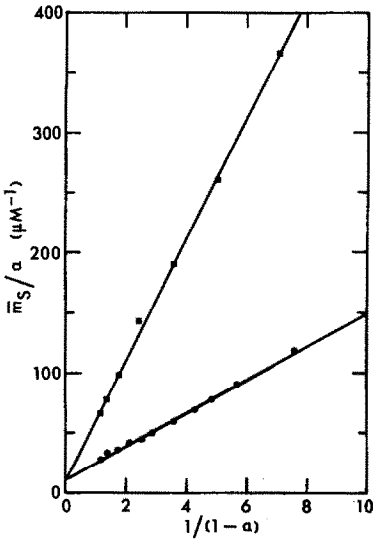

Fig. 2. Evaluation of the stoichiometry and strength of the interactions of chloroquine with uroporphyrin (●) and coproporphyrin (■), the results from Fig. 1 being plotted according to equation 1 with $\alpha = \Delta A/\Delta A_m$.

ordinate intercept of 10 μM, corresponding to the product $n\bar{m}_A$ in these experiments with 10 μM porphyrin, clearly signifies a 1:1 stoichiometry for the two porphyrin–chloroquine complexes. Least-squares calculations yield $n = 1.0$ (± 0.1) and $K_d = 14.0$ (± 0.3) μM for the uroporphyrin interaction; and $n = 1.0$ (± 0.3) and $K_d = 50$ (± 1) μM for the coproporphyrin–chloroquine system. The greater uncertainty in the estimate of n for the latter system reflects the fact that $\bar{m}_A << K_d$ for the coproporphyrin–chloroquine system [6, 7]. We note that the present results confirm the assumed value of unity for n in an earlier study of the coproporphyrin–chloroquine system [4], and hence validate the consequent value of 1 μM for K_d under conditions much lower in regard to ionic strength (0.05 M Tris–HCl, pH 7.2). The much smaller dissociation constant under those conditions indicates that an electrostatic interaction between the positively charged chloroquine molecule and the porphyrin carboxylate groups is a contributing factor to the strength of complex formation.

The most important feature of this investigation is the use of difference spectroscopy to elucidate the stoichiometry of complex formation between chloroquine and two porphyrins, the major difference between which is the existence of four and eight carboxyl groups on coproporphyrin and uroporphyrin respectively. Thus, although electrostatic factors affect the extent of the spectral changes [1] and the strength of the interactions (as discussed above in relation to the coproporphyrin–chloroquine system), the stoichiometry of the interaction between the cationic chloroquine and porphyrin is unaffected by the presence of the additional four negatively charged carboxyl groups on uroporphyrin: the strength of the interaction is, understandably, increased some threefold. Finally, although the stoichiometry cannot be established unequivocally for the interaction of chloroquine with haematoporphyrin, which possesses two carboxyl groups, the results are certainly consistent with a value of unity for n. Moreover, from the slope of the Scatchard plot (Fig. 1), the interaction is still weaker ($K_d = 250$ (± 8) μM), a finding that also fits in with the concept that the number of negatively charged carboxyl groups on the tetrapyrrole structure has an important bearing on the strength of chloroquine–porphyrin interactions.

It is hoped that these quantitative investigations may lead to a better understanding of the therapeutic use of chloroquine in the treatment of porphyrias [3]; and also to an awareness of potential effects in the use of haematoporphyrin derivatives (HPD) in conjunction with phototherapy [9] to treat cancer in subjects also receiving antimalarial doses of chloroquine.

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Aging and stereospecific reactivation of mouse erythrocyte and brain acetylcholinesterases inhibited by soman

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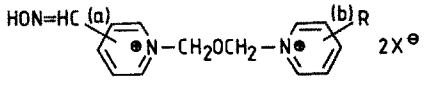
Certain bispyridinium mono-oximes, so-called Hagedorn oximes (see Fig. 1 for chemical structures of oximes), when combined with atropine, have been reported as effective antidotes against intoxication by the nerve agent soman (1,2,2-trimethylpropyl methylphosphonofluoridate) in rodents [1–5] and dogs [6]. These oximes, although being effective in restoring *in vitro* the neuromuscular function of soman-poisoned rat diaphragm, hardly show any activity in restoring the neuromuscular function of human intercostal muscle after *in vitro* incubation with soman [7, 8]. In order to get more insight into the mechanism underlying the differences in effectiveness of the oximes between various



Compound	a	b	R	X
HI-6.H_2O	2	4	$C(O)NH_2$	Cl
HS-6.$2H_2O$	2	3	$C(O)NH_2$	Cl
HGG-21	2	3	$C(O)CH_3$	I
HGG-42	2	3	$C(O)C_6H_{11}$	I
HGG-52.$2H_2O$	2	3	$C(O)CH_2CH(CH_3)_2$	Cl
Obidoxime	4	4	CH = NOH	Cl

Fig. 1. Chemical structures of oximes used in this study.

species, we have previously performed *in vitro* studies on aging and oxime-induced reactivation of soman-inhibited acetylcholinesterase (AChE, acetylcholine hydrolase, EC 3.1.1.7) from erythrocytes of some mammals [9, 10]. The results show only slight differences in the ability of the inhibited enzymes to be reactivated. The rate constants of aging, however, may vary up to one order of magnitude.

In order to make a reliable extrapolation from these *in vitro* results to antidote efficacy of the oximes, ultimately in man, additional information should be available. Particularly, information is lacking on whether reactivation and aging of soman-inhibited AChE from erythrocytes are representative for the corresponding reactions of soman-inhibited AChEs from other organs of the same species, e.g. brain or muscles. Therefore, we studied in the present work aging and oxime-induced reactivation of three soman-inhibited AChEs, i.e. of mouse erythrocyte AChE, high salt soluble (HSS) and detergent soluble (DS) brain AChE.

Reactivation studies with soman-inhibited AChE may be complicated by the chiral structure of the organophosphate. Due to two chiral centers, soman consists of four stereoisomers (see Fig. 2). Only two isomers, C(+)P(−) and C(−)P(−), are potent inhibitors of AChE [11, 12]. In this work reactivation and aging of the two inhibited enzymes formed upon reaction with these isomers were studied separately by using C(+)- and C(−)-soman (see Fig. 2).

Materials and methods

The isolation of AChE from erythrocytes of (BCBA) F_1 mice was performed as described previously for rat erythrocyte AChE [10]. High salt soluble (HSS) and detergent soluble (DS) brain AChE were isolated from whole mouse brain as a 10% homogenate in 0.01 M phosphate buffer, pH 7.4, containing 1 M NaCl and 0.05 M $MgCl_2$, and in 0.01 M phosphate buffer, pH 7.4, containing 0.144 M NaCl and 1% Triton X-100, respectively, according to Sørensen *et al.* [13]. The HSS brain AChE was subsequently dialyzed against 0.01 M phosphate buffer, pH 7.4, containing 0.144 M NaCl. The enzyme preparations were stable for several months when stored at −20°. Soman preparations, oximes and other reagents were obtained as previously described [10].

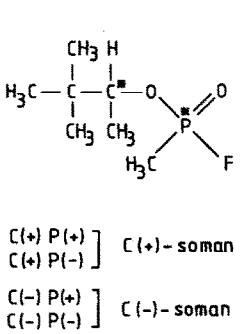


Fig. 2. Four stereoisomers of soman. C stands for the chiral α-carbon atom in the pinacolyl moiety (C*) and P for the chiral phosphorus atom (P*).

Reactivation and aging experiments were carried out as described previously for rat erythrocyte AChE [10], except for the following minor alterations. Inhibited brain enzyme was formed with an 11-fold and three-fold dilution of the preparations of DS and HSS brain AChE, respectively, in 0.01 M phosphate buffer, containing 0.144 M NaCl. The conditions for the incubation of three enzymes with C(+)- and C(−)-soman are given in Table 1. Aging or reactivation with simultaneous aging at pH 7.5 and 25° were started by addition of one volume of inhibited enzyme solution to one volume of 0.05 M veronal buffer, pH 7.1, (erythrocyte) 0.05 M phosphate buffer, containing 0.144 M NaCl, pH 7.33 (DS brain) or pH 7.39 (HSS brain), without or with added oxime, respectively. In reactivation experiments samples of 0.7 or 0.8 ml (erythrocyte) of the latter reaction mixtures were taken after 45 min of incubation and filled up to 5 ml with buffer containing 0.05% Triton X-100 in case of DS brain and erythrocyte AChE. To determine the rate of aging about 10 samples of 0.7 or 0.8 ml of the former reaction mixtures were taken during 60 min, added to 50 μl of an HI-6 solution, incubated for 45 min at 25° and filled up to 5 ml. In these aging experiments final HI-6 concentrations were 0.03 and 0.1 mM for C(+)-soman inhibited erythrocyte and brain AChE, respectively, and 0.3, 0.3 and 0.5 mM for C(−)-soman inhibited erythrocyte, HSS and DS brain AChE, respectively.

Enzyme activities were assayed in triplicate as described previously [10] by incubating enzyme samples with a substrate (acetylthiocholine) solution for 20 min (DS brain) or 40 min.

In calculations of percentages reactivation corrections were made for incomplete inhibition, 50 or 70–80% (DS brain AChE inhibited by C(+)-soman), at the start of reactivation, for about 5% (erythrocyte) or 15% decrease of enzyme activity taking place at conditions used for formation of inhibited enzyme and for the effect of oxime on enzyme activity. For this purpose appropriate blanks were run in addition to blanks for spontaneous and oxime-induced hydrolysis of the substrate.

Results and discussion

A minor percentage (15%) of the mouse brain AChE activity was solubilized in high salt solution; the major fraction of the activity was solubilized by treatment with detergent. Similar observations were made for brain AChE activity from rat [14] and man [13].

Results of reactivation and aging experiments carried out with these enzymes and with erythrocyte AChE, after inhibition with C(+)- and C(−)-soman, are summarized in Table 2. Reactivation data represent percentages of maximum reactivation. In a previous study [9] we showed that incomplete reactivation of soman-inhibited bovine erythrocyte AChE is due to aging simultaneously taking place. In this way, complete recovery of the enzyme activity is precluded.

Our results show a striking similarity for the three mouse AChEs with regard to the reactivating potencies of the various oximes (Table 2). The AChEs inhibited with C(−)-soman are much more refractory to oxime reactivation than the C(+)-soman inhibited enzymes. HI-6 is the superior reactivator for all inhibited enzymes, whereas the reactivating potency of obidoxime is very weak in all cases. The other oximes show a moderate activity towards the C(+)-soman inhibited enzymes and are as inactive as obidoxime towards the C(−)-soman inhibited enzymes. The inhibited enzymes age rather slowly. In contrast with the reactivation reaction, the configuration around the α-carbon atom of the pinacolyl moiety of the phosphonyl group has hardly any effect on the rate of aging. The similarity of the ability

Table 1. Conditions for inhibition of mouse AChEs with C(+)- and C(−)-soman

Enzyme	Concn (nM) C(+)-soman	Concn (nM) C(−)-soman	pH	Time of incubation (hr)
Erythrocyte AChE	0.7	2	10.0	1
DS brain AChE	0.75	0.8	9.0	1
HSS brain AChE	0.6	1.5	9.5	2*

* C(−)-soman: incubation for 1 hr and subsequent mixing with the adsorbens XAD-2 (200 mg/ml) on a vortex mixer for 10 sec and centrifugation in order to obtain a complete removal of excess inhibitor.

Table 2. Percentages of maximum reactivation* induced by 0.03 mM oxime (pH 7.5, 25°) in mouse erythrocyte acetylcholinesterase and in detergent soluble (DS) and high salt soluble (HSS) mouse brain acetylcholinesterase after inhibition with C(+)- and C(−)-soman

Oxime	Erythrocyte AChE inhibited with C(+)-soman	Erythrocyte AChE inhibited with C(−)-soman	DS brain AChE inhibited with C(+)-soman	DS brain AChE inhibited with C(−)-soman	HSS brain AChE inhibited with C(+)-soman	HSS brain AChE inhibited with C(−)-soman
HI-6	90	25 ± 4(4)	60 ± 1	11 ± 2	64 ± 2	13 ± 3(4)
HS-6	41 ± 3(4)	7 ± 1(4)	16 ± 1	<5	14 ± 1	<5
HGG-21	38 ± 3(4)	<5	16 ± 1	<5	16 ± 2	<5
HGG-42	25 ± 1	<5	11 ± 1	<5	9 ± 1	<5
HGG-52	30 ± 2(4)	<5	11 ± 1	<5	11 ± 1	<5
Obidoxime	<5	<5	<5	<5	<5	5 ± 2(4)
k_{aging}† (10^{-2} min^{-1})	2.3–2.5	2.3–2.4	1.9–2.0	1.5–1.5	2.3–2.6	2.2–2.5

Rate constants of aging (k_{aging}, pH 7.5, 25°) of the inhibited enzymes are also given.
* Mean values ± S.D.; averages of three determinations or of the number of determinations denoted within parentheses.
† Duplo experiments.

to be reactivated as well as of the tendency to age of soman-inhibited AChEs from various sources within one species is analogous with previous observations on other properties of erythrocyte and brain AChE from mouse [15], and also from rabbit [16], viz. with respect to substrate hydrolysis and sensitivity to inhibitors. Supporting evidence to these findings is afforded by the observation of immunological cross reactivity between various AChEs within one species, i.e. human erythrocyte and intercostal muscle AChE [17], human erythrocyte, DS and HSS brain AChE [18], and rat DS and HSS brain AChE [19].

In a previous study we compared erythrocyte AChE from some mammals, inhibited with soman, with respect to reactivation and aging [10]. We found that the relative reactivating potencies of various oximes towards the inhibited enzymes of these species hardly differ, whereas rate constants of aging may vary considerably. Our present results with regard to the relative reactivating potencies of the oximes tested towards the various inhibited mouse enzymes are in agreement with our previous results, extending the previous observation. The rate of aging of the inhibited mouse enzymes ($t_{\frac{1}{2}}$ 30–35 min) is much lower than that of the human enzyme ($t_{\frac{1}{2}}$ 1.5 min), but comparable with that of the rat enzyme ($t_{\frac{1}{2}}$ 21 min) suggesting relatively slow aging of soman-inhibited rodent AChEs to be a more general phenomenon.

In summary, the correspondence found between properties of soman-inhibited mouse AChEs with respect to oxime-induced reactivation and aging suggests that various AChEs from one species yield similar results when used in an *in vitro* test for antidote efficacy of oximes against soman intoxication. Moreover, additional information is presented indicating comparable abilities of human and rodent soman-inhibited AChE to be reactivated, but significantly different rates of aging.

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Energy metabolism of cardiac cell cultures during oxygen deprivation: effects of creatine and arachidonic acid

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In recent years, many studies have shown that oxygen deficiency in heart results in a decline of ATP production and cessation of contractile activity [1, 2].

Myocardial cell cultures are a useful model for studying myocardial nucleotide metabolism during oxygen deprivation. In the myocyte, such deprivation induces modifications of cell membranes, cytoplasmic enzyme leakage and lysosomal damage [3–5]; ATP, derived from glycolytic pathway, might play an important role in membrane maintenance [6]. The aim of the present study was to determine whether creatine or arachidonic acid could reduce the energy deficiency due to oxygen and glucose deprivation in cardiac cell cultures.

As regards creatine, it is now well documented that the creatine–PCr–mitochondrial creatine kinase (CK, EC 2.7.3.2) system, in heart cells, serves for regulatory function in the coupling of energy production (mitochondrial site) to energy utilization (myofibrillar site) [7, 8]. Creatine, added to the culture medium, was already shown to induce, in normoxic conditions, an increase in phosphocreatine (PCr) [9] and in mitochondrial CK [10] in myocardial cells. Thereby, it was of interest to investigate if creatine might induce, in normoxic conditions, an increase in contractile activity simultaneous with an increase in PCr synthesis in myocardial cells and, in oxygen deprived conditions, might protect cells against the fall of PCr synthesis already observed in ischemic whole heart [2].

On the other hand, arachidonic acid, as a component of membrane phospholipids, has been suggested to be implicated in membrane integrity and fluidity [1]. Moreover, we showed previously, that non esterified arachidonic acid disappeared from the myocyte when oxygen and glucose deprived cells stopped beating [11]. Consequently, it was suggested that arachidonic acid could protect cellular membrane, thereby reduce enzyme leakage and maintain intracellular energy content.

Materials and methods

Cultures of heart cells. Cultures of cardiac cells were prepared from 3 day-old rats (Sprague–Dawley), according to the method of Harary and Farley [12] with some modifications [13]. Cells were grown in Eagle's minimum essential medium with 10% calf serum and antibiotics.

Experimental procedure. On day 8, just before the beginning of the experiment, glucose concentration in the culture medium was controlled to be near 0 by glucose-oxydase method (Biochemica-Test Combination, Boehringer, Mannheim). Thereafter, half of the flasks were supplemented with glucose to obtain final concentration of 10^{-3} M. Each of the two groups, with or without glucose, was divided into two parts, one of which was gassed with air 95% + CO_2 5%, the other with nitrogen 95% + CO_2 5%, for 55 min at 37°. Air-gassed cultures had average pO_2 values of 117 ± 6 mm Hg and oxygen deprived cultures had pO_2 values of 25 ± 2 mm Hg; pO_2 remained at this level for the 3 h experiment. In air- as well as in nitrogen(N_2)-gassed cultures, we obtained thereby four groups: air or N_2 without glucose, air or N_2 with glucose, air or N_2 with treatment, air or N_2 with glucose and treatment. Creatine (5.10^{-3} M) was added to the treated cultures once a day for 8 days, the latter addition being made just before gassing. Arachidonic acid was dissolved, under N_2, in Na_2CO_3 solution (10^{-1} M); 12.5 μl of the solution 10^{-3} M (i.e. 1‰ of the volume of medium) was added to each culture flask to give a final concentration of 10^{-6} M. Treatment was applied once in control and oxygen deprived cultures just after gassing.

Beating rate was measured at 37° under an inverted microscope at a magnification of 300 in 5 randomly selected microscopic fields for each culture flask, at least 3 times before starting the experiment, then every half hour. The results were expressed as percentages of the mean pre-hypoxic values.