AFFINITY OF BIS-QUATERNARY PYRIDINEDIALDOXIMES FOR THE ACTIVE CENTRE OF INTACT AND ISOPROPYLMETHYLPHOSPHONYLATED ACETYLCHOLINESTERASE

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The effect of eight members of series of the N,N'-polymethylene-bis(4-pyridinaldoxime) dibromides on intact and isopropylmethylphosphonylated acetylcholinesterase was examined. A relationship between the kinetic constants characterizing this interaction, the chemical structure of the compounds used and their lipophilic nature was sought.

According to the present concepts the active centre of acetylcholinesterase is formed by an esteratic and by one[1] or two[2] anionic sites. Acetylcholine which is the substrate of the enzyme is bound by electrostatic forces between the quaternary nitrogen and one of the anionic sites while the esteratic site which is formed most likely by the serine hydroxyl[3,4] is being acetylated. The acetylated enzyme is then rapidly hydrolyzed by water[5]. The inhibitory effect of organophosphoric compounds on acetylcholinesterase is accounted for by the phosphorylation of the esteratic site in the active centre of the enzyme[6,7]. Hydrolysis of the phosphorylated enzyme is very slow in contrast with the hydrolysis of the acetylated enzyme so that the reaction leading to the phosphorylated enzyme may be considered as irreversible. The phosphorylated enzyme may be reactivated through the action of compounds more nucleophilic than water[8], the most potent being aldoximes of the pyridine series containing a quaternary nitrogen in the molecule[9,10]. The compounds are designated as cholinesterase reactivators. Through their action, the inhibited enzyme is dephosphorylated and its activity is restored[11].

Recently, principal attention has been devoted to the relationship between the chemical structure of the reactivators and their effect[12–14] which was also the aim of this work. The effect of the reactivators pivots on the presence of the quaternary nitrogen atom at a suitable distance from the oxime group[15] through which it is bound to the anionic site of the active centre and thus facilitates the orientation of the reactivator molecule to a position suitalbe for a nucleophilic attack. The affinity of these compounds for the anionic site of the active centre of acetylcholinesterase causes them to behave as reversible inhibitors[16–18] toward the intact, *i.e.* non-phosphorylated enzyme, in common with other quaternary ammonium bases[1,19–21].

EXPERIMENTAL

Chemicals. The reactivators used and some of their physicochemical properties are shown in Table I. N,N'-Monomethylene- to N,N'-tetramethylene-bis(4-pyridinaldoxime) dibromides were

prepared in this laboratory by Dr J. Janoušek[22]; the other oximes were obtained from Dr P. Milojević, Medical Faculty, University of Belgrade, Yugoslavia.

Acetylcholinesterase was prepared from washed bovine erythrocytes from which the enzyme was released by an osmotic shock and adsorbed to a calcium phosphate gel. After removing hemoglobin by washing the gel with distilled water, the enzyme was released into 0·2M phosphate buffer of pH 7·6 and precipitated with ammonium sulfate (45%). After dialysis and freeze-drying, the enzyme preparation had a specific activity of about 250 mU/mg. The biochemical properties of the enzyme were identical with the commercial preparation of bovine erythrocyte acetylcholinesterase of Koch-Light.

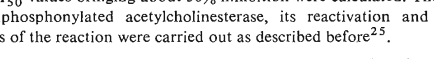
The activity of acetylcholinesterase was estimated by the electrometric method[23] using a previously described apparatus[24]. As substrate served acetylcholine iodide in a final concentration of $4{\cdot}76 \cdot 10^{-3}$M. The measurement took place at 25°C in a 0·015M veronal–phosphate buffer (pH 8·0) made isotonic with sodium chloride. Unless stated otherwise all the compounds used in the reaction were dissolved in this buffer. The inhibition constants of the oximes toward acetylcholinesterase were measured as follows: The incubation mixture containing 18 ml buffer, 1 ml enzyme (5 mg/ml) and 1 ml reactivator was left for 10 min at 25°C (the inhibition is not progressive and prolonging the period did not increase it). Thus 1 ml of 0·1M aqueous acetylcholine iodide was added and the remaining activity was established. By plotting the per cent of inhibition against the molarity of the reactivator in a probit-logarithmic transformation straight lines were obtained from which the I_{50} values bringing about 50% inhibition were calculated. The preparation of the isopropylmethylphosphonylated acetylcholinesterase, its reactivation and estimation of the kinetic constants of the reaction were carried out as described before[25].

Paper chromatography of the reactivators was done in the descending direction at room temperature on Whatman No 1 paper in 1-butanol–acetic acid–water (10 : 2·4 : 10). The spots were

TABLE I

Chemical Structure of the Reactivators Used and Some of Their Physico-Chemical Properties

The melting points have not been corrected, the dissociation constants were determined potentiometrically.



Reactivator	n	M.p., °C	M.p. reported, °C	pK'_{a_1}	pK'_{a_2}
C-1	1	235	—	7·13	7·79
C-2	2	298	300[a], 285[b], 260[c]	7·60	8·75
C-3	3	241	238–241[a], 222[b]	7·70	8·77
C-4	4	242–243	239–241[a], 245–246[d]	7·95	8·72
C-5	5	210–212	208–210[a]	7·96	8·70
C-6	6	216	212–214[b], 208–209[c]	8·00	8·70
C-8	8	228–230	—	8·02	8·70
C-10	10	223–225	219–223[a], 210–212[b]	8·05	8·72

[a] Ref.[22], [b] ref.[32], [c] ref.[33], [d] ref.[34].

detected either under a UV lamp (254 nm) or using Dragendorff's reagent[26]. The partition coefficients of the reactivators for cyclohexanol–water were estimated as follows: 10 ml 10^{-3}M solution of the reactivator in 0·02M phosphate buffer of pH 7·2 was agitated on Kahn's shaker with 50 ml cyclohexanol at 20°C for 20 min. The phases were separated by centrifuging for 15 min at 3000 *g* and the concentration of the reactivator was estimated spectrophotometrically at the maximum of the betaine band (Zeiss UR-2).

RESULTS AND DISCUSSION

The dissociation constant K_i which indicates the affinity of the inhibitor for the enzyme was calculated according to

$$K_i = I_{50}/(1 + [S]/K_m + [S]/K_m K_{s2}) .$$

The K_m for acetylcholine as substrate was estimated as $5{\cdot}8 . 10^{-4}$M, as compared with the literature value[27] of $6{\cdot}0 . 10^{-4}$M or[28] $5{\cdot}5 . 10^{-4}$M. K_{s2} was $1{\cdot}70 . 10^{-2}$M, as compared with reference[27] $1{\cdot}5 . 10^{-2}$M. For [S] $4{\cdot}76 . 10^{-3}$M and K_m and K_{s2} as found here, the K_i results as $0{\cdot}087 . I_{50}$. The interaction of the reactivator R with phosphorylated acetylcholinesterase EP may be described by the equation[11]

$$EP + R \overset{K_R}{\rightleftharpoons} EPR \xrightarrow{k_R} E + PR ,$$

where EPR is the transition complex, E the regenerated enzyme, PR the phosphorylated reactivator. The reaction is characterized by the dissociation constant K_R and by the degradation rate constant k_R. The ratio of the two constants k_R/K_R is the bimolecular rate constant which defines the overall efficiency of the reactivator. The kinetic constants of inhibition and of reactivation are shown in Table II.

TABLE II

Kinetic Constants Characterizing the Interaction of Reactivators with Intact and with Isopropylmethylphosphonylated Acetylcholinesterase

The constants are shown as average ± confidence interval for *P* 0·95.

Reactivator	K_i M	K_R M	k_R min^{-1}	k_r $l\ mol^{-1}\ min^{-1}$
C-1	$2{\cdot}97 \pm 0{\cdot}33 . 10^{-4}$	$4{\cdot}10 \pm 0{\cdot}80 . 10^{-3}$	$0{\cdot}835 \pm 0{\cdot}185$	$2{\cdot}04 \pm 0{\cdot}86 . 10^{2}$
C-2	$2{\cdot}95 \pm 0{\cdot}35 . 10^{-4}$	$4{\cdot}00 \pm 0{\cdot}42 . 10^{-4}$	$0{\cdot}500 \pm 0{\cdot}063$	$1{\cdot}25 \pm 0{\cdot}30 . 10^{3}$
C-3	$3{\cdot}82 \pm 0{\cdot}36 . 10^{-4}$	$6{\cdot}40 \pm 1{\cdot}29 . 10^{-5}$	$0{\cdot}243 \pm 0{\cdot}018$	$3{\cdot}80 \pm 0{\cdot}73 . 10^{3}$
C-4	$1{\cdot}83 \pm 0{\cdot}32 . 10^{-4}$	$2{\cdot}28 \pm 0{\cdot}36 . 10^{-4}$	$0{\cdot}370 \pm 0{\cdot}029$	$1{\cdot}58 \pm 0{\cdot}36 . 10^{3}$
C-5	$8{\cdot}35 \pm 0{\cdot}25 . 10^{-5}$	$2{\cdot}22 \pm 0{\cdot}40 . 10^{-4}$	$0{\cdot}134 \pm 0{\cdot}062$	$6{\cdot}10 \pm 2{\cdot}61 . 10^{2}$
C-6	$2{\cdot}95 \pm 0{\cdot}65 . 10^{-5}$	$4{\cdot}80 \pm 0{\cdot}80 . 10^{-4}$	$0{\cdot}098 \pm 0{\cdot}016$	$2{\cdot}05 \pm 0{\cdot}64 . 10^{2}$
C-8	$4{\cdot}87 \pm 0{\cdot}86 . 10^{-6}$	$4{\cdot}00 \pm 0{\cdot}25 . 10^{-4}$	$0{\cdot}016 \pm 0{\cdot}003$	$4{\cdot}10 \pm 0{\cdot}95 . 10^{1}$
C-10	$1{\cdot}48 \pm 0{\cdot}18 . 10^{-6}$	$4{\cdot}32 \pm 1{\cdot}20 . 10^{-4}$	$0{\cdot}014 \pm 0{\cdot}002$	$3{\cdot}18 \pm 0{\cdot}63 . 10^{1}$

A correlation was found between the chemical structure of the reactivators (Table I) and the measured kinetic constants. As may be seen from Fig. 1*A*, the dependence of the inhibition constants (pK_i) on the number of carbon atoms in the connecting polymethylene chain of the reactivators is linear, with the exception of the first two members. The correlation coefficient is 0·998 and the correlation is significant for $P > 0{\cdot}99$. The degradation rate constant k_R is a logarithmic function of the number of carbon atoms in the connecting chain (Fig. 1*B*). By plotting $\log k_R$ against n one obtains a linear function with a correlation coefficient of 0·972 (the correlation is significant for $P > 0{\cdot}99$). The dependence of the bimolecular rate constant of reactivation on the number of carbon atoms has a biphasic character and shows a pronounced maximum for $n = 3$ (Fig. 1*C*).

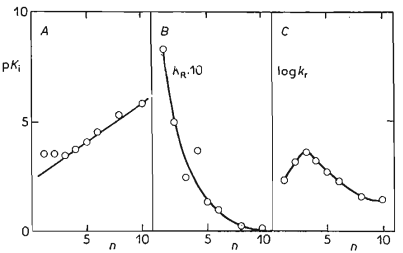


FIG. 1

Dependence of Some Kinetic Constants of the Reactivators on the Number of Methylene Groups (n) in the Molecule

A Dependence of the inhibition constants (pK_i) on n, *B* dependence of the degradation rate constant k_R on n; *C* dependence of the bimolecular rate constant of reactivation k_r on n.

TABLE III

Constants Characterizing the Lipophilic Character of the Reactivators Used

α Partition coefficient for cyclohexanol–water, R_M was calculated from R_F.

Reactivator	α	R_M	Reactivator	α	R_M
C-1	$6{\cdot}58 \cdot 10^{-4}$	0·50	C-5	$8{\cdot}12 \cdot 10^{-3}$	0·39
C-2	$5{\cdot}90 \cdot 10^{-4}$	0·48	C-6	$5{\cdot}00 \cdot 10^{-3}$	0·37
C-3	$2{\cdot}77 \cdot 10^{-3}$	0·43	C-8	$1{\cdot}89 \cdot 10^{-2}$	0·18
C-4	$6{\cdot}35 \cdot 10^{-3}$	0·41	C-10	$8{\cdot}49 \cdot 10^{-2}$	−0·02

The lipophilic character of the reactivators is expressed by the partition coefficients α estimated for a system of cyclohexanol–water, as well as by the values of R_M calculated from the magnitude of R_F according to $R_M = \log(1/R_F - 1)$. A correlation exists also between the lipophilic character and the chemical structure of the reactivators. The negative logarithm of the partition coefficient ($p\alpha$) is directly proportional to the number of carbon atoms in the connecting chain of the reactivators (Fig. 2*A*). The correlation coefficient is 0·956 and the correlation is significant for $P > 0{\cdot}99$. Similarly, the dependence of R_M on the number of carbon atoms is linear but is biphasic (Fig. 2*B*). The correlation coefficient for the first part of the function for $n = 1-6$ is 0·985 and the correlation is significant for $P > 0{\cdot}99$.

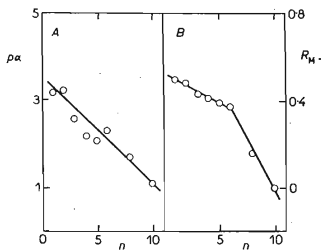


FIG. 2

Dependence of the Lipophilic Character of the Reactivator on the Number of Methylene Groups (n) in the Molecule

A Dependence of the partition coefficient α on n; *B* dependence of R_M on n.

The group of reactivators studied here represents a homologous series of compounds differing only in the length of the connecting polymethylene chain between the two quaternary nitrogens of the reactivator. With increasing number of carbon atoms the lipophilic character of the compound increases, as indicated by the increase of the partition coefficient for cyclohexanol–water and by the decreasing values of R_M as obtained by paper chromatography in 1-butanol–acetic acid–water. At the same time, the dissociation ability of the two oxime groups increases.

Changes in the length of the reactivator chain are simultaneously reflected in the ability of these compounds to react with the active centre of intact as well as of isopropylmethylphosphonylated acetylcholinesterase. A linear relationship was found between the magnitude of K_i and the number of methylene groups in the connecting chain of the reactivators, only the first two members of the series being an exception (Fig. 1*A*). The active site of acetylcholinesterase contains most probably two anionic sites[2] capable of interacting with two positively charged nitrogens of the reactivator. If the two quaternary nitrogens are separated by one or two methylene groups, no interactions can occur with the two anionic sites. Only a three-membered and

longer chain brings about greater flexibility of the molecule which can better adapt itself to the active centre of the enzyme. Each methylene group of the connecting chain brings an increment ΔpK_i which is associated with a change of the binding energy $\Delta(\Delta F)$ amounting to 0·481 ± 0·129 kcal/mol. This change in the binding energy per methylene group lies within the range of energies characteristic of interactions[29] under the influence of van der Waals forces (0·36 – 0·95 kcal/mol/CH_2). Similar values of $\Delta(\Delta F)$ were obtained *e.g.* during interaction of homologues of the N^1-alkylnipecotamides with butyrylcholinesterase[30] or of n-alkyltrimethylammonium halogenides and polymethylene-bis-trimethylammonium halogenides with acetylcholinesterase and butyrylcholinesterase[31]. The change in the length of the connecting polymethylene chain in the reactivator molecule is also apparent during interaction of these compounds with isopropylmethylphosphonylated acetylcholinesterase. All the compounds studied behave as reactivators of the phosphonylated enzyme. Their reactivation efficiency expressed by the bimolecular rate constant k_r shows a maximum for the trimethylene derivative (Fig. 1*C*).

The k_r constant is the ratio of the degradation constant k_R and the dissociation constant K_R. While the first constant k_R decreases with increasing number of methylene groups in the reactivator molecule (Fig. 1*B*) the dissociation constant K_R shows an opposite trend; however, for the last members of the series it does not change (Table II). The biphasic character of the dependence of k_r on the number of methylene groups is thus due to both relationships.

The stability of the transition complex of the phosphonylated enzyme with the reactivator is directly related to the lipophilic nature of the reactivator. The following relationship was found between the k_R constant and the partition coefficient α $\log k_R = -0{\cdot}7765 + 0{\cdot}8315\alpha$. The correlation coefficient is 0·904, for $P > 0{\cdot}99$. Similarly, the k_R is related to R_M through $\log k_R = -1{\cdot}3127 + 6{\cdot}4224R_M$ with a correlation coefficient of 0·933 for $P > 0{\cdot}99$.

The results obtained indicate that during binding of the compounds studied to the active centre of acetylcholinesterase both electrostatic forces and hydrophobic interactions come into play. According to the present concepts the active surface of the enzyme contains in the vicinity of the esteratic site a hydrophobic region which plays a significant role during interactions of a whole series of compounds with this enzyme[35–40]. According to Bracha[41] the region is about 10 Å from the esteratic site.

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