REACTIVATION OF ISOPROPYL-METHYLPHOSPHONYLATED RAT BRAIN ACETYLCHOLINESTERASE BY OXIMES

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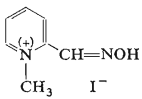
The paper deals with the reactivating effect of oximes on rat brain acetylcholinesterase inhibited by isopropyl-methylphosphonofluoridate *in vitro*. As reactivators the following compounds were used: 1-methyl-2-pyridiniumaldoxime iodide (pralidoxime), N,N'-trimethylene-bis(4-pyridiniumaldoxime)dibromide (trimedoxime), 1,3-bis(4-pyridiniumaldoxime)-2-oxapropane dichloride (obidoxime) and 1-(4-pyridiniumaldoxime)-3-diethylmethylammoniumpropane dibromide. The time characteristic of the reactivation process and its dependence on the reactivator concentration are described.

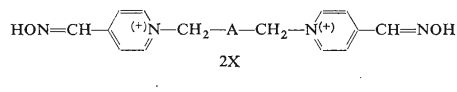
The interaction of cholinesterases with organic phosphorus compounds results in the formation of inactive phosphorylated enzymes[1]. The enzymes thus phosphorylated are irreversibly inhibited but their activity can be restored by treatment with nucleophilic agents, the most potent of these being quaternary derivatives of 2- and 4-pyridinaldoxime[2,3].

Literature reports contain a number of data on the kinetics of reactivation of phosphorylated cholinesterases by the action of oximes[4–10]. As the source of the enzyme, in most cases human or horse plasma butyrylcholinesterase[8,11,12] or human or bovine erythrocyte acetylcholinesterase[5–7,9,10,12,13] are used. Only few authors have used brain acetylcholinesterase[14–16] since its properties are not fully identical with those of the erythrocyte enzyme[17]. Since the brain acetylcholinesterase is a key enzyme during organophosphorus intoxications and the degree of its damage is directly related to the clinical state of the organism[17,18] it is important to understand the kinetic parameters of reactivation of this phosphorylated enzyme for a possible confrontation with therapeutic results.

The present work is concerned with the study of the reactivating effect of four oximes of the pyridine series on rat brain acetylcholinesterase inhibited by isopropyl-

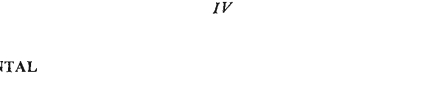


I



II, A = CH_2, X = Br
III, A = 0, X = Cl

methylphosphonofluoridate. As reactivators, the following compounds were used: 1-methyl-2-pyridiniumaldoxime iodide (pralidoxime, *I*), N,N'-trimethylene-bis(4-pyridinumaldoxime) dibromide (trimedoxime, *II*), 1,3-bis-(4-pyridiniumaldoxime)-2-oxapropane dichloride (obidoxime, *III*) and 1-(4-pyridiniumaldoxime)-3-diethylmethylammoniumpropane dibromide (BI-100, *IV*). The first three oximes are the best understood reactivators which found their application even in human therapy of organophosphorus intoxications[19-23]. Compound *IV* was found to be an efficient reactivator of isopropyl-methylphosphonylated bovine erythrocyte acetylcholinesterase[24].



IV

EXPERIMENTAL

Enzyme preparation. As a source of the enzyme we used a homogenate of whole rat brain (rats of Wistar strain) without sex preference, of individuals weighing 180—220 g. The animals were killed by cutting the carotids and the brains were excised, rinsed in physiological saline and stored at −18°C. Directly before use they were homogenized in an Ultra-Turrax (Germany) homogenizer in a veronal–phosphate (1 : 9) buffer of pH 8·0 made isotonic with sodium chloride[25].

Reagents. Compound *I*, m.p. 219°C (decomp.), ref.[26] gives a m.p. of 218—219°C (decomp.); compound *II*, m.p. 241°C (decomp.), ref.[27] gives a m.p. of 238—241°C (decomp.). Both were made by Léčiva, Prague. Compound *III* was prepared according to Lüttringhaus and Hagedorn[23] and melted at 212°C (decomp.), ref.[28] gives a m.p. of 212°C (decomp.). Reactivator *IV* was prepared according to a previously described procedure[29] and melted at 209—211°C (decomp.), ref.[30] gives a m.p. of 180—192°C (decomp.). The oximes were dissolved in a veronal–phosphate buffer of pH 8·0 to 0·01M solutions and were processed on the same day. The other chemicals were of analytical purity.

Preparation of phosphonylated acetylcholinesterase and its reactivation. One ml 10% brain homogenate was mixed with 1 ml 10^{-8}M isopropyl-methylphosphonofluoridate in a veronal–phosphate buffer and the mixture was incubated at 25°C for 30 min (about 90% inhibition). Then 1 ml of a reactivator solution at a suitable concentration was added and, after time *t*, the reaction mixture was made up to the final volume of 20 ml with the veronal–phosphate buffer[25]. After adding 1 ml 0·1M acetylcholine iodide the enzyme activity was assayed.

Activity of acetylcholinesterase was determined by the electrometric method[25] using a semiautomatic equipment with direct registration[31]. The measurement took place at 25°C, using acetylcholine iodide at a final concentration of 4·76 mM as substrate. In each series, both the activity of the reactivated enzyme (a_r) and of the uninhibited enzyme (a_0) when 1 ml of the buffer was added instead of the isopropyl-methylphosphonofluoridate, as well as that of the inhibited enzyme (a_i) when 1 ml buffer was added instead of the reactivator, was estimated. The percentage of the reactivated enzyme was estimated from the following formula:

$$\% \text{ reactivation} = 100 - 100(a_0 - a_r)/(a_0 - a_i) \qquad (1)$$

and the fraction of reactivation r from the formula:

$$r = 1 - (a_0 - a_r)/(a_0 - a_i) \qquad (2)$$

Calculation of the kinetic constants: The mathematical processing of the results was done on a Minsk-22 computer. Regression analysis was used for constructing straight lines through the experimental points.

RESULTS

In a series of measurements we followed the dependence of fractional reactivation r on the time of preincubation of the reactivator with the phosphonylated enzyme. The final value r which did not change with prolonged preincubation was designated as r_f. By plotting the value of $\log(r_f - r)$ against t_r, series of straight lines were obtained (Figs 1–4) from which the monomolecular rate constants k_1 were calculated according to

$$k_1 = -2{\cdot}303 \operatorname{tg} \alpha\,, \qquad (3)$$

where $\operatorname{tg} \alpha$ is the slope of the regression line. By dividing the monomolecular rate

TABLE I

Monomolecular (k_I) and Bimolecular (k_{II}) Rate Constants of Reactivation of Isopropyl-methylphosphonylated Rat Brain Acetylcholinesterase by Oximes *in vitro*

Oxime	Concentration M	k_I min^{-1}	k_{II} $l\,mol^{-1}\,min^{-1}$
I	$3 . 10^{-4}$	1·66	$5{\cdot}55 . 10^3$
	$2{\cdot}5 . 10^{-4}$	1·29	$5{\cdot}16 . 10^3$
	$1{\cdot}5 . 10^{-4}$	0·99	$6{\cdot}60 . 10^3$
	$1 . 10^{-4}$	0·66	$6{\cdot}60 . 10^3$
II	$5 . 10^{-5}$	1·17	$2{\cdot}35 . 10^4$
	$2{\cdot}5 . 10^{-5}$	1·15	$4{\cdot}60 . 10^4$
	$1 . 10^{-5}$	1·01	$1{\cdot}01 . 10^5$
	$5 . 10^{-6}$	0·80	$1{\cdot}60 . 10^5$
III	$5 . 10^{-4}$	4·85	$9{\cdot}70 . 10^3$
	$1 . 10^{-4}$	1·86	$1{\cdot}86 . 10^4$
	$5 . 10^{-5}$	1·08	$2{\cdot}16 . 10^4$
	$1 . 10^{-5}$	0·70	$9{\cdot}90 . 10^4$
IV	$2{\cdot}5 . 10^{-4}$	1·55	$6{\cdot}20 . 10^3$
	$1 . 10^{-4}$	1·06	$1{\cdot}06 . 10^4$
	$2{\cdot}5 . 10^{-5}$	0·89	$3{\cdot}55 . 10^4$

TABLE II

Kinetic Parameters of Reactivation of Isopropyl-methylphosphonylated Rat Brain Acetylcholinesterase by Oximes *in vitro*

R_{10}, R_{50}, R_{90} are the concentrations of the reactivator capable of restoring 10, 50 and 90% of the inhibited enzyme within 5 min, n_H Hill coefficient.

Oxime	R_{10} M	R_{50} M	R_{90} M	R_{90}/R_{10}	n_H[a]
I	$2{\cdot}10 \cdot 10^{-5}$	$1{\cdot}40 \cdot 10^{-4}$	$1{\cdot}10 \cdot 10^{-4}$	52·5	1·08
II	$2{\cdot}80 \cdot 10^{-7}$	$3{\cdot}55 \cdot 10^{-6}$	$4{\cdot}00 \cdot 10^{-5}$	144·0	0·89
III	$5{\cdot}40 \cdot 10^{-6}$	$4{\cdot}00 \cdot 10^{-5}$	$2{\cdot}80 \cdot 10^{-4}$	52·0	1·08
IV	$3{\cdot}50 \cdot 10^{-6}$	$5{\cdot}50 \cdot 10^{-5}$	$8{\cdot}60 \cdot 10^{-4}$	245·0	0·81

[a] In view of the fact that the source of the enzyme is a crude homogenate of rat brain one cannot exclude a nonspecific binding of the reactivator to ballast protein. This may affect the magnitude of n_H.

constants by the corresponding reactivator concentration the bimolecular rate constants k_{II} were obtained ($k_{II} = k_I/[R]$).

The computed values of the two rate constants are shown in Table I. The values of k_{II} are not independent of the reactivator concentration $[R]$ as would correspond to second-order kinetics but rise with decreasing concentration of the reactivator.

Dependence of the degree of reactivation on the reactivator concentration. The reactivator was preincubated for 5 min with the phosphonylated enzyme which is long enough for attaining dynamic equilibrium. The dependence of fractional reactivation on $[R]$ is a sigmoid function which gives a straight line in a probit-logarithmic transformation (Fig. 5). From the regression line the reactivator concentrations producing 10%, 50% and 90% reactivation effect were estimated and designated with R_{10}, R_{50} and R_{90}. The values of these constants are shown in Table II, together with the values of the ratio R_{90}/R_{10} and with the values of the Hill coefficient n_H which was obtained from the ratio R_{90}/R_{10} using a nomogram constructed on the basis of published data[32]. The construction of the nomogram is based on the fact that $1/\log(R_{90}/R_{10})$ is directly proportional to the Hill coefficient n_H.

DISCUSSION

It follows from the data obtained that the most efficient reactivator of isopropyl-methylphosphonylated rat brain acetylcholinesterase is trimedoxime and the least efficient reactivator is pralidoxime. The efficiency of *III* and *IV* is about the same and lies between that of *II* and *I*. The results are in agreement with the finding of Back[16] who found a greater reactivation effect of obidoxime as compared with pralidoxime

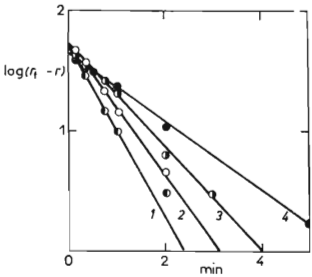


FIG. 1

Time Dependence of Reactivation of Isopropyl-methylphosphonylated Rat Brain Acetylcholinesterase with Pralidoxime

1 $3 \cdot 10^{-4}$, *2* $2{\cdot}5 \cdot 10^{-4}$, *3* $1{\cdot}5 \cdot 10^{-4}$, *4* 10^{-4} M reactivator. t_r Duration of reactivation, $\log (r_f - r)$ logarithm of the loss of inhibited enzyme.

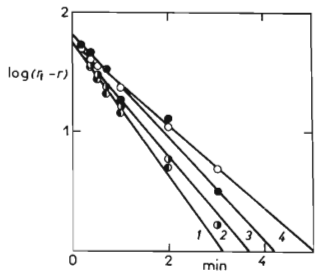


FIG. 2

Time Dependence of Reactivation of Isopropyl-methylphosphonylated Rat Brain Acetylcholinesterase with Trimedoxime

1 $5 \cdot 10^{-5}$, *2* $2{\cdot}5 \cdot 10^{-5}$, *3* 10^{-5}, *4* $5 \cdot 10^{-6}$ M reactivator. t_r Duration of reactivation, $\log (r_f - r)$ logarithm of the decrease of inhibited enzyme.

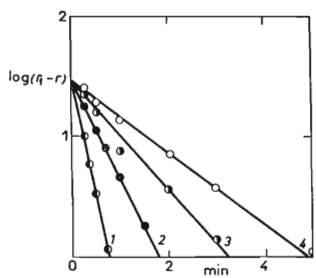


FIG. 3

Time Dependence of Reactivation of Isopropyl-methylphosphonylated Rat Brain Acetylcholinesterase with Obidoxime

1 $5 \cdot 10^{-4}$, *2* 10^{-4}, *3* $5 \cdot 10^{-5}$, *4* 10^{-5} M reactivator. t_r Duration of reactivation, $\log (r_f - r)$ logarithm of the decrease of inhibited enzyme.

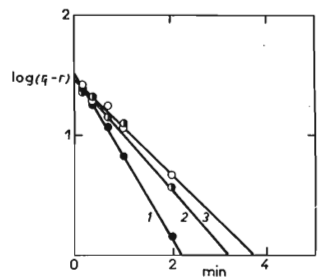


FIG. 4

Time Dependence of Reactivation of Isopropyl-methylphosphonylated Rat Brain Acetylcholinesterase with *IV*

1 $2{\cdot}5 \cdot 10^{-4}$, *2* 10^{-4}, *3* $2{\cdot}5 \cdot 10^{-5}$ M, reactivator. t_r Duration of reactivation, $\log (r_f - r)$ logarithm of the decrease of inhibited enzyme.

if rat brain acetylcholinesterase inhibited with isopropyl-methylphosphonofluoride was used as the phosphonylated enzyme.

The magnitude of the bimolecular rate constant k_{II} depends on the reactivator concentration as was observed several times before[5,7,33]. As the reactivator concentration decreases the value of k_{II} approaches the limit of k_r which is the bimolecular rate constant for $[R] \ll K_R$ where K_R is the dissociation constant of the transition complex phosphorylated enzyme-reactivator given by[8] $K_R = [EP] \,.\, [R]/[EPR]$ where EP is the phosphorylated enzyme, R is the reactivator and EPR the transition complex. This complex then breaks down to the regenerated enzyme E and to the reaction product P so that the overall reactivation scheme can be written as[5,8]

$$EP + R \underset{}{\overset{K_R}{\rightleftharpoons}} EPR \xrightarrow{k_R} E + P$$

Under these conditions the bimolecular rate constant k_r is given by the ratio k_R/K_R.

In the present case the k_{II} constant depends on the reactivator concentration and increases with its decreasing concentration which is in full agreement with what has been said above. A certain exception to this case is formed by pralidoxime with which the k_{II} is practically independent of reactivator concentration. Its mean value is $5{\cdot}98 \pm 1{\cdot}04 \,.\, 10^3$ l mol^{-1} min^{-1} (mean $\pm$ confidence interval for $P = 0{\cdot}95$). The reactivator concentrations used are probably lower than the dissociation constant K_R so that k_{II} approaches the value of k_r. In contrast with the other reactivators studied pralidoxime shows a high value of K_R which indicates a low affinity of this reactivator for isopropyl-methylphosphonylated acetylcholinesterase. Similar results were obtained during reactivation of isopropyl-methylphosphonylated bovine erythrocyte acetylcholinesterase (unpublished) where for pralidoxime a value

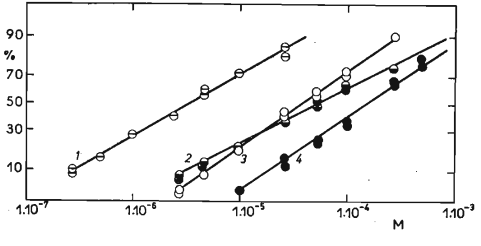


FIG. 5
Dependence of Reactivation of Isopropyl-methylphosphonylated Rat Brain Acetylcholinesterase on Reactivator Concentration

1 Trimedoxime, *2 IV*, *3* obidoxime, *4* pralidoxime. % reactivation in probit scale, M molarity of reactivator.

of K_R of $1{\cdot}00 \cdot 10^{-3}$M was obtained while for the other reactivators the values of K_R were much lower ($6{\cdot}40 \cdot 10^{-5}$M for *II*; $5{\cdot}00 \cdot 10^{-5}$M for *III* and $4{\cdot}35 \cdot 10^{-5}$M for *IV*).

A comparison of the efficiencies of reactivators on the basis of concentrations bringing about the desired effect depends to a certain extent on the magnitude of the selected effect if the regression lines of the dependence of the per cent reactivation on reactivator concentration are not parallel as the case is here. Most convenient for the comparison appears to be a concentration bringing about 10% reactivation (R_{10}). It is believed that the value of R_{10} might be in best correlation with the therapeutical efficiency of the compounds since reactivator doses bringing about a 10% increase in activity of certain brain regions of intoxicated animals are therapeutically fully active. According to J. Bajgar the drop of activity below this limit results in intoxication symptoms of all animals.

The magnitude of R_{10} appears to be more suitable for estimating the reactivation efficiency than the value R_{50} which was used *e.g.* by Nishimura and coworkers[34]. The relative efficiency of the reactivators referred to pralidoxime and expressed by the ratio of R_{10} may be expressed as follows: *I* 1, *II* 75, *III* 3·9 and *IV* 6.

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