KINETICS OF REACTIVATION OF ISOPROPYL-METHYLPHOSPHONYLATED BOVINE ERYTHROCYTE ACETYLCHOLINESTERASE BY BISQUATERNARY PYRIDINEMONOALDOXIMES*

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The kinetics of reactivation of isopropyl-methylphosphonylated acetylcholinesterase by thirteen different bisquaternary pyridinemonoaldoximes *in vitro* was studied. From the dependence of the reactivation rate on the concentration of the aldoxime the dissociation constants (K_R), the decomposition rate constants (k_R), and the bimolecular rate constants (k_r) were calculated for all the products studied. The relative effectiveness of these reactivators with respect to 2-pyridinealdoxime methiodide was calculated.

Acetylcholinesterase and similarly also cholinesterase, which had been inhibited by organophosphorus compounds, can be regenerated by certain nucleophilic agents, such as hydroxylamine, choline, hydroxamic acids, and above all oximes[1,2]. Among the most effective reactivators of cholinesterases belong aldoximes of the pyridine series which contain in their molecule quaternary nitrogen. These products have found use also as antidotes administered after poisoning by organophosphorus compounds. The best known products which fall into this group are, *e.g.* 2-pyridinealdoxime methiodide, N,N'-trimethylene-bis-(4-pyridinealdoxime) dibromide, and 1,3-bis-(4-pyridinealdoxime)-2-oxapropane dichloride (obidoxime, Toxogonin^R).

Earlier studies[3–6] have shown that the kinetics of reactivation of the phosphorylated enzyme by an oxime is analogous to the kinetics of interaction of the enzyme with the substrate. The reactivator forms with the phosphorylated enzyme a labile complex which decomposes to the regenerated enzyme and a reaction product, most likely the phosphorylated oxime[7,8]. The reaction can thus be characterized by two constants: by the dissociation constant of the intermediary labile complex phosphorylated enzyme-reactivator and by its decomposition rate constant.

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Studies dealing with the determination of these constants for known reactivators of the oxime series are very scarce. An investigation of this kind has been carried out, *e.g.* with 2- and 4-pyridinealdoxime methiodide and isonitrosoacetophenone on human erythrocyte acetylcholinesterase[4], inhibited by isopropyl-methylphosphonofluoridate and tetraethylpyrophosphate, and on human plasmatic cholinesterase[6], inhibited by diethyl-*p*-nitrophenyl phosphate. The data on the remaining reactivators are limited mostly to values of the bimolecular reactivation rate constant[9,10] or even to mere information on the per cent of reactivation per a certain time unit at one concentration of reactivator[11–13].

In this study the dissociation rate constants, decomposition constants, and bimolecular rate constants have been determined for thirteen reactivators of the pyridinealdoxime series, which can be characterized as bis-quaternary pyridinemonoaldoximes. Some of these compounds have been prepared earlier and already tested as reactivators[14–16]. Bovine erythrocyte acetylcholinesterase inhibited by isopropylmethylphosphonofluoridate served as a source of the phosphorylated enzyme.

EXPERIMENTAL

Chemicals. The chemical structure of the tested reactivators and some of their physico-chemical constants are given in Table I. These compounds have been prepared by Bielavský of this Institute and their synthesis has been described elsewhere[17]. 2-Pyridinealdoxime methiodide (Léčiva Prague), m.p. 219° (dec.) (recorded data[18] m.p. 210–220°, dec.), pK_a 7·84 (recorded data 7·82 (ref.[18]) and 7·84 (ref.[6])). The stock solutions of the reactivators were prepared in the buffer. used for the measurement of acetylcholinesterase activity (pH 8·0) and kept in a refrigerator at +4° for no longer than 2 days. Acetylcholine iodide (Lachema Brno), used as substrate, was dissolved to 0·1M solutions in the same buffer. The substrate solution was prepared fresh every day.

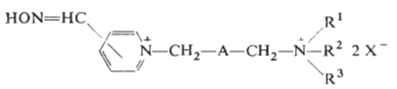
Acetylcholinesterase was prepared from bovine erythrocytes[19] and kept as a stable lyophilized preparation of specific activity 250 mU/mg. Measured K_m $5{\cdot}8 \cdot 10^{-4}$M (recorded data[20] $6{\cdot}0 \cdot 10^{-4}$M). The enzyme solution (5 mg/ml) was prepared fresh every day in Michel veronal-phosphate buffer[21] at pH 8·0.

Inhibition of acetylcholinesterase and reactivation of phosphorylated enzyme. The solution of acetylcholinesterase (1 ml = 5 mg of lyophilized preparation) was mixed with 1 ml of $2 \cdot 10^{-9}$M isopropyl-methylphosphonofluoridate (final concentration $1 \cdot 10^{-9}$M) and incubated 30 min. at 25° (approximately 80% inhibition). The reaction mixture was then diluted with the Michel buffer[21] to 19 ml, 1 ml of the reactivator solution was added, and the mixture was incubated at the same temperature for 1,2,3,4, and 5 min. respectively. The 0·1M solution of acetylcholine iodide (1 ml) was added afterwards (final concentration 4·76 mM) and the residual activity of the enzyme was measured.

Measurement of enzymatic activity. The activity of acetylcholinesterase was measured by the electrometric method[21] with the aid of a semiautomatic device described earlier[22]. Acetylcholine iodide (final concentration 4·76 mM) was used as substrate. The measurement was carried out at 25°C. Together with the testing of each reactivator, the activity was measured of the intact enzyme (a_0), of the inhibited enzyme (a_i), and of the reactivated enzyme (a_r) for several reactivator concentrations.

TABLE I

Chemical Structure of Tested Oximes and Some of Their Physico-Chemical Properties



Compound	Position of aldoxime group	A	R^1	R^2	R^3	X	m.p. °C	Recorded m.p., °C	pK_a
I	4	$-CH_2-$		pyridine		Br	242	212[a]	8·17
II	4	$-CH_2-$	Et	piperidine		Br	232–234	225[b] 209[c]	8·17
III	4	$-CH_2-$	Me	morpholine		Br	222–224	213[b] 216[c]	8·17
IV	4	$-CH_2-$	Me	piperidine		Br	227–230	217[b]	8·19
V	4	$-CH_2-$	Me	Me	Me	Br	168–169	159–160[b]	8·16
VI	4	$-(CH_2)_2-$	Me	Me	Me	Br	231–233	—	8·29
VII	4	$-(CH_2)_4-$	Me	Me	Me	Br	143–149	—	8·29
VIII	4	$-CH_2-$	Me	Me	C_2H_5OH	Br	142–144	—	8·18
IX	4	$-CH_2-$	Me	Et	Et	Br	209–211	180–192[b]	8·17
X	4	$-CH_2-$	Et	Et	Et	Br	238–240	223[b,c]	8·18
XI	4	—O—	Me	Me	Me	Cl	135–136	—	7·90
XII	4	—O—	Me	morpholine		Cl	196–200	—	7·86
XIII	2	—O—	Me	Me	Me	Cl	173–174	—	7·62

[a] ref.[14]; [b] ref.[15]; [c] ref.[16].

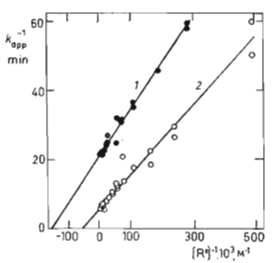


FIG. 1

Dependence of Reciprocal Values of k_{app} on Reciprocal Concentration Values of Dissociated Form of Reactivator $[R^{\pm}]$

1-(4-pyridiniumaldoxime)-3-trimethylammonium-propane dibromide (compound *V*); 1-(4-pyridiniumaldoxime)-3-diethylmethylammonium-propane dibromide (compound *IX*).

RESULTS AND DISCUSSION

The kinetics of the reactivation process can be described schematically by the equation

$$EI + R \overset{K_R}{\rightleftharpoons} EIR \xrightarrow{k_R} E + P$$

where EI is the phosphorylated enzyme, R the reactivator, EIR the intermediary complex, P the reaction product, and E the regenerated enzyme. K_R is the dissociation constant of complex EIR and k_R the decomposition rate constant of this complex.

For the calculation of these constants a relation has been derived[4,6] which is identical with the equation characterizing the relation between the rate of the enzymatic reaction and the concentration of the substrate under the conditions of Michaelis kinetics[23]. This equation in the following form

$$\frac{1}{k_{app}} = \frac{1}{k_R} + \frac{K_R}{k_R[R]}$$

permits the calculation of K_R and k_R and also of the bimolecular rate constant

TABLE II

Kinetic Constants of Tested Oximes and Their Relative Effectiveness with Respect to 2-Pyridinealdoxime Methiodide

Compound	k_R min^{-1}	K_R mM	k_r 10^3 liter. mol^{-1} min^{-1}	Relative effectiveness
2-PAM[a]	0·250 ± 0·022	0·670 ± 0·052	0·375 ± 0·030	1·0
I	0·218 ± 0·011	0·114 ± 0·034	1·910 ± 0·180	5·1
II	0·327 ± 0·063	0·141 ± 0·055	2·326 ± 0·380	6·2
III	0·074 ± 0·002	0·018 ± 0·007	4·141 ± 0·272	11·0
IV	0·102 ± 0·008	0·032 ± 0·007	3·160 ± 0·605	8·5
V	0·048 ± 0·004	0·007 ± 0·002	6·502 ± 0·280	17·4
VI	0·251 ± 0·023	0·213 ± 0·019	1·180 ± 0·120	3·2
VII	0·142 ± 0·021	0·035 ± 0·006	4·050 ± 0·050	10·8
VIII	0·124 ± 0·009	0·285 ± 0·058	0·435 ± 0·012	1·2
IX	0·182 ± 0·014	0·016 ± 0·002	11·400 ± 1·505	30·4
X	0·044 ± 0·006	0·019 ± 0·002	2·306 ± 0·112	6·3
XI	0·100 ± 0·003	2·500 ± 0·051	0·040 ± 0·004	0·1
XII	0·662 ± 0·026	2·650 ± 0·520	0·255 ± 0·043	0·7
XIII	0·041 ± 0·006	0·470 ± 0·036	0·097 ± 0·007	0·3

[a] 2-Pyridinealdoxime methiodide.

of reactivation $k_r = k_R/K_R$ for $[R] \ll K_R$, when we plot the reciprocal values of k_{app} *versus* the reciprocal values of $[R]$.

The apparent rate constant k_{app} can be calculated from

$$k_{app} = \frac{2{\cdot}303}{t} \log \frac{a_0 - a_r}{a_0 - a_i}$$

where t stands for reactivation time.

This approach to the evaluation of reactivators was used also in our study. Each experiment was carried out at least with 5 and at the most with 15 different reactivator concentrations. Since the effective form of the reactivator is only its dissociated ion[3-6], the concentrations of the reactivators were expressed as the concentration of the dissociated form at pH 8·0, calculated from the Henderson-Hasselbach equation. The interpolation of the lines through experimental points and the calculation of the corresponding constants were carried out on MINSK 22 computer using an modified program for the calculation of Michaelis constant[22,24].

The dependence of $1/k_{app}$ on $1/[R]$ for 1-(4-pyridiniumaldoxime)-3-trimethylammonium-propane dibromide (compound *V*) and 1-(4-pyridiniumaldoxime)-3-diethylmethylammonium-propane dibromide (compound *IX*) is shown by way of example in Fig. 1.

The calculated kinetic constants for all the thirteen reactivators tested are given in Table II. These constants are valid for the dissociated forms of the oximes at pH 8·0. The size of the bimolecular rate constant k_r serves as a measure of the reactivation effect. The size of these constants is also expressed with respect to 2-pyridinealdoxime methiodide, whose reactivation effectiveness was taken as one unit.

The obtained results indicate that all the oximes tested reactivate isopropyl-methylphosphonyl acetylcholinesterase. Their relative effectiveness, however, considerably differs. It follows from the comparison of the values of the relative effectiveness that except for compounds *XI*, *XII*, and *XIII*, all the remaining oximes show an effect better or comparable to that of 2-pyridinealdoxime methiodide. The best effect show compound *IX* and also compound *V*, which has given good results when employed for the treatment of experimental isopropyl-methylphosphonofluoridate intoxication in mice[25].

Effective reactivators among compounds of this type have been revealed also by Japanese authors[15,16]. The effect of these products *in vitro* has been evaluated with human erythrocyte acetylcholinesterase inhibited by tetraethylpyrophosphate[16] and a measure of the effect was the concentration of oxime producing 50% reactivation in five minutes (RC_{50}). Since the effect was evaluated from a different viewpoint and on different models, these and our results cannot be compared.

Both our results and the results of Japanese authors[16] confirm Wilson's hypothesis of the unnecessariness of the second oxime group in the reactivator molecule if the second quaternary nitrogen atom[1] is retained at proper distance. The substitution

of this nitrogen affects rather considerably the reactivation effect. A relation between the character of the substituent and the effect has not been found. It would appear that more effective are products with substituents which are less bulky. A different relationship, however, has been observed by Nishimura and coworkers[16].

The effect of the chain length between the two quaternary nitrogens has been investigated only with two higher homologs of compound *V* (the tetramethylene homolog, compound *VI*, and the hexamethylene homolog, compound *VII*) and no unambiguous conclusions could be drawn. The replacement of the middle methylene group in the three-carbon atom chain by an oxygen atom (compounds *XI* and *XII*), which in the case of N,N'-trimethylene-bis-(4-pyridinealdoxime) dibromide leads to the more effective Toxogonin® (ref.[26]), results in a decrease of the reactivation effectiveness.

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