 (4)	0.12 ± 0.04 (6)
110	C	0.41 ± 0.04 (3)	0.14 ± 0.08 (3)

* ±Standard deviation; within parenthesis the number of sampling points.

Figure 2 shows that for each epimeric pair the P(−)-isomers phosphonylate much more rapidly than their respective P(+)-counterparts. The mean values of the ratios of phosphonylation rate constants of C(+)- and C(−)-epimers are given in Table 2 for various batches of human serum and various initial concentrations of soman epimers. The mean values of the ratios of rate constants are calculated from Table 2, resulting in a set of two equations

$$k_{C(-)P(+)} = 0.31 k_{C(-)P(-)} \quad (4)$$

$$k_{C(+)P(+)} = 0.15 k_{C(+)P(-)} \quad (5)$$

In a second approach degradation rate constants of soman epimers were compared in preincubated and in not preincubated serum. Figure 3 shows that no

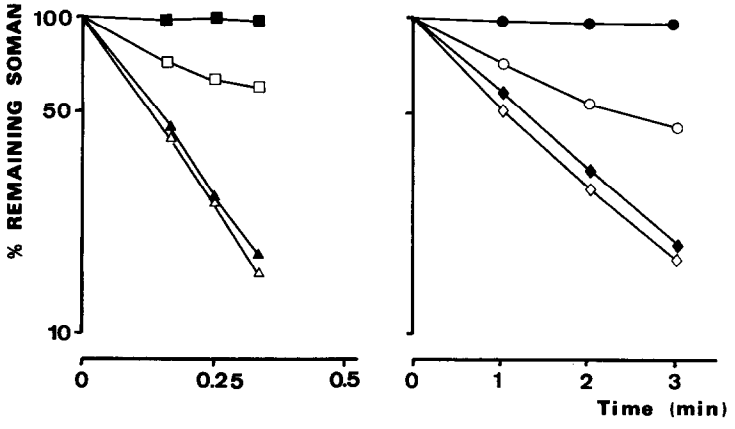

Fig. 3. Degradation of C(−)P(±)- and C(+)P(±)-soman in preincubated (closed symbols), not-preincubated (open symbols) human serum at pH 7.4 and 25°. Initial C(+)P(±)- and C(−)P(±)-soman concentration: 165 nM. △, ▲, C(+)P(+); □, ■, C(+)P(−); ◇, ◆, C(−)P(+); ○, ●, C(−)P(−).

Table 3. Bimolecular phosphonylation rate constants (±95% confidence limits) of human serum proteins by C(−)P(−)- and C(+)P(−)-soman at 25° and pH 7.4

Initial concentration of C(+)P(±)- or C(−)P(±)-soman (nM)	Batch	Rate constant	
		C(−)P(−) ($10^{-7}.M^{-1}.min^{-1}$)	C(+)P(−) ($10^{-7}.M^{-1}.min^{-1}$)
55	A	0.60 ± 0.13	n.d.
55	B	0.59 ± 0.08	4.6 ± 1.3
55	C	0.78 ± 0.15	5.0 ± 0.9
110	A	0.65 ± 0.14	n.d.
110	B	0.47 ± 0.11	3.4 ± 2.6
110	C	0.63 ± 0.09	2.1 ± 0.6
165	B	0.60 ± 0.20	3.4 ± 1.1
165	C	0.57 ± 0.04	3.1 ± 0.7

n.d. = not determined.

significant difference exists for the P(+)-isomers. On the other hand the hydrolysis rates of the P(−)-isomers in preincubated serum are negligible as compared with their phosphonylation rates. The bimolecular phosphonylation rates were determined according to the theory of bimolecular reactions, for various batches and various initial concentrations of soman epimers (Table 3). Mean values were calculated: $3.6 \times 10^7\ M^{-1}.min^{-1}$ for C(+)P(−) and $0.61 \times 10^7\ M^{-1}.min^{-1}$ for C(−)P(−)-soman. These two values can be combined with equations (4) and (5) to obtain a relative ranking of phosphonylation rates of the four isomers of soman (Table 4).

DISCUSSION

Proteins that are rapidly phosphonylated may play a role as organophosphorus antagonists by trapping these compounds. In such a way proteins act like a "sponge" [17]. Human serum cholinesterase, in analogy with horse serum cholinesterase [11], can be expected to be phosphonylated in a rapid and irreversible way by soman. In addition to butyrylcholinesterase, other phosphonylation sites could be present in human serum. The concentration of phosphonylation sites, using the method described in this paper, can be compared, however, with the concentration of butyrylcholinesterase in human serum, measured by Myers [18], who used the theoretical relation between the concentrations of enzyme and of a competitive reversible inhibitor, on the one hand, and the remaining fractional activity, on the other hand [19]. His value of 79 nM was found to be within the 95% confidence interval of the mean value we obtained for the concentration of phosphonylation sites in the sera of six individuals (Table 1) and in agreement with the value of 88 nM* found for a pool of human serum, representing some forty individuals (Fig. 1). This similarity suggests that the only significant phosphonylation sites that exist in human serum belong to butyrylcholinesterase.

In determining the stereoselectivity of the phosphonylation of human serum proteins, presumably butyrylcholinesterase, by soman the qualitative and quantitative results of our first approach are valid provided the combination of EDTA and aluminon is efficient in blocking the enzymatic degradation and does not stereoselectively influence phosphonylation. The effectiveness of the blocking agents at physiological pH is demonstrated in a previous paper [14]. Since EDTA and aluminon are achiral molecules, there is no *a priori* reason to expect a stereoselective interference. The general conclusion of this first approach, i.e. that P(−)-soman phosphonylates more rapidly than its epimeric P(+)-counterpart, is in agreement with the results of Nordgren *et al.* [13], obtained with a similar method using purified horse serum cholinesterase.

Using such measurements with epimers a conclusion on the relative phosphonylation ranking of all four stereoisomers cannot be drawn. Therefore, in another set of experiments, advantage is taken from the observation that P(+)-soman in untreated human serum is hydrolysed much more rapidly and, in turn, phosphonylates more slowly than its epimeric P(−)-isomer: the ratios of rate constants of

* The value of 120 nM we reported previously [20] was an erroneous transcription, not detected during the correction of the printing proofs.

Table 4. Relative contribution by the four stereoisomers of soman to phosphonylation of human serum proteins

Isomer	C(+)P(−)	C(−)P(−)	C(+)P(+)	C(−)P(+)
Relative contribution	18	3	3	1

The phosphonylation rate constant by C(−)P(+)-soman is arbitrarily assigned a value of 1.

C(+)P(+)- and C(+)P(−)-soman for hydrolysis and phosphonylation are 240 [14] and 0.15 (Eqn 5), respectively; the corresponding ratios of C(−)P(+)- and C(−)P(−)-soman are 16 [14] and 0.31 (Eqn 4). Therefore the degradation of the P(−)-isomer in untreated serum is mainly phosphonylation. The values of the bimolecular phosphonylation rate constants of both isomers are within the same order of magnitude as the values reported earlier on horse serum cholinesterase [11].

The ranking of the phosphonylation of human serum butyrylcholinesterase by the four isomers of soman (Table 4) shows a strong stereoselectivity towards the C(+)P(−)-isomer. This is in agreement with the results obtained by Keijer and Wolring [11] on purified horse serum cholinesterase using inhibition kinetics. In disagreement with these results is the ranking of the P(+)-isomers, which, however, might be explained by species difference, since for human brain and erythrocyte acetylcholinesterase too the C(+)P(+)-isomer phosphonylates faster than C(−)P(+)-soman [12].

The phosphonylation of acetylcholinesterase, the primary target of soman, is also highly stereoselective towards the P(−)-isomers [11, 12], which accounts for their high *in vivo* toxicity [21]. The existence of additional soman binding sites such as butyrylcholinesterase and aliesterase, of which the inhibition does not seem to result in physiological disturbances, might be important for detoxification. Using our estimated concentration of phosphonylation sites of 73 nM, the available serum sites in man, having 0.045 l serum/kg body weight [22], can be estimated at 3.3 nmol/kg. For the rat, the concentration of phosphonylation sites in serum, mainly due to aliesterase [6, 7], was reported to be 2.8 μM [23] and, with a serum volume of 0.04 l/kg body weight [24], the available sites in rat serum are estimated to be 112 nmol/kg. Although the process in serum may reflect only part of the fate of soman in the whole body, its toxicity in man can, on the basis of the difference in "sponge" sites, be expected to be much higher than in the rat. As was already mentioned in the introduction, the LD_{50} of soman in rats is greatly reduced when the aliesterases are blocked by pretreatment with TOCP [8] and CBDP [9].

Acknowledgement—We thank Dr H. P. Benschop and Dr L. P. A. De Jong, Prins Maurits Laboratory—TNO, for helpful discussions.

REFERENCES

1. R. F. Hudson and L. Keay, *J. Chem. Soc.* 1859 (1960).
2. B. J. Jandorf and B. D. McNamara, *J. Pharmac. exp. Ther.* **98**, 77 (1950).
3. J. A. Cohen and M. G. P. J. Warringa, *J. clin. Invest.* **33**, 459 (1954).
4. B. V. Ramachandran, *Biochem. Pharmac.* **15**, 169 (1966).
5. B. R. Martin, *Toxicol. appl. Pharmac.* **77**, 275 (1985).
6. P. J. Christen, *De stereospecifieke hydrolyse van sarin in plasma.* Thesis, Leiden (1967).
7. R. L. Polak and E. M. Cohen, *Biochem. Pharmac* **18**, 813 (1969).
8. B. Bošković, *Archs. Toxic.* **42**, 207 (1979).
9. J. G. Clement, *Biochem. Pharmac.* **33**, 3807 (1984).
10. M. L. Reynolds, P. J. Little, B. F. Thomas, R. B. Bagley and B. R. Martin, *Toxic. appl. Pharmac.* **80**, 409 (1985).
11. J. H. Keijer and G. Z. Wolring, *Biochim. biophys. Acta* **185**, 465 (1969).
12. L. P. A. De Jong and S. P. Kossen, *Biochim. biophys. Acta* **830**, 345 (1985).
13. I. Nordgren, G. Lundgren, G. Puu and B. Holmstedt, *Archs. Toxic.* **55**, 70 (1984).
14. H. C. J. V. De Bisschop, W. A. P. De Meerleer, P. R. J. Van Hecke and J. L. Willems, *Biochem. Pharmac.* **36**, 3579 (1987).
15. H. C. De Bisschop, J. G. Mainil and J. L. Willems, *Biochem. Pharmac.* **34**, 1895 (1985).
16. J. C. Jungers, *Cinétique Chimique Appliquée*, p. 142. Société des Editions Technip, Paris (1958).
17. A. G. Karczmar, *Fundam. appl. Toxic.* **5**, S270 (1985).
18. D. K. Myers, *Biochem. J.* **51**, 303 (1952).
19. A. Goldstein, *J. gen. Physiol.* **27**, 529 (1944).
20. H. C. De Bisschop, E. E. Van Driessche, M. L. M. Alberty and J. L. Willems, *Fundam. appl. Toxic.* **5**, S175 (1985).
21. H. P. Benschop, C. A G. Konings, J. Van Genderen and L. P. A. De Jong, *Toxic. appl. Pharmac.* **72**, 61 (1984).
22. H. A. Harper, V. W. Rodwell and P. A. Mayes, *Review of Physiological Chemistry*, p. 189. Lange Medical Publications, CA (1979).
23. L. P. A. De Jong and C. Van Dijk, *Biochem. Pharmac.* **33**, 663 (1984).
24. H. J. Baker, J. R. Lindsey and S. H. Weisbroth, *The Laboratory Rat. Volume I. Biology and Diseases*, Appendix 1. Academic Press, New York (1979).