INHIBITION OF ACETYLCHOLINESTERASE AND BUTYRYLCHOLINESTERASE BY O-ISOPROPYL S-(2-DIISOPROPYLAMINOETHYL) METHYLTHIOPHOSPHONATE

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The kinetics of the inhibition of acetylcholinesterase and butyrylcholinesterase by O-isopropyl S-(2-diisopropylaminoethyl) methylthiophosphonate at 20°C and pH 8·0 was investigated and the affinity (K_a), phosphorylation (k_p), and bimolecular (k_i) constants of this interaction were determined.

The organophosphorus compounds of type (*I*) where R_1, R_2, R_3, and R_4 are alkyls were prepared first by Ghosh and Newman[1]. These compounds show high anticholinesterase activity and high toxicity[2,3]. Unlike the fluorophosphates they are not degraded by alkylphosphofluoridases[4] in the organism and are also more resistant to hydrolysis[5], thus increasing their toxic effect. Only a few representatives of the

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great number of theoretically possible compounds of this type have been prepared and examined. All information on these compounds is mostly represented only by the knowledge of their toxicity and affinity for cholinesterase as expressed by the value of I_{50}. In this study, the kinetics of interaction of O-isopropyl S-(2-diisopropylaminoethyl) methylthiophosphonate (*I*: R_1 = Me, $R_2 = R_3 = R_4$ = iPr) with acetylcholinesterase and butyrylcholinesterase was investigated and the affinity, phosphorylation, and bimolecular constants of this interaction were determined.

EXPERIMENTAL

Material. Acetylcholinesterase was prepared from bovine erythrocytes[6]; it was a lyophilized preparation of specific activity 0·26 μmol of acetylcholine hydrolyzed by 1 mg of the preparation

per 1 min (0·26 U) at pH 8·0 and 25°C. Butyrylcholinesterase was prepared from horse serum[7]; the specific activity of the lyophilized preparation was 4·6 μmol of butyrylcholine hydrolyzed by 1 mg of the preparation per 1 min (4·60 U) at pH 8·0 and 25°C. Both enzymes were dissolved in the veronal–phosphate buffer[8] of Michel at pH 8·0 to give solutions containing 1 U/ml. Acetylcholine iodide and butyrylcholine iodide were purchased from Lachema, Brno. Acetylcholine iodide was dissolved in distilled water to a 5 mM solution, butyrylcholine iodide to a 10 mM solution.

Measurement of esterase activity. The initial rate of the enzymatic hydrolysis of the two substrates (v) was measured titrimetrically at 20°C and pH 8·0 in the Radiometer pH-stat equipped with Autoburet ABU 12, titrating assembly TTA 3, titrator TTT 11, pH-meter PHM 26, and recorder SBR 2c. The titration was effected by 0·05M-NaOH. Acetylcholine and butyrylcholine were used for the determination of the activity of acetylcholinesterase and butyrylcholinesterase, respectively. The enzyme solution (0·5 ml) and the inhibitor solution (0·5 ml) at appropriate concentrations were incubated for the period measured (t, 1 min at the most) at 20°C and pH 8·0. The inhibition reaction was then discontinued by the addition of 20 ml of the substrate solution and the residual enzymatic activity was determined.

Determination of kinetic constants. The K_a-, k_p- and k_i-values were obtained by the procedure of Main and Ivorsen[9]. The rate of the inhibition of the two enzymes at various inhibitor concentrations was calculated from the log v *versus* t plot. The slope of these lines has a value of $2{\cdot}303\ \Delta\ .\ \log v/\Delta t$ and was calculated by regression analysis. The values thus obtained were used to draw the plot of $\Delta t/2{\cdot}303\ \Delta \log v$ *versus* $1/[I]$, where [I] stands for the concentration of O-isopropyl S-(2-diisopropylaminoethyl) methylthiophosphonate from equation $\Delta t/2{\cdot}303\ \Delta \log v = K_a/k_p . 1/[I] + 1/k_p$ (transformation A), the plot of $[I]\ \Delta t/2{\cdot}303\ \Delta \log v$ *versus* [I] from equation $[I]\ \Delta t/2{\cdot}303\ \Delta .\ \log v = [I]/k_p + K_a/k_p$ (transformation B), and the plot of $2{\cdot}303\ \Delta \log v/\Delta t$ *versus* $2{\cdot}303\ \Delta \log .\ v/[I]\ \Delta t$ from equation $2{\cdot}303\ \Delta \log v/\Delta t = k_p - K_a\ (2{\cdot}303\ \Delta \log v/[I]\ \Delta t)$ (transformation C). The interpolation of the straight lines by the regression analysis and the calculations of the individual constants were carried out in Minsk-22 computer.

RESULTS AND DISCUSSION

In the first stage of the interaction of organophosphorus inhibitors with cholinesterases an intermediary complex is formed which is subsequently converted into the phosphorylated enzyme[10]. This can be expressed by the following equation

$$E + PX \underset{}{\overset{K_a}{\rightleftharpoons}} EPX \xrightarrow{k_p} EP\,,$$

where E is the enzyme, P the alkylphosphoryl group, X the leaving group of the organophosphate, EPX the reversible complex, and EP the phosphorylated enzyme. The first, reversible part of the reaction is characterized by the affinity constant K_a, the second, irreversible part of the reaction by the phosphorylation constant k_p. The bimolecular rate constant of the inhibition is given by $k_i = k_p/K_a$.

The individual constants were calculated from three transformations of the same relation so that the calculated constant was always a part of the expression characterizing the slope. The bimolecular rate constant k_i (slope $1/k_i$) was calculated from

TABLE I

Affinity (K_a), Phosphorylation (k_p), and Bimolecular (k_i) Constants of Inibition of Acetylcholinesterase (AChE) and Butyrylcholinesterase (BuChE) by O-Isopropyl S-(2-diisopropylaminoethyl) Methylthiophosphonate at 20°C and pH 8·0

Enzyme	$K_a \pm$ S.D. M	$k_p \pm$ S.D. min^{-1}	$k_i \pm$ S.D. $M^{-1}\ min^{-1}$
AChE	$(1{\cdot}36 \pm 0{\cdot}18) \cdot 10^{-6}$	$2{\cdot}840 \pm 0{\cdot}381$	$(2{\cdot}08 \pm 0{\cdot}27) \cdot 10^{6}$
BuChE	$(4{\cdot}02 \pm 0{\cdot}34) \cdot 10^{-8}$	$0{\cdot}225 \pm 0{\cdot}021$	$(5{\cdot}60 \pm 0{\cdot}49) \cdot 10^{6}$

transformation A, the phosphorylation constant k_p (slope $1/k_p$) from transformation B, and the affinity constant K_a (slope K_a) from transformation C. This method was used because the calculation of the slope and of its standard deviation is more exact than the calculation of the size of the intersections of the linear function and of the axes[11]. The constants calculated are given in Table I. The results show that the bimolecular rate constants of the inhibition k_i are almost identical for the two enzymes in spite of the fact that there are considerable differences between constants K_a and k_p. The affinity of O-isopropyl S-(2-diisopropylaminoethyl) methylthiophosphonate for acetylcholinesterase is smaller than its affinity for butyrylcholinesterase. The half-life of the conversion of the complex EPX into the phosphorylated enzyme EP, calculated from the formula $t_{0,5} = 0{\cdot}6933/k_p$, is 3·10 min for butyrylcholinesterase and 0·245 min for acetylcholinesterase, *i.e.* approximately 13-times lower.

A comparison of the results obtained with data recorded in the literature is extremely difficult since the vast majority of studies on the determination of the affinity and phosphorylation constants of the inhibition of cholinesterase have been carried out with other type of compounds[12–15] and at a lower temperature (5°C). The constants, however, which have been obtained, *e.g.* by Bracha[16] for the inhibition of acetylcholinesterase by O,O-diethyl S-(3-etho-1-pentyl) thiophosphate (pH 7·4, 25°C), are comparable. Their values are K_a $(6{\cdot}16 \pm 0{\cdot}53) \cdot 10^{-5}$M, k_p $2{\cdot}58 \pm$ $\pm$ 0·13 min. Kinetic data on thiophosphonates are published relatively scarcely and concern mostly the size of the bimolecular rate constants only. The inhibition of human erythrocyte acetylcholinesterase by O-ethyl S-(2-dimethylaminoethyl) methylthiophosphonate (*I*, $R_1 = R_3 = R_4 = Me$, $R_2 = Et$) is characterized[3] by bimolecular constant k_i $(7{\cdot}13 \pm 2{\cdot}96) \cdot 10^{8} M^{-1}\ min^{-1}$ (pH 8·0, 25°C). The same compound inhibits butyrylcholinesterase less markedly[17], k_i $3{\cdot}08 \cdot 10^{6} M^{-1}\ min^{-1}$ (pH 8·0, 25°C). The inhibition of butyrylcholinesterase by the diethyl analog of this organophosphate (*I*, $R_1 = Me$, $R_2 = R_3 = R_4 = Et$) is characterized[17] by approximately the same bimolecular constant, k_i $1{\cdot}07 \cdot 10^{6} M^{-1}\ min^{-1}$ (pH 8·0, 25°C).

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