Acknowledgements—We thank Professor J. P. Campion for providing liver samples, Dr. R. Voges (Sandoz, Biopharmaceutical Department, Basle) for providing radiolabelled ketotifen, Mrs. C. Julien-Larose (Sandoz Research Center, Rueil) for her help in GLC–MS analysis and Mrs. O. Houzelle for typing the manuscript. This work was supported by INSERM (grant: ATP 77 79 109), MRT (Mission des Biotechnologies) and Sandoz S.A.R.L.

JEAN M. BEGUE*‡
JEAN F. LE BIGOT†
CHRISTIANE GUGUEN-GUILLOUZO*
JEAN R. KIECHEL†
ANDRÉ GUILLOUZO*§

*Unité de Recherches Hépatologiques
U 49 de l'INSERM
Hôpital Pontchaillou
35011 Rennes Cédex
France

†Centre de Recherches Pharmacocinétiques
Laboratoires Sandoz
S.A.R.L.
14 Bd Richelieu
92506 Rueil-Malmaison Cédex
France

REFERENCES

1. A. Rane and G. Tomson, *Eur. J. Clin. Pharmac.* **18**, 9 (1980).
2. P. Kremers, F. Goujon, F. De Graeve, J. P. Confort and J. Gielen, *Eur. J. Biochem.* **116**, 67 (1981).
3. B. E. Lake and S. O. Gangolli, in *Concepts in Drug Metabolism* (Eds. P. Jenner and B. Testa) part B, p. 167. M. Dekker, New York (1981).
4. K. N. Jeejeebhoy and M. J. Phillips, *Gastroenterology* **71**, 1086 (1976).
5. A. E. Sirica and H. C. Pitot, *Pharmac. Rev.* **31**, 205 (1980).
6. D. E. Rollins, C. Von Bahr, H. Glaumann, P. Moldeus and A. Rane, *Science N.Y.* **205**, 1414 (1979).
7. A. Guillouzo, J. F. Le Bigot, C. Guguen-Guillouzo and J. R. Kiechel, *Biochem. Pharmac.* **31**, 2427 (1982).
8. C. Guguen-Guillouzo, J. P. Campion, P. Brissot, D. Glaise, B. Launois, M. Bourel and A. Guillouzo, *Cell Biol. Intern. Rep.* **6**, 625 (1982).
9. D. M. Bissell and P. S. Guzelian, *Archs Biochem. Biophys.* **192**, 569 (1979).
10. G. M. Decad, D. P. H. Hsieh and J. L. Byard, *Biochem. Biophys. Res. Commun.* **78**, 279 (1977).
11. A. J. Paine, L. J. Hockin and R. F. Legg, *Biochem. J.* **184**, 461 (1979).
12. C. Guguen-Guillouzo, B. Clement, G. Baffet, C. Beaumont, E. Morel-Chany, D. Glaise and A. Guillouzo, *Expl. Cell Res.* **143**, 47 (1983).
13. C. Guguen-Guillouzo, G. Baffet, B. Clement, J. M. Begue, D. Glaise and A. Guillouzo, in *Isolation, Characterization and Use of Hepatocytes* (Eds. R. A. Harris and N. W. Cornell). Elsevier, New York (in press).
14. E. Morel-Chany, C. Guillouzo, G. Trincal and M. F. Szajnert, *Eur. J. Cancer* **14**, 1341 (1978).
15. M. Guerret, C. Julien-Larose and D. Lavene, in *Proc. First Eur. Cong. Biopharm. Pharmacokin.* Clermont-Ferrand, Vol. I, p. 317. Edit. Technique et Documentation (1981).
16. C. Guguen, C. Gregori and F. Schapira, *Biochimie* **57**, 1065 (1975).
17. C. Guguen-Guillouzo, L. Tichonicky, M. F. Szajnert, F. Schapira and J. Kruh, *Biol. Cell.* **31**, 225 (1978).
18. A. E. Sirica, W. Richards, Y. Tsukada, C. Sattler and H. C. Pitot, *Proc. natn. Acad. Sci. U.S.A.* **76**, 283 (1979).

‡ Present address: Laboratoire de Physiologie, Faculté de Médecine, 35013 Rennes Cédex, France.
§ To whom requests should be addressed.

Biochemical Pharmacology, Vol. 32, No. 10, pp. 1646–1649, 1983.
Printed in Great Britain.

0006-2952/83 $03.00 + .00

Cholinesterase and carboxylesterase activities in soman poisoned rats treated with bispyridinium mono-oximes HI-6 and HS-6

(*Received* 17 *August* 1982; *accepted* 4 *November* 1982)

Traditional reactivators (oximes) used in the therapy of organophosphate poisoning have limited effect against the organophosphorus nerve agent soman [1], in accordance with their poor ability to reactivate acetylcholinesterase inhibited by soman [2, 3]. The newer bispyridinium mono-oximes HI-6 and HS-6, however, are effective in the treatment of soman poisoning in rodents [4–11], dog [12] and monkey [13], but their mechanism of action has remained unclear. Although both HS-6 and HI-6 are reported to reactivate soman inhibited acetylcholinesterase *in vitro* [2, 3], this has not been unambiguously shown to take place *in vivo*. In addition a direct inactivation of soman by the oximes [9], and/or ganglion blocking properties of HI-6 and HS-6 [14], have been suggested to be important for their antidotal effect. Recently, we found that detoxification of soman in rodents is strongly influenced by the enzyme carboxylesterase (= aliesterase = tributyrinase) which binds and, therefore, neutralizes a high proportion of soman [15–17]. Reactivation of inhibited carboxylesterases could explain the antidotal effect of those newer oximes [18]. The present investigation was undertaken to measure the effect of HI-6 and HS-6 on cholinesterase and carboxylesterase activities in different rat tissues during poisoning by soman and by tri-ortho-cresyl phosphate (TOCP), a carboxylesterase inhibitor.

Materials and methods

Animals. Male Wistar rats (weight 200–250 g) were purchased from the National Institute of Public Health.

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

HI-6 and HS-6. The bispyridinium mono-oximes HI-6, ({[4-(amino-carbonyl)pyridino]methoxy}methyl}-2-[(hydroxyimino)methyl]-pyridinium dichloride) and HS-6, ({{[3 - (amino - carbonyl)pyridino]methoxy}methyl}-2[(hydroximino)methyl]-pyridinium dichloride) were synthesized by the method of Hagedorn *et al.* [19] in this laboratory, and assessed to be more than 98% pure.

Injection procedures. Soman, atropine sulphate (Norsk Medisinaldepot) and oximes were dissolved in physiological saline immediately before use. Soman (100–200 μg/kg) was administered subcutaneously and atropine (17.5 mg/kg) intramuscularly 10 sec thereafter. HI-6 or HS-6 was given intraperitoneally (50–100 mg/kg) 20 sec after soman if not otherwise indicated. In some cases TOCP (tri-ortho-cresyl phosphate, K&K Laboratories) dissolved in 1,2-propanediol was given subcutaneously (100 mg/kg) 20 hr before soman. Previous experiments have shown that this will inhibit carboxylesterase more than 90% for 36 hr [15]. Injected vols. were in all cases 0.5 ml/kg body weight.

Determination of cholinesterase activities. At a period of 1 hr after administration of oxime the animals were decapitated and erythrocytes, plasma, brain and diaphragm tissues were prepared in 20 mM sodium phosphate buffer, pH 7.4, as previously described [15, 20]. A 10% (w/v) homogenate of liver tissue in 25 mM $NaHCO_3$ was prepared with a Potter–Elvehjem homogenizer, and diluted with an equal vol. of 20 mM sodium phosphate buffer, pH 7.4, before assay. A 10% (w/v) homogenate of lung tissue in 25 mM $NaHCO_3$ was prepared with an Ultra-Turrax homogenizer, and diluted as above before assay. Acetylcholinesterase (EC 3.1.1.7, acetylcholine hydrolase) and butyrylcholinesterase (EC 3.1.1.8, acylcholine acylhydrolase) activities were determined by the radiochemical method of Sterri and Fonnum [21] as previously described [15, 22] with acetylcholine as the substrate. A quantity of 0.2 mM ethopropazine was included to inhibit butyrylcholinesterase activities in brain and diaphragm.

Determination of carboxylesterase activities. Carboxylesterase (EC 3.1.1.1, carboxylic-ester hydrolase) activities in plasma and lung were examined with tributyrine as the substrate, using the Warburg technique as described by DuBois *et al.* [23] with minor modifications [15]. Undiluted plasma (0.2 ml) or 0.2 ml of 10% homogenate of lung tissue was used in the assay mixture (2.0 ml total vol).

Results and discussion

The effect of HI-6 and HS-6 was investigated at concentrations when they had a distinct therapeutic effect *in vivo*

Table 1. Effect of bispyridinium mono-oximes HI-6 and HS-6 on mortality of soman poisoned rats*

Oxime dose (mg/kg)	Mortality (dead/total)		
	HI-6	HS-6	No oxime
50	0/3	1/3	14/19
100	1/5	1/8	

*Soman (200 μg/kg) was given subcutaneously, atropine (17.5 mg/kg) intramuscularly 10 sec thereafter, and oxime intraperitoneally 20 sec after soman. Mortality was recorded after 1 hr.

Table 2. Effect of bispyridinium mono-oximes HI-6 and HS-6 on cholinesterase and carboxylesterase activities in soman poisoned rats*

Soman dose (μg/kg)	Oxime dose (mg/kg)	Time after soman (sec)	Enzyme activity (% of control)								
			Acetylcholinesterase			Butyrylcholinesterase			Carboxylesterase	
			Erythrocytes	Brain	Diaphragm	Plasma	Liver	Lung	Plasma	Lung
100	No oxime	—	3 ± 1	47 ± 7	49 ± 8	8 ± 1	109 ± 10	8 ± 1	25 ± 3	43 ± 7
100	HI-6 50	20	26 ± 10	56 ± 6	82 ± 8	34 ± 9	115 ± 11	21 ± 4	30 ± 5	42 ± 2
100	HS-6 50	20	20 ± 5	52 ± 9	81 ± 5	27 ± 3	102 ± 12	20 ± 3	31 ± 5	40 ± 6
200	No oxime	—	1 ± 1	1 ± 1	2 ± 1	2 ± 1	80 ± 12	3 ± 1	6 ± 1	26 ± 2
200	HI-6 50	20	6 ± 2	1 ± 1	26 ± 8	10 ± 4	88 ± 6	7 ± 1	3 ± 1	22 ± 1
200	HS-6 50	20	1 ± 1	1 ± 1	4 ± 1	7 ± 3	93 ± 7	5 ± 2	7 ± 3	16 ± 1
200	HI-6 100	20	—	—	16 ± 5	—	—	—	—	—
200	HS-6 100	20	—	—	14 ± 2	—	—	—	—	—
150	No oxime	—	1 ± 1	29 ± 11	32 ± 6	9 ± 1	91 ± 5	8 ± 1	20 ± 1	40 ± 4
150	HI-6 100	3600	2 ± 1	34 ± 10	36 ± 5	14 ± 1	85 ± 9	10 ± 1	26 ± 5	40 ± 12
150	HS-6 100	3600	—	—	26 ± 2	—	—	—	—	—

* Soman was given subcutaneously, atropine (17.5 mg/kg) intramuscularly 10 sec thereafter, and oxime intraperitoneally at the times indicated. Animals were taken 1 hr after oxime administration, or immediately after death within 1 hr, and analysed for enzyme activities. Results are mean values of 3–19 animals ± S.E.M. Control = 100% activity = animals receiving atropine only.

Table 3. Effect of bispyridinium mono-oxime HI-6 on cholinesterase and carboxylesterase activities in TOCP treated soman poisoned rats*

Soman dose (μg/kg)	HI-6 dose (mg/kg)	Enzyme activity (% of control)							
		Acetylcholinesterase			Butyrylcholinesterase			Carboxylesterase	
		Erythrocytes	Brain	Diaphragm	Plasma	Liver	Lung	Plasma	Lung
No soman	No oxime	—	—	—	—	—	—	6 ± 1	26 ± 1
No soman	50	—	—	—	—	—	—	5 ± 1	31 ± 1
30	No oxime	3 ± 1	46 ± 5	25 ± 4	6 ± 1	74 ± 7	9 ± 1	10 ± 1	24 ± 2
30	50	36 ± 3	40 ± 8	55 ± 8	43 ± 8	79 ± 7	21 ± 1	9 ± 1	25 ± 1

* TOCP (tri-ortho-cresyl phosphate) (100 mg/kg) was given subcutaneously 20 hr before soman. Soman was given subcutaneously, atropine (17.5 mg/kg) intramuscularly 10 sec thereafter, and oxime intraperitoneally 20 sec after soman. Animals were taken for enzyme analysis 1 hr after oxime administration. Results are mean values of 3–6 animals ± S.E.M. Control cholinesterase = 100% activity in animals receiving TOCP and atropine. Control carboxylesterase = 100% activity in animals receiving atropine only.

(Table 1). Rats treated with HI-6 or HS-6 immediately after administration of 100 μg/kg soman showed significantly higher cholinesterase activities in erythrocytes, diaphragm, plasma and lung than rats which did not receive oxime (Table 2). The brain cholinesterase activity, however, was not significantly affected by the oxime treatment (Table 2). In the case of 200 μg/kg soman most animals died within 1 hr without oxime treatment (Table 1). In contrast, oxime treatment saved most animals for 1 hr after soman administration (Table 1), i.e. the time when the animals were killed for biochemical investigation. In this case a higher cholinesterase level were found in the rat diaphragm only (Table 2). HI-6 or HS-6 administered 1 hr after soman had under our experimental conditions no effect on cholinesterase activities (Table 2). Treatment with HI-6 or HS-6 did not affect the tributyrine hydrolysing capacity in plasma or lung of neither soman poisoned (Table 2) nor TOCP poisoned (Table 3) animals. Inhibition of the carboxylesterases by TOCP increased the anticholinesterase effect of soman considerably (compare 100 μg soman, Table 2, with 30 μg soman, Table 3).

The enhanced activities of peripheral, but not cerebral, cholinesterase (Table 2) are in accordance with previous findings for blood [9, 18], whole brain [10, 18] and diaphragm [10, 11] cholinesterase activities in soman poisoned rodents treated with bispyridinium mono-oximes HI-6 and HS-6. The results suggest the increased enzyme activities to be due to reactivation of soman inhibited peripheral cholinesterase by the oximes. The inactivation of soman by a direct reaction with oxime as proposed by Erdmann [9], or reactivation of inhibited carboxylesterase which will allow it to bind more soman, as might be suggested by findings of Bosković [18], is less likely. A direct reaction between soman and oxime should make a smaller amount of soman available for inhibition of enzymes both centrally and peripherally. Therefore, also the cholinesterase activity in brain as well as the peripheral carboxylesterase activities should have been enhanced. This was not the case (Table 2). Similarly, carboxylesterase reactivation does not appear to be a target for HI-6 or HS-6, since the tributyrine hydrolysing capacities were not affected by oxime treatment in either soman (Table 2) or TOCP (Table 3) treated animals.

In vivo reactivation of soman inhibited cholinesterase by bispyridinium mono-oximes, is in accordance with results *in vitro* showing that the rate of reactivation by HI-6 is fast enough to be able to compete with the very rapid rate of dealkylation (aging) of soman inhibited cholinesterase [3]. The lack of effect of HI-6 and HS-6 when administered 1 hr after soman (Table 2), is in accordance with results of Wolthuis and Kepner [4] that HI-6 can not reactivate soman inhibited cholinesterase which has already undergone aging.

The results indicate a slightly better effect of HI-6 than HS-6 on diaphragm cholinesterase activity in case of the highest soman dose (Table 2). This is in accordance with previous biochemical and physiological results comparing the antidotal effect of HI-6 vs HS-6, in rodents [4, 5].

In conclusion, the results indicate that soman inhibited peripheral cholinesterase may be reactivated by treatment with HI-6 or HS-6, whereas soman inhibited carboxylesterase is not reactivated by the oximes.

Norwegian Defence Research Establishment
Division for Toxicology
N-2007 Kjeller
Norway

SIGRUN H. STERRI*
SYNNØVE LYNGAAS
FRODE FONNUM

REFERENCES

1. E. Heilbronn and B. Tolagen, *Biochem. Pharmac.* **14**, 73 (1965).
2. K. Schoene, *Biochem. Pharmac.* **22**, 2997 (1973).
3. L. P. A. DeJong and G. Z. Wolring, *Biochem. Pharmac.* **29**, 2379 (1980).
4. O. L. Wolthuis and L. A. Kepner, *Eur. J. Pharmac.* **49**, 415 (1978).
5. L. A. Kepner and O. L. Wolthuis, *Eur. J. Pharmac.* **48**, 377 (1978).
6. O. L. Wolthuis, H. J. Clason-van der Wiel and R.P.L.S. Visser, *Eur. J. Pharmac.* **39**, 417 (1976).
7. B. Bosković and P. Stern, *Archs Toxic.* **26**, 306 (1970).
8. H. Oldiges and K. Schoene, *Archs Toxic.* **26**, 293 (1970).
9. W. D. Erdmann, *Naunyn-Schmiedbergs Arch. exp. Path. Pharmak.* **263**, 61 (1969).
10. J. G. Clement, *Fundam. appl. Toxic.* **1**, 193 (1981).
11. J. G. Clement, *Biochem. Pharmac.* **31**, 1283 (1982).
12. J. Schenk, W. Löffler and N. Weger, *Archs Toxic.* **36**, 71 (1976).
13. J. Lipp and T. Dola, *Archs int. Pharmacodyn. Ther.* **246**, 138 (1980).
14. P. L. Lundy and K. P. Tremblay, *Eur. J. Pharmac.* **60**, 47 (1979).
15. S. H. Sterri, S. Lyngaas and F. Fonnum, *Acta pharmac. toxic.* **49**, 8 (1981).
16. S. H. Sterri, *Acta pharmac. toxic.* **49** (Suppl. V), 67 (1981).
17. F. Fonnum and S. H. Sterri, *Fundam. appl. Toxic.* **1**, 143 (1981).
18. B. Bosković, *Archs Toxic.* **42**, 207 (1979).

* To whom correspondence should be addressed.

19. I. Hagedorn, I. Stark, K. Schoene and H. Schenkel, *Arzneimittel-Forsch./Drug Res.* **28**, 2055 (1978).
20. S. H. Sterri, S. Lyngaas and F. Fonnum, *Acta pharmac. toxic.* **46**, 1 (1980).
21. S. H. Sterri and F. Fonnum, *Eur. J. Biochem.* **91**, 215 (1978).
22. S. H. Sterri, B. Rognerud, S. E. Fiskum and S. Lyngaas, *Acta pharmac. toxic.* **45**, 9 (1979).
23. K. P. DuBois, F. K. Kinoshita and J. P. Frawley, *Toxic. appl. Pharmac.* **12**, 273 (1968).

Biochemical Pharmacology, Vol. 32, No. 10, pp. 1649–1651, 1983.
Printed in Great Britain.

0006-2952/83 $03.00 + .00

Reactivation of soman inhibited acetylcholinesterase *in vitro* and protection against soman *in vivo* by bispyridinium-2-aldoximes

(*Received* 25 *August* 1982; *accepted* 17 *November* 1982)

Conventional oxime therapy against organophosphate poisoning is unsuccessful in the case of intoxications by soman (1,2,2'-trimethylpropylmethylphosphonofluoridate). It is generally assumed, that the failure of oxime therapy is due to the rapid transformation of the soman-inhibited enzyme acetylcholinesterase (EC 3.1.1.7, AChE) into a non-reactivatable form [1]. This secondary process consists in a dealkylation of the trimethylpropyl residue and is called aging. The reported data for the half life of aging *in vivo* range from 1.5 to 16 min [2–4]. These figures mean that 10 min after inhibition still 1–65%, respectively, of the phosphonylated AChE should be available for reactivation by an oxime.

In the recent decade, several pyridinium oximes, synthesized in the laboratory of Hagedorn, Freiburg, have been reported to exhibit considerable protective effects against soman in mice [5], rats [6], dogs [7] and monkeys [8]. Their protective mechanism is still unknown.

Findings described by Wolthuis and Kepner indicate that already 1–2% reactivation of the original AChE activity in the tissue can result in a partial recovery of the neuromuscular transmission, sufficient to sustain spontaneous respiration. Thus, it seems reasonable to assume that the beneficial activity of the new oximes could, at least in part, result from their reactivating action. In order to get more information about this question, we found it worthwhile to compare the protective effects of some of these oximes *in vivo* to their reactivating potencies *in vitro*.

In 1980, DeJong and Wolring [9] analysed the kinetics of the competing reactions reactivation and aging on soman-inhibited AChE *in vitro*. At 25°, pH 7.4 the rates of both reactions were found to be in a similar range. According to the complicated reaction mechanisms rather sophisticated experimental arrangements and calculations had to be applied. For testing a series of oximes, for the present primarily upon their reactivating potency, we decided to simplify the system by minimizing the interference of the aging reaction. According to previous experiences [10], this was done by lowering the temperature to 5° and increasing the pH to 8.0. In spite of the fact that this is far away from physiological conditions, it should be worthwhile to compare the gradation of the reactivating potencies with the protective effects *in vivo* within selected series of oximes. Eventual correlations would give valuable hints for the further investigation of the protective mechanism of these oximes.

The phosphonylation of AChE, with equimolar amounts of the fast reacting isomers of soman, was performed at 5° and pH 9.0 in order to keep the loss of reactivatable activity by aging during the inhibition period as low as possible. Control experiments were run to take account of the inhibitory effect of the oximes themselves. The reactivation was carried out with 1 mM oxime in absence of substrate at 5°, pH 8.0, and determined by activity measurements in samples drawn from the reaction mixture at appropriate time intervals.

Materials and methods

Materials. Bovine red cell acetylcholinesterase (AChE, EC 3.1.1.7) was purchased from Sigma (München) and contained 6.5×10^{-12} mol of active sites per mg, as determined by 'titration' with 97% pure soman [11, 12]. Solutions of acetylthiocholine iodide and 5,5'-dithiobis-(2-nitrobenzoic acid) from Serva (Heidelberg) were made up according to the usual Ellman procedure [13]. Standard buffers from Ingold were used to calibrate the glass electrodes.

All pyridinium oximes were gifts from Professor I. Hagedorn, Freiburg/Br. We received the HGG-compounds as iodides; their poor solubility often did not allow to make up of the stock solution in the required concentration of 5 mM. Therefore, they were converted into the chlorides by ion exchange on Dowex 2 × 10. This was done immediately before the enzyme experiments, using the eluate from the exchange column directly after filling up to a defined vol. The concentration of the 5 mM stock solutions was controlled spectrophotometrically (in 0.1 M NaOH); the yield of the ion exchange procedure was 100% in each case.

Phosphonylation of AChE. A quality of 1 ml of a 1.3×10^{-8} M enzyme solution in 0.155 M NaCl was cooled to 5°, adjusted to pH 9.0 with 1 M NaOH and mixed with 4 μl of a solution of soman in ethanol to give 2.6×10^{-8} M as the final soman concentration. This corresponds to a concentration of 1.3×10^{-8} M with regard to the fast reacting soman isomers [14]. After 15 min the completion of the reaction was controlled by assaying a 10 μl sample in the Ellman test [13] for its residual enzymatic activity. This stock solution was used immediately after the 15 min inhibition period for the reactivation experiments as described below.

Aging during the phosphonylation period. A solution of inhibited enzyme as described above was diluted with 4.5 ml 0.155 M NaCl, kept at 5° and pH 9.0, the latter being controlled by a pH-stat arrangement. After appropriate time intervals, 100 μl samples were transferred from the aging mixture into test tubes containing 0.9 ml 0.155 NaCl and 1.0 ml 0.1 M 2-hydroxyiminomethyl-1-methylpyridinium iodide as the reactivating agent, adjusted to pH 8.0. The reactivating mixture was kept overnight at 25°. After 24 hr, 1.8 ml of the solution was used to determine the enzymatic activity by pH-stat titration at 25°, pH 7.0, with 20 mM NaOH in the presence of 55 mM acetylcholine