KINETIC AND THERMODYNAMIC PARAMETERS OF REACTIVATION OF O-ISOPROPYL-METHYLPHOSPHONYLATED RAT BRAIN ACETYLCHOLINESTERASE BY ASYMMETRIC BIS-QUATERNARY 4-PYRIDINE ALDOXIMES

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The equilibrium kinetics was studied of reactivation of O-isopropyl-methylphosphonylated rat brain acetylcholinesterase by asymmetric bis-quaternary 4-pyridine aldoximes. The kinetic constants were calculated characterizing this interaction, *i.e.* the concentration of reactivator causing a 50% reactivation, the dissociation constant of the EIR_n complex, and Hill's coefficient indicating the number of reactivator molecules in this complex. The standard changes in free energy of reactivator binding to the phosphonylated enzyme were determined from the values of the dissociation constant of the EIR_n complex, calculated per concentration of the ionized form of the oxime at pH 8·0.

The finding that oximes can reactivate acetylcholinesterase inhibited by organophosphorus compounds[1] led to the synthesis of a great number of these products which were shown to possess a reactivating effect both *in vitro* and *in vivo* (*e.g.*[2-8]). The most effective reactivators were discovered among the quaternary derivatives of 2- and 4-pyridine aldoxime[2,9]. The first compound of practical applicability in the therapy of organophosphorus poisoning was 2-pyridinium aldoxime methoiodide (2-PAM), independently synthetized by Childs and coworkers[1] and Wilson and Ginsburg[2]. Reactivators of the bis-quaternary 4-pyridine aldoxime group[3,10], which were even more effective than 2-PAM, were prepared later. As predicted by Wilson[11] and confirmed by other workers later[12-14], the factor decisive for the high effect of reactivators of this type is not the presence of a second aldoxime group but the presence of a second quaternary nitrogen atom at an appropriate distance from the aldoxime group. Reactivators of this type are usually designated as asymmetric bisquaternary aldoximes.

This paper deals with the reactivating effect of six asymmetric bis-quaternary 4-pyridine aldoximes in which two quaternary nitrogen atoms are linked together by a $—CH_2—CH_2—CH_2—$ or $—CH_2—O—CH_2—$ chain. The reactivating effect of these compounds was tested *in vivo* on rat brain acetylcholinesterase inhibited by O-isopropyl-methylphosphonofluoridate.

EXPERIMENTAL

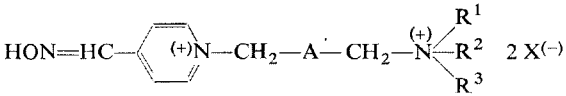
Chemicals. The chemical structure of the reactivators used and some of their physico-chemical constants are given in Table I. 1-(4-Pyridiniumaldoxime)-3-trimethylammonium-propane dibromide (*Ia*), 1-(4-pyridiniumaldoxime)-3-trimethylammonium-2-oxapropane dichloride (*Ib*), 1-(4-pyridiniumaldoxime)-3-methylmorpholinium-propane dibromide (*IIa*), 1-(4-pyridiniumaldoxime)-3-methylmorpholinium-2-oxapropane dichloride (*IIb*), and 1-(4-pyridiniumaldoxime)-3-diethylmethylammonium-propane dibromide (*IIIa*) were prepared in this Institute and their synthesis was described elsewhere[15]. 1-(4-Pyridiniumaldoxime)-3-diethylmethylammonium-2-oxapropane dichloride (*IIIb*) was prepared as follows. Bis-chloromethyl ether, 100 ml, (1·2 mol) in 400 ml of a mixture of acetone and ether (1 : 1, v/v) was treated with stirring dropwise with 26·1 g (0·3 mol) of diethylmethylamine in 200 ml of ether at −20 to −30°C. The reaction mixture was stirred 3 h at −20°C and then set aside for 3 days at +4°C in a cold room. The product which had separated was allowed to settle repeatedly in five subsequent portions of a mixture of ether and acetone (2 : 1, v/v). The solvents were decanted off and the product was dried *in vacuo*. The crude product was dissolved in 500 ml of dimethylformamide containing 36 g (0·3 mol) of 4-pyridine aldoxime. The solution was allowed to stand two days at room temperature. The crystalline product which had separated was filtered off, washed with dimethylformamide and acetone, and recrystallized from 250 ml of 2-propanol. The yield was 50 g (49% of theory calculated for diethylmethylamine) of a product, m.p. 127–129°C. Analysis for $C_{13}H_{23} . Cl_2N_3O_2 + 1/3\ C_3H_8OCl$: calculated 20·60%, found 20·51%. Diethylmethylamine was prepared from diethylamine by the Leuckart–Wallach reaction. 4-Pyridine aldoxime was purchased from Schuchardt, acetylcholine iodide and the remaining common chemicals from Lachema. O-Isopropyl-methylphosphonofluoridate was a generous gift of Dr J. Vachek.

Preparation of O-isopropyl-methylphosphonylated acetylcholinesterase and its reactivation. A homogenate of whole brain of Wistar rats (weight 180–220 g) of both sexes served as a source of acetylcholinesterase. The animals were sacrificed by scission of carotid arteries, the brains were rapidly excised, washed with physiological saline, and kept at −20°C. The brains were homogenized with physiological saline to a 10% homogenate (w/v) immediately before use. The 10% brain homogenate (0·5 ml) was mixed with 0·5 ml of $1 . 10^{-8}$M O-isopropyl-methylphosphonofluoridate and the mixture was incubated 30 min at 25°C (about 90% of inhibition). Subsequently 1 ml of the reactivator solution at appropriate concentration was added, the pH was adjusted to 8·00 (Radiometer) and the mixture was incubated 5 min at 25°C. This time is sufficient for the reactivation to achieve equilibrium conditions even at the lowest reactivator concentrations. After 5 min of contact of inhibited enzyme with the reactivator, the volume of the reaction mixture was made up to 20 ml with physiological saline and its enzymatic activity was measured after the addition of 1 ml of 0·1M acetylcholine iodide.

The activity of acetylcholinesterase was measured in Radiometer pH-stat consisting of autoburet ABU 12, titration assembly TTA 3, titrator TTT 11, pH-meter PHM 26, and recorder SBR 2c. The substrate was 4·76 mM acetylcholine iodide and acetic acid released by enzymatic hydrolysis was titrated with 0·05M-NaOH. The measurement was made at 25°C and pH 8·0. The activity of the enzyme was expressed as initial rate of hydrolysis of substrate. In each series of measurement, the activity of the inhibited (a_i) and reactivated (a_r) sample was measured in addition to the sample of uninhibited enzyme (a_0). When measurements of a_0 were made, the mixture contained the reactivator at the same concentration as that used for reactivation to eliminate the inhibitory effect of the reactivator itself. The latter, however, was observed only with the highest concentrations used. Fractional reactivation, r, was calculated from the formula $r = (a_r-a_i)/(a_0-a_i)$. The constants characterizing the reactivation process under equilibrium conditions were calculated as described earlier[16]. All the calculations were made in MINSK-22 computer.

TABLE I

Chemical Structure and Some Physico-Chemical Constants of Reactivators Used



Compound	A	R^1	R^2	R^3	X	M.p., °C[a]	pK_a[b]
Ia	$-CH_2-$	CH_3	CH_3	CH_3	Br	168–169	8·16
Ib	—O—	CH_3	CH_3	CH_3	Cl	135–136	7·90
IIa	$-CH_2-$	CH_3	morpholine		Br	222–224	8·17
IIb	—O—	CH_3	morpholine		Cl	196–200	7·86
IIIa	$-CH_2-$	CH_3	C_2H_5	C_2H_5	Br	209–211	8·17
IIIb	—O—	CH_3	C_2H_5	C_2H_5	Cl	127–129	7·68

[a] The melting points were determined on a Kofler block and were not corrected. Except for compound *IIIb*, all substances melted with decomposition. [b] The dissociation constant of the oxime group was measured spectrophotometrically.

TABLE II

Kinetic and Thermodynamic Constants Characterizing Reactivation of O-isopropyl-methylphosphonylated Rat Brain Acetylcholinesterase by Asymmetric Bis-quaternary 4-Pyridine Aldoximes Under Equilibrium Conditions

The constants were determined at 25°C and are given as arithmetic means ± standard deviation. *n*, Hill's coefficient indicating the number of reactivator molecules in the EIR_n complex, pR_{50}, negative decadic logarithm of reactivator concentration which causes a 50% reactivation pK_r negative decadic logarithm of dissociation constant of EIR_n complex, $pR_{50}^{\pm}$ and $pK_r^{\pm}$, constant calculated per concentration of ionized form of reactivator at pH 8·0, ΔG° standard change in free energy calculated from the formula $\Delta G^\circ = 2{\cdot}303\, \boldsymbol{RT}\, pK_r^{\pm}$. Standard deviation (S.D.) of ΔG° calculated from formula[18] $S.D.(\Delta G^\circ) = \boldsymbol{RT}[S.D.(K_r^{\pm})]/K_r^{\pm}$, δ, ratio of ΔG° of compound indexed *a* to ΔG° of compound indexed *b*.

Compound	*n*	pR_{50}	pK_r	$pR_{50}^{\pm}$	$pK_r^{\pm}$	ΔG° kcal/mol	δ
Ia	0·98 ± 0·04	3·34 ± 0·09	3·26 ± 0·09	3·73 ± 0·10	3·66 ± 0·10	−4·90 ± 0·04	1·65
Ib	0·58 ± 0·06	3·42 ± 0·11	1·98 ± 0·07	3·70 ± 0·13	2·23 ± 0·10	−2·98 ± 0·06	
IIa	1·30 ± 0·04	3·84 ± 0·07	4·98 ± 0·10	4·24 ± 0·10	5·36 ± 0·14	−7·15 ± 0·04	1·78
IIb	0·59 ± 0·02	3·02 ± 0·05	1·78 ± 0·04	3·26 ± 0·07	3·00 ± 0·09	−4·02 ± 0·04	
IIIa	0·79 ± 0·04	4·22 ± 0·10	3·32 ± 0·09	4·62 ± 0·12	3·71 ± 0·11	−4·98 ± 0·04	1·76
IIIb	0·70 ± 0·07	2·77 ± 0·12	1·94 ± 0·10	2·94 ± 0·14	2·11 ± 0·11	−2·83 ± 0·07	

RESULTS

The kinetics of reactivation of acetylcholinesterase, which has been blocked by an organophosphorus inhibitor, EI, by a reactivator, R, can be described[17] by a general equation

$$EI + nR \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EIR_n \xrightarrow{k_2} E + P,$$

where EIR_n is a temporary complex containing n molecules of bound reactivator, E the regenerated enzyme, and P the reaction product; k_1, k_{-1}, and k_2 are the corresponding rate constants. The equilibrium kinetics of reactivation describes equation (*1*)

$$\frac{1 - r}{r} = \frac{k_1[R]^n}{k_{-1} + k_2}. \qquad (1)$$

Eq. (*1*) can be transformed logarithmically to the equation of a straight line[16]

$$\log [(1 - r)/r] = n \log [R] - \log K_r, \qquad (2)$$

where $K_r = (k_{-1} + k_2)/k_1$.

By plotting $\log [(1 - r)/r]$ *versus* $\log [R]$ a straight line is obtained whose slope indicates the order of the reaction, n, the so-called Hill's coefficient, the intersection with the ordinate the negative logarithm of the dissociation constant, pK_r, and the intersection with the abscissa the negative logarithm of reactivator concentration at which 50% reactivation occurs, pR_{50}.

The dependence of reactivation degree on reactivator concentration in the Hill plot for all six compounds studied is given in Fig. 1. The values obtained experimentally comply with a linear equation, as evidenced by the high values of correlation coefficients, varying over the range 0·96–0·99. The correlation is in all cases significant for $P > 0{\cdot}99$. The constants calculated, which characterize the reactivation process under equilibrium conditions, are summarized in Table II. Since the ionized form of the oxime is the only effective form of the reactivator, the values of pR_{50} and pK_r were calculated by means of the Henderson–Hasselbach equation for the concentrations of the ionized form at pH 8·0. These constants are marked by symbols $pR_{50}^{\pm}$ and $pK_r^{\pm}$ and are also given in Table II.

DISCUSSION

The results obtained provide evidence showing that all the asymmetric bis-quaternary 4-pyridine aldoximes tested reactivate rat brain acetylcholinesterase inhibited by O-isopropyl-methylphosphonofluoridate. Propane derivatives are generally better

reactivators than 2-oxapropane derivatives. The values of Hill's coefficient are smaller than one, except for compound *IIa*. We may thus use the term of negative cooperativity[19] in this case yet we must consider the fact that the Hill's coefficient measured experimentally merely indicates the apparent number of ligands bound to the enzyme molecule[20]. The stoichiometry of this reaction then corresponds to a situation where one reactivator molecule reacts with one molecule of the phosphonylated enzyme[16].

Since the measurement is made under equilibrium conditions after the quantity of enzyme reactivated has achieved its maximum and no longer changes, $k_2 = 0$ and hence $k_{-1} \gg k_2$. Constant K_r is then given by expression $K_r = k_{-1}/k_1$ and represents the true dissociation constant of complex EIR_n. We can therefore calculate from its value the magnitude of standard change in free energy of the bond ΔG° from the formula $\Delta G^\circ = -RT \log (1/K_r)$, where the reciprocal value of K_r represents the equilibrium constant of complex EIR_n.

From the knowledge of values of ΔG° we can imagine the equilibrium distance, d_e, between the reactivator and the inhibited enzyme during their interaction, leading to the formation of complex EIR_n. The character of this interaction is predominantly electrostatic; the interaction involves the quaternary nitrogen atom of the reactivator and the ionized carboxyl group which represents the anionic center of

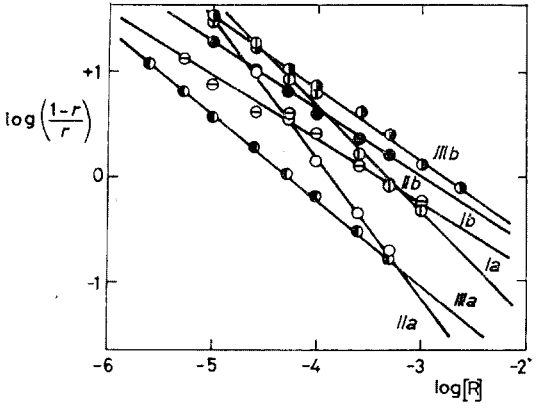


FIG. 1

Dependence of Degree of Reactivation of O-Isopropyl-methylphosphonylated Rat Brain Acetylcholinesterase on Molar Concentration of Reactivator in Hill Plot

Measured at 25°C and pH 8·0. Each point represents a mean value of three independent measurements. The interpolation of straight lines in experimental points was effected by regression analysis in the computer. The constants calculated are summarized in Table II. Designation of straight lines: ◌ compound *Ia*, ◌ compound *Ib*, ○ compound *IIa*, ● compound *IIb*, ◐ compound *IIIa*, and ◑ compound *IIIb*.

acetylcholinesterase[21]. If we disregard the influence of ionic atmosphere, a value of d_e equal 3·4 to 4·7 Å corresponds to the binding energies of ΔG° in the range of approximately[22] 3 to 7 kcal/mol.

The second quaternary nitrogen atom of the oxime molecule, *i.e.* the one which is not a component of the pyridine ring, plays the main role in the binding of the bis-quaternary reactivator to the active surface of phosphonylated acetylcholinesterase. This follows from the finding that the binding energy is strongly affected by the character of substituents on this nitrogen atom. More bulky substituents prevent the $-\overset{+}{N}\equiv$ group from a closer contact with the $-COO^{(-)}$ group thus increasing the value of d_e and decreasing the value of ΔG°. Appearingly electrostatic Coulomb forces are not the only ones which participate on the interaction of the reactivator with phosphorylated acetylcholinesterase. This is indicated by the fact that the ΔG°-values measured by us are on the average slightly higher that the corresponding binding energy values measured in experiments with the interaction of tetraalkylammonium salts with acetylcholinesterase[23]. This difference could be ascribed to the presence of two quaternary nitrogen atoms in one type of reactivator, yet the changes in binding energy caused by the introduction of an oxygen atom into the chain linking the two quaternary nitrogen atoms provide evidence of participation of this chain on the bond. Van der Waals forces, whose binding energy per one methylene group lies in the range[24] 0·36–0·95 kcal/mol, play a role in this hydrophobic interaction. The importance of hydrophobic interactions for the binding of reactivators to intact and phosphonylated acetylcholinesterase has been demonstrated before[25]. The replacement of one methylene group of the linking reactivator by an oxygen atom leads to a decrease of the ΔG°-value. The ratio of ΔG° for a compound with the $-CH_2-CH_2-CH_2-$-chain to ΔG° for a compound with the $-CH_2-O-CH_2-$ is constant for all three pairs of compounds (*cf.* Table II) and its mean value is 1·72.

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