Arh. hig. rada, 24 (1973) 11.

REACTIVATION OF PHOSPHORYLATED CHOLINESTERASES IN VITRO AND PROTECTING EFFECTS IN VIVO OF SOME PYRIDINIUM AND QUINOLINIUM OXIMES

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(Received for publication May 22, 1973)

Six pyridinium and quinolinium oximes (I-VI) were tested as reactivators of serum and erythrocyte cholinesterases inhibited with O-ethyl-S--(2-dimethylaminothioethyl)-methylphosphonate (medemo) and O-ethyl p--nitrophenyl-ethylphosphonate (armin). They were also tested as protective agents in rats poisoned with 1-methyl-2,2-dimethylpropylmethylphosphonofluoridate (soman) and medemo. The results obtained were compared with the effect of TMB-4.

Oxime IV [1,3 acetone-bis (4-pyridinium aldoxime)dichloride] proved to be a good reactivator of erythrocyte cholinesterase inhibited by medemo. Used as a protective agent together with atropine in rat poisoning it increased LD_{50} of medemo 48 times. Oximes I (1-benzyl-4-pyridinium aldoxime chloride) and III (1-phenacyloxime-2-pyridinium aldoxime chloride) were good reactivators of serum cholinesterase inhibited by armin and medemo. Oximes I and III used together with atropine raised LD_{50} in rats poisoned with medemo 20 times. Oximes III and II (1-phenacyloxime-2-pyridinium aldoxime chloride) used together with atropine increased LD_{50} in rats poisoned with soman 2.0 (oxime II) and 1.8 times (oxime III) respectively. Oximes V and VI (1-phenacyloxime-quinolinium chloride and 1-phenacyloxime-*iso*quinolinium chloride) were very toxic and did not reactivate inhibited cholinesterases at all.

The effects of oximes tested were equal to or weaker than those of TMB-4 both *in vitro* and *in vivo* studies.

It is well known that oximes are reactivators of cholinesterases inhibited by organophosphorus compounds. Since this property of oximes was observed numerous compounds of different structure have been synthetised and tested. Several new pyridinium and quinolinium oximes (Table 1) have been tested in our laboratories and the results are given in this paper. Some of the oximes tested have been recently described (1–9) and the antidotal effect of some of them (compounds I, IV, V and

VI, Table 1) investigated in mice poisoned with paraoxon and sarin (1, 3, 7). The compound IV was extensively investigated by *Binenfeld et al.* (3, 4, 5) and our results confirm and complete the results of these authors. The characteristics of oximes II and III have not been described as far as we know. The aim of this work was to acquire more information about the reactivating effect of these compounds upon phosphorylated cholinesterases as well as to observe their protecting effects on rats poisoned by various organophosphates.

MATERIALS AND METHODS

The oximes are listed in Table 1; TMB-4 was used as a reference compound. All oximes were dissolved in water immediately before the experiment.

For the *in vitro* experiments native human erytrocytes and horse serum cholinesterase were used; the *in vivo* experiments were done on albino rats.

The following organophosphorous compounds were used: O-ethyl-S--(2-dimethylaminothioethyl)-methylphosphonate (medemo), O-ethyl-p--nitrophenyl-ethylphosphonate (armin), 1-methyl-2,2-dimethylpropyl-methylphosphonofluoridate (soman) and *iso*propylmethylphosphonofluoridate (sarin). Stock solutions (1 mM) of the inhibitors were prepared in propylene glycol except for armin, which was prepared in water.

The activities of cholinesterases were measured by the manometric method using Warburg apparatus, with acetylcholine as substrate at 37°C and bicarbonate buffer pH 7.4. The details of the procedure were described earlier (10–12). The concentration of acetylcholine during the measurement of enzyme activity was 27.6 and 13.8 mM for serum and erythrocyte cholinesterase respectively.

Inhibition of cholinesterases. The inhibitory power of oximes (I–VI) was measured on erythrocyte and serum cholinesterases. Substrate and oxime were added together to the enzyme and the activity was measured as described above. The degree of inhibition was expressed as $100 - (a_i \times 100/a_k)$ where a_i is the activity of sample inhibited by oxime and a_k is the activity of the enzyme in the absence of oxime.

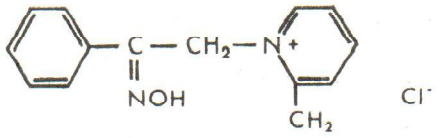
Reactivation of cholinesterase. The reactivating power of oximes (I–IV) was measured on serum cholinesterase inhibited by armin (1.5 μM) or medemo (0.5 μM) and on erythrocyte cholinesterase inhibited by medemo (40 μM). The enzyme samples were inhibited to about 70%. The reaction mixture was diluted and the reaction with oximes was performed. The oximes were left in contact with the enzyme for 30, 60 and 90 min. before addition of the substrate and measurement of enzyme activity. The degree of reactivation was calculated according to the equation: % reactivation $= (a_r - a_i) \times 100/a_k - a_i$, where a_r is the

Table 1.

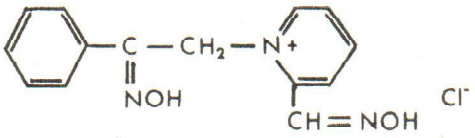
Chemical names and structures of oximes used

1-benzyl-4-pyridinium-aldoxime chloride
(I)

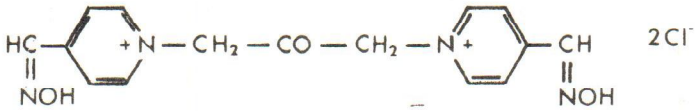
1-phenacyloxime-2-methyl-pyridinium chloride
(II)



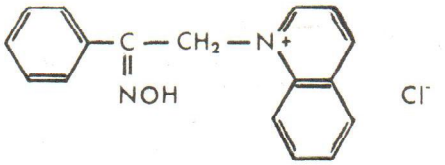
1-phenacyloxime-2-pyridinium-aldoxime chloride
(III)



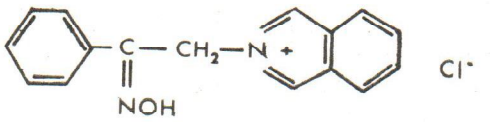
1,3-acetone-bis (4-pyridinium-aldoxime) dichloride
(IV)



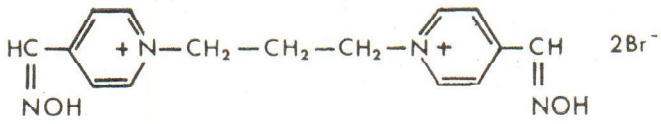
1-phenacyloxime-quinolinium chloride
(V)



1-phenacyloxime-*iso*quinolinium chloride
(VI)



1,3-trimethylene-bis (4-pyridinium aldoxime) dibromide (TMB-4)
(VII)



activity of the inhibited sample after the addition of oxime, a_i is the activity of inhibited enzyme and a_k is the activity of control sample without inhibitor but in the presence of oxime of the same concentration as in sample a_r.

Oxime V was tested also as a protecting agent *in vitro* against the inhibition of erythrocyte cholinesterase by medemo, sarin and soman. The enzyme, inhibitor and oxime were incubated for 60 min., before the substrate was added, and cholinesterase activity measured as described above. The final concentration of oxime V was 25 μM, medemo 40 nM, soman 2.6 nM and sarin 45 nM.

Toxicity. For the toxicity measurements oximes (I–VI) were injected to rats intraperitoneally. LD_{50} values were calculated according to *Thompson* and *Weil* (13, 14) on the basis of a 24-hours lethality.

Protecting effect in vivo. The protecting effect of oximes (I–IV) was tested on rats poisoned by medemo and soman. The poisons were injected subcutaneously in increasing doses (factor 1.26) the first dose being 50 μg/kg and 100 μg/kg for medemo and soman respectively. Immediately after the injection of the poison, the oximes were injected intraperitoneally. All oxime solutions were made in equimolar concentrations in a mixture with atropine. The antidotal mixture containing 0.1 mmole of the oxime per ml and 5 mg of atropine sulphate per ml was injected to rats intraperitoneally in a dose of 2 ml/kg. The animals were observed during 24 hours, and LD_{50} values calculated as described above.

RESULTS AND DISCUSSION

The degree of cholinesterase inhibition by the oximes is given in Table 2. The inhibition was not very pronounced when the concentration of oximes was 10 μM (about 10% inhibition) except for oximes V and VI which at this concentration inhibit about 50% of serum cholinesterase. In a concentration of 100 μM, all oximes, except oxime IV, are more potent inhibitors of cholinesterases than TMB-4 (Table 2). The degree of inhibition obtained by oximes I and IV is in accordance with the results of *Milošević et al.* and *Binenfeld et. al.* (1, 3) and the inhibition produced by oximes V and VI confirms earlier findings that these compounds belong to the strongest inhibitors of cholinesterases among oximes tested (7).

The results after 60 min. reactivation of inhibited cholinesterases are given in Table 2. The percentage of reactivation after 30 min. is lower than after 60 min, but after 90 min. it is only slightly higher than after 60 min. Such effect of oximes is in accordance with the results obtained by other authors (12, 15, 16).

The degree of reactivation measured on erythrocyte and serum cholinesterases inhibited by medemo shows that these oximes except oxime IV. are better reactivators of serum cholinesterase and also that serum cho-

Table 2.

Inhibition of human erythrocyte acetylcholinesterase (AChE) and horse serum cholinesterase (ChE) by oximes and the degree of reactivation (60 min) of these enzymes inhibited by medemo and armin. The percentages given in the table are the mean values obtained from the results of at least three measurements.

Oxime	(μM)	% Inhibition AChE	% Inhibition ChE	% Reactivation AChE-medemo	% Reactivation ChE-medemo	% Reactivation ChE-armin
I	100	34	19	46	63	37
	10	19	< 5	17	30	11
II	100	27	34	0	11	10
	10	6	< 5	< 5	0	< 5
III	100	26	45	30	57	45
	10	6	14	< 5	42	18
IV	100	11	< 5	86	40	20
	10	< 5	< 5	55	< 5	< 5
V	100	44	82	0	0	0
	10	8	48	0	0	0
VI	100	54	86	0	0	0
	10	9	52	0	0	0
VII	100	11	2*	—	—	64*
	10	0	—	—	29	16*

* E. Reiner, personal communication

linesterase inhibited by medemo is reactivated more rapidly than when inhibited by armin (Table 2). The same effect was observed by *Maksimović et. al.* (4) on rat plasma cholinesterase inhibited by edemo (ethyl derivative of medemo) and armin. These authors found that 50% of reactivation was achieved for rat plasma inhibited by armin with 20 μM, and for the same enzyme inhibited by edemo with 3.5 μM of oxime IV.

In our experiments oximes I and III (Table 2) are found to be better reactivators of serum cholinesterase inhibited by medemo and armin than other oximes tested and their reactivating effect is very similar to the effect obtained with TMB-4. Compound IV is the best reactivator of erythrocyte cholinesterase inhibited by medemo. The degree of reactivation of erythrocyte cholinesterase with oxime IV is in good accordance with the results of *Maksimović et al.* (4) and *Binenfeld et al.* (3) obtained on erythrocyte cholinesterase inhibited by edemo and paraoxon.

Oximes V and VI do not reactivate inhibited cholinesterases at all. When oxime V was added to erythrocyte cholinesterase at the same time as the inhibitor, no protecting effect against medemo, sarin and soman

was observed. This finding is opposite to the results of *Malatesta et al.* (6) who found that this compound protects acetylcholinesterase against the inhibition by sarin.

The acute intraperitoneal toxicity of oximes in rats expressed as LD_{50} values in mg/kg or mmoles of oxime/kg is given in Table 3. The signs of poisoning with all the oximes were similar to those of anticholinesterase intoxication. In addition to a slight tremor respiratory disorders in the form of dyspnea were manifested. Strong convulsions occurred before death. In animals treated with large doses of oximes the symptoms appeared 4–20 min after the application while in those which survived after treatment with lower doses the symptoms lasted for approximately 1–2 hours and then disappeared.

Table 3.

Acute intraperitoneal toxicity (LD_{50}) of oximes for male rats and protecting effect of oximes (0.2 mmoles/kg) and atropine sulphate (10 mg/kg) applied intraperitoneally to male rats. The protecting effect is expressed as the ratio of LD_{50}'s for oxime-treated and -untreated animals. The LD_{50} values for untreated rats are 63 μg/kg and 147 μg/kg for medemo and soman respectively

Oxime	LD_{50} i. p.		Protecting effect	
	mg/kg	mmol/kg	medemo	soman
(I)	100	0.40	19	1.4
(II)	71	0.27	9	2.0
(III)	94	0.32	20	1.8
(IV)	212	0.57	48	1.5
(V)	35	0.12	—	—
(VI)	32	0.11	—	—
(VII)	178	0.40	126	1.9

The toxicity of the above oximes agrees with their inhibitory properties on serum and erythrocyte cholinesterase *in vitro*. Compound IV is the least toxic and is the weakest inhibitor and compounds V and VI are the most toxic and the strongest inhibitors.

The results of testing the effect of an intraperitoneal application of oximes I–IV and atropine in rats poisoned by medemo and soman are given in Table 3. The protecting effect of oximes V and VI was not studied *in vivo* because of their high toxicity. Compound IV showed the best antidotal effect against medemo among the oximes studied, increasing its LD_{50} value about 48 fold. For comparison, TMB-4 applied with atropine increased the LD_{50} of medemo 126 times. The therapeutic effect of oximes is of the same order as the reactivating power of these compounds measured on erythrocyte cholinesterase inhibited by medemo *in vitro*. The intraperitoneal application of oximes and atropine

after a subcutaneous application of soman shows that oximes II, III and TMB-4 produce similar protective effect by increasing the LD_{50} value about twice, while the protecting effects of oximes I and IV are less pronounced.

As far as we know the action of oxime III has not yet been described in literature, but on the basis of our results its potential protective properties should be taken into consideration in poisoning by other anticholinesterases.

ACKNOWLEDGEMENTS

The authors are greatly indebted to Prof. M. Laćan, Faculty of Technology, University of Zagreb, for the syntheses of oximes II to VI, and to Prof. Z. Binenfeld, Chemical Plant »Chromos-Katran-Kutrilin« Zagreb, for the syntheses of oxime I. Thanks are also due to the technical staff of Laboratories for Biochemistry and Toxicology, Institute for Medical Research and Ocupational Health, Zagreb. This work was partly supported by the Comission for Medical Research, Belgrade, Yugoslavia.

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Sažetak

REAKTIVACIJA FOSFORILIRANIH KOLINESTERAZA *IN VITRO* I ZAŠTITNI UČINCI *IN VIVO* NEKIH PIRIRIDINIJEVIH I KINOLINIJEVIH OKSIMA

Šest piridinijevih i kinolinijevih oksima (I–VI) bili su testirani kao reaktivatori eritrocitne i serumske kolinesteraze inhibirane s 0-etil-(2-dimetilaminotioetil/-metilfosfonatom (medemo) i O-etil-p-nitrofenil-etilfosfonatom (armin), te kao zaštitni agensi u trovanju štakora s 1-metil-2,2-dimetilpropilmetilfosfonofluoridatom (soman) i medemom. Dobiveni rezultati uspoređeni su s učinkom TMB-4.

Oksim IV [1,3 aceton-bis(4-piridinij-aldoksim) diklorid] pokazao se kao dobar reaktivator eritrocitne kolinesteraze inhibirane medemom, a upotrebljen zajedno s atro-

pinom kao zaštitno sredstvo u trovanju štakora povisuje LD_{50} medema 48 puta. Oksim I (1-benzil-4-piridinij-aldoksim klorid) i oksim III (1-fenacil-oksim-2-piridinij-aldoksim klorid) dobro reaktiviraju serumsku kolinesterazu inhibiranu arminom i medemom. Aplicirani zajedno s atropinom povisuju LD_{50} štakora otrovanih medemom 20 puta. Oksimi III i II (1-fenaciloksim-2-piridinij-aldoksim klorid) upotrebljeni zajedno s atropinom povećavaju LD_{50} somana 2.0 puta (oksim II) odnosno 1.8 puta (oksim III). Oksimi V i VI (1-fenaciloksim-kinolinij klorid i 1-fenaciloksim-*iso*kinolinij klorid) bili su vrlo toksični i uopće nisu reaktivirali inhibirane kolinesteraze.

Učinci testiranih oksima bili su jednaki ili slabiji od učinaka TMB-4 u istraživanjima *in vitro* i *in vivo*.

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Primljeno 22. V 1973.