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

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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

chloride as the substrate.

Reactivation of soman inhibited AChE. Immediately after the 15 min phosphonylation period, 100 μl aliquots from the inhibition batch were transferred into previously prepared, to 5° thermostated test tubes. The test tubes contained 700 μl 0.1 M sodium phosphate buffer, pH 8.0, and 200 μl of 5 mM bispyridinium-2-aldoxime solution, the final concentration of oxime being 1 mM. After appropriate time intervals, at least after 0.5, 1.5 and 20 hr, samples of 10 or 20 μl were drawn to determine the enzymatic activity using the Ellman procedure.

Stability of oximes. Aqueous 0.05 mM solutions of the oximes in 0.1 M sodium phosphate buffer pH 8.0, were kept at 5° for 20 hr. At appropriate time intervals the UV-spectra were recorded. The changes, if any, in the absorption maxima at 305–310 and 355 nm, are given as percent of the absorbance at time zero in Table 1. With all oximes, except HGG 81, these changes were <5% in the first 1.5 hr, so that they could be neglected in the later calculation. With HGG 81, the absorbance both at 310 and 355 nm shifted to higher values. This behaviour was unique in the series studied here and had never been observed before with other pyridinium oximes. Because the effect upon the reactivity of the oxime group (to which usually the absorbance at 355 nm is attributed) remained uncertain, we did not correct the oxime concentration on account of this change.

Inhibition of the enzyme activity by oximes. Solutions of identical composition, except soman, as the reactivation mixture were prepared with all oximes and kept at 5°, pH 8.0, for 20 hr. At different time intervals samples were drawn for measuring the enzymatic activity. The relevant results are included in Table 1.

Evaluating of the ED_{50} *and* LD_{50}. Female NMRI-mice (23 g av. weight) received s.c. 280 μg/kg (LD_{95}) soman in 155 mM NaCl containing 2% (v/v) ethanol. At 1 min after poisoning 10 mg/kg atropine sulphate and oxime in 155 mM NaCl were administered i.m. Collectives of eight mice were used in each experiment. Based upon the number of deaths within the following 24 hr, the ED_{50} was calculated according to the method of Litchfield and Wilcoxon. The LD_{50} data for i.m. application of the oximes were evaluated in the same way.

Results and discussion

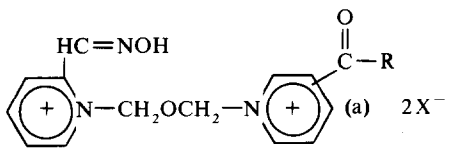
The rate constant of phosphonylation as it resulted from the active site determination [12] was $1.0 \times 10^8\ M^{-1}\ min^{-1}$.

During the phosphonylation at 5°, pH 9.0, the aging proceeded with a rate constant of $1.82 \times 10^{-3}\ min^{-1}$, according to a loss of maximally 2.7% of reactivatable enzyme during the 15 min inhibition period. When calculating the maximal reactivatable amount of enzyme activity, this value of 2.7% was taken into account.

As a further correction, the enzyme inhibition by the oximes themselves had to be considered when calculating the maximal achievable enzyme recovery. The figures in Table 1 indicate that the degree of inhibition is time dependent. The mechanism of this process has not yet been investigated in detail; obviously it is more prevalent with bispyridinium-2-aldoximes than with their 4-isomers.

After these corrections had been applied, the percentage of recovered enzyme activity was calculated. The results are given in Table 1 as "% reactivation" after 0.5, 1.5 and 20 hr reactivation time. These values are not corrected for the additional loss of enzyme activity which occurs due to aging during the reactivation period. The rate constant of aging of soman inhibited AChE under the conditions

Table 1. *In vitro* and *in vivo* properties of bispyridinium-2-aldoximes



Code	a	R	% spectral change after 20 hr	% inhibition (after hr) 0.5	1.5	20	% reactivation (after hr) 0.5	1.5	20	ED_{50} (μmol/kg) i.m.	LD_{50} (μmol/kg) i.m.
HS 6	3	NH_2	<5	5	5	7	10	19	35	25	960
HJ 6	4	NH_2	<5	8	10	13	75	87	90	43	1260
HGG 12	3	C_6H_5	10	10	26	31	12	21	37	1.4	846
HGG 9	4	C_6H_5	<5	12	26	27	61	70	74	25	300
HGG 54	3	$CH_2C_6H_5$	15	10	24	36	50	60	61	2.9	730
HGG 20	4	$CH_2C_6H_5$	<5	5	13	21	66	70	71	51	213
HGG 21	3	CH_3	<5	5	10	20	44	62	62	8.0	100
HGG 73	3	CH_2CH_3	18	10	25	30	31	53	62	2.0	111
HGG 58	3	$(CH_2)_2CH_3$	20	9	23	29	33	47	57	1.1	114
HGG 60	3	$(CH_2)_3CH_3$	15	12	27	33	33	47	52	0.5	312
HGG 69	3	$(CH_2)_4CH_3$	21	15	19	31	29	47	50	0.4	329
HGG 70	3	$(CH_2)_5CH_3$	10	16	28	42	65	72	73	0.3	252
HGG 93	3	$(CH_2)_6CH_3$	15	18	34	53	69	78	82	0.5	300
HGG 82	3	$(CH_2)_7CH_3$	18	34	52	78	80	89	94	15	620
HGG 81	3	$(CH_2)_9CH_3$	$t_{1/2} \sim 4$ hr	48	60	63	52	62	64	15	>640

Per cent spectral change in absorbance at 300–310 and 355 nm at pH 8.0, 5°. Per cent inhibition of AChE by 1 mM oxime at pH 8.0, 5°. Per cent in reactivation of soman-inhibited AChE at pH 8.0, 5°. Corrected for side effects as described in the text. ED_{50} in female mice, the oximes being given i.m. together with atropine sulphate (10 mg/kg) 1 min after soman (LD_{95}, s.c.). LD_{50} of the oximes, given i m. to female mice.

applied here, but in absence of oxime, was determined to be $3 \times 10^{-3}\,min^{-1}$ [10]. The kinetical analysis using this value and the measured time course of reactivation, however, did not lead to satisfactory results. This irregular behaviour was likewise found by DeJong and Wolring and its possible reasons are exhaustively discussed in their paper [9]. Nevertheless, the percentage figures in Table 1 allow a rough estimation: with HS 6 or HGG 12, for instance, reactivation and aging appear to proceed with similar rates, whereas with HJ 6 the reactivation rate constant should be more than 10 times higher.

Group 1 compounds comprises three pairs of isomers with respect to the location of the substituent in the non-oxime ring ('ring 2'). *In vitro*, reactivation is optimal with the substituent located in the 4-position. For the compounds HS 6 and HJ 6 DeJong and Wolring [9] reported analogous results. Accordingly, we found throughout in previous investigations, that bovine red cell AChE, when inhibited by paraoxon, tabun and sarin [15, 16], prefers the substitution in position 4 of this 'second' pyridine ring. The same holds for the respective ED_{50} data from mice [16], except soman; against soman the 3-substituted (with respect to the 'ring 2') bispyridinium-2-aldoximes in general lead to lower ED_{50} values than the 4-isomers [5, 17, 18]. The same gradation for HGG 12/HGG 9 was found by Maksimovic *et al.* [19] using mice and by Weger and Szinicz with beagles for HS 6/HJ 6 [20]. Kepner and Wolthuis [21], however, found HJ 6 to be superior to HS 6 in rats; Clement and Lockwood [22] came to the same result with mice. Their experimental procedures are not so different from those used here, and by the other authors, that this fact alone could explain the differing results. In view of a possible extrapolation to the behaviour of the oximes in man this discrepancy needs further investigation.

The second group of oximes represents a series of homologous compounds differing only in the number of methylene groups in the side chain of the 'second' pyridine ring. The ED_{50} values reach a minimum with the hexyl compound, whereas the maximal reactivating potency *in vitro* was found with the octyl-homologue.

In a previous paper [18], we reported the protective effects of homologous bis-(4-tert-butylpyridinium)alkylene salts against soman in mice and towards bovine red cell AChE. There we found optimal protection *in vitro* with the five-membered, and *in vivo* with the hexylene chain.

The reaction mechanisms *in vitro* are obviously different for the homologous oximes and the oxime-free salts, one being reactivation and the other protection of the active site. From the *in vivo* results, however, regarding the discrepancy to the reactivation studies and the remarkable coincidence in favouring a six-membered alkyl chain, no reliable conclusion can be drawn about the mode of action of the oximes.

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β-Adrenoreceptors display different efficiency on lymphocyte subpopulations

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Catecholamines modulate cellular activity via specific receptors on the cell surface. Such receptors were first identified on cell membrane preparations [1] and more recently the availability of ligands of high affinity and high specific activity has made possible their quantitation on intact cells including lymphocytes [2–5]. The binding of a β-agonist to its receptor triggers an increase in adenylate cyclase activity with a resultant increase in the synthesis of