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AROMATIC PHOSPHORYL THIOCHOLINES. III.* THE KINETICS OF INHIBITION OF PURIFIED HORSE SERUM CHOLINESTERASE

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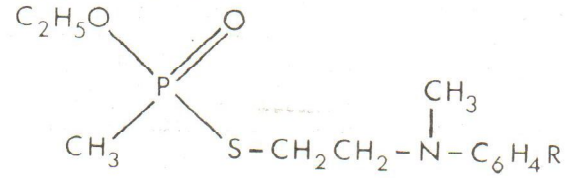
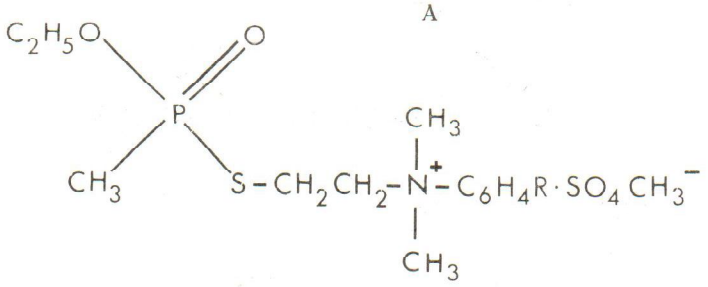
Kinetics and thermodynamics of inhibition of purified horse serum cholinesterase by seven O-ethyl-S-[(2-N-methyl-N-arylamino) ethyl]methyltiophosphonates and seven O-ethyl-S[(2-N-methyl-N-arylammonium)ethyl]methylthiophosphonate methosulphates of the general formula



A



B

where R = H, —CH_3, —OCH_3 and —Cl, in *m* and *p* positions

For compounds A the k_i varied from (0,056—0,284) · 10^5 M^{-1}. min^{-1}, and for compounds B from (1,95 — 29,4) · 10^5 ($M^{-1} \cdot min^{-1}$). Activation energy (E) for the former class varied from 4,2 to 14,38 kcal/mol, and for the latter from 6,87 to 10,06 kcal/mol. The preexponential factor for the two classes (log A) varied from 7,53 to 14,22 and from 11,16 to 13,7 respectively.

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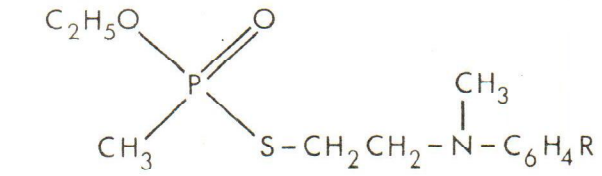
It was demonstrated that not only nitrogen atom and the presence of benzene ring influence the anticholinesterase activity of examined compounds, but also the position of the ring substituent affects the steric properties of thioethylarylamino and thiothylarylammonium groups in the molecule.

The cholinesterase (ChE) inhibition by organophosphorus compounds is a chemical reaction between the enzyme and the organophosphorus ester (1, 2). The inhibitory power mostly depends on the reactivity of the organophosphorus ester (3), but the steric properties of the inhibitor's molecule (4), or the hydrophobic action of the enzyme (5) may greatly influence the interaction between the organophosphorus compound and ChE.

In this connection the derivatives of thiophosphonic acid in which the basis of the acyl group is the thioethylamino group, so called »phosphorylthiocholines« (6) are very interesting, because of significantly increased anticholinesterase effects when the nitrogen atom passes into a cationic form. Such type of compounds reacts bifunctionally with the catalytic center of ChE, because the cationic side of the inhibitor takes place towards the anionic side of the enzyme (7).

A nonspecific adsorption of the thioethylammonium group may also occur on the hydrophobic parts of ChE bettering the orientation of the inhibitor's molecule towards the active surface of the enzyme (5).

In order to determine how these factors influence ChE inhibition, we synthetised seven new O-ethyl-S[(2-N-methyl-N-arylamino)ethyl]methylthiophosphonates (A) and seven methosulphates of their dimethylammonium analogues (B) corresponding to the general formulas:



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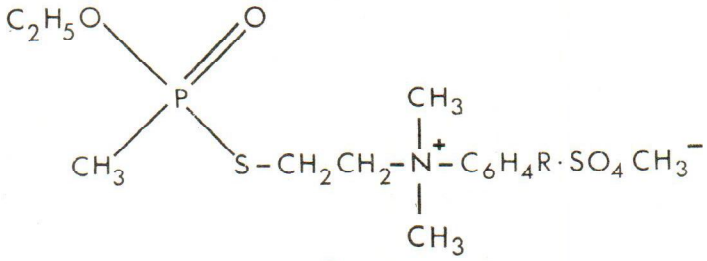


B

where R = H, —CH_3, —OCH_3 and —Cl in *m* and *p* positions (8).

It was supposed that the changes in electronegativity of the benzene ring owing to the kind and position of ring substituents would induce

changes in hydrophobic adsorption of thioethylarylamino and thioethylarylammonium groups on the active surface of ChE and at the same time alter anticholinesterase properties of the whole inhibitor's molecule being a tertiary or a quaternary one.

In a previous work (8) the second order rate constants of inhibition (k_i) of human erythrocyte ChE with the above compounds were determined. All compounds strongly inhibited acetylcholinesterase (AChE) in which the quaternary compounds (B) with $k_i = (0{,}12 - 1{,}8) \cdot 10^7$ M^{-1} min^{-1} were about 100 times more potent than the tertiary (A) with $k_i = (0{,}77 - 4{,}91) \cdot 10^5$ M^{-1} min^{-1}. A definite proof of the influence of the kind and position of benzene ring substituents on AChE inhibition was found neither in class A nor in class B.

To better understand the interaction between the enzyme and this class of organophosphorus compounds we studied the kinetics and thermodynamics of purified horse serum ChE (SChE) inhibition.

MATERIAL AND METHODS

The organophosphorus compounds used for kinetic and thermodynamic studies are given in Table 1. Each compound was dissolved in isopropanol, to give a 1% stock solution, and solutions were kept at 4°C.

Enzyme. Purified horse SChE (Schuhardt) was dissolved in 0,1 M phosphate buffer (pH = 7,0) in dilution of 1 microgram of enzyme preparation in 3 ml of buffer.

SChE activity. Continuous monitoring of the activity of enzyme in, or without, the presence of inhibitor was performed by the Ellman's method (9). The measurements were done on Ratio Recording Spectrophotometer Beckmann DK-2A. The substrate was butyrilthiocholine iodide (final concentration 5.4 mM).

Kinetic and thermodynamic constants. The inhibition rate was determined by the second order rate constant of inhibition (k_i) with inhibitor in excess. The measurements were made at four different temperatures between 20 and 35°C. The following equation was used:

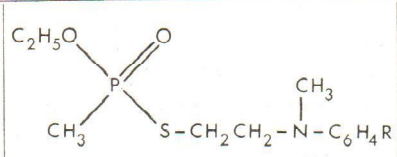
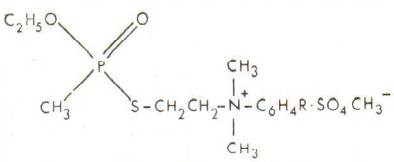
$$k_i = \frac{2{,}303}{[I] \cdot t} \log \frac{v_o}{v_i}$$

where [I] – initial molar inhibitor concentration, v_o = enzyme activity in the absence of inhibitor, v_i = enzyme activity in the presence of inhibitor in time t. The obtained k_i are mean values of at least six k_i determinations.

Activation energy (E) and preexponential factor (A) were calculated from the plot of temperature dependence upon enzyme reaction rate in Arrhenius coordinates.

Table 1

Structure, first-order rate constants, activation energy and preexponential factor for the SChE inhibition of studied compounds (T 298°K)

No. of compound (R)*	Biological data	 O-ethyl-S-[(2-N-methyl-N-aryl-amino)ethyl]methylthiophosphonate		 O-ethyl-S-[(2-N-methyl-N-aryl-ammonium)ethyl]methyl-thiophosphonate methosulphate	
1	$k_i \cdot 10^5$ ($M^{-1} \cdot min^{-1}$)	0,095		1,95	
2		0,066		22,5	
3		0,056		29,4	
4		0,135		21,2	
5		0,284		14,5	
6		0,193		7,7	
7		0,175		13,6	
1	E(Kcal/mol) and log A	9,24	10,75	9,09	11,95
2		14,21	14,22	9,21	13,1
3		14,38	14,21	7,7	12,1
4		12,58	13,35	10,06	13,7
5		4,2	7,53	8,28	12,23
6		8,58	10,57	8,02	11,76
7		10,33	11,8	6,87	11,16

* 1. R = H (phenyl); 2. R = m—CH_3 (m-tolyl); 3. R = m—OCH_3 (m-methoxy); 4. R = m—Cl (m-chloro); 5. R = p—CH_3 (p-methyl); 6. R = p—OCH_3 (p-methoxy) and 7. R = p—Cl (p-chloro).

RESULTS AND DISCUSSION

The relationship between the logarithm of the inhibition rate constant and temperature was linear for both classes of compounds (A and B) in the examined temperature range (Figure 1) and corresponded to the Arrhenius equation ($\log k = \log A - \frac{E}{RT}$).

All quaternary compounds (B) inhibit SChE at a higher rate than the corresponding tertiary ones (A), the ratio of k_i values varying from

20,5 (R = H) to 525 (R = m-methoxy) (see Table 1). This is in accordance with the results obtained in human erythrocyte AChE inhibition by the same compounds (8), where the inhibition rate ratios varied from 80 (R = m-methoxy) to 800 (R = m-methyl). Both results confirm the predominant influence of nitrogen quaternization on the inhibition rate in this class of compounds, the inhibition rate being faster for AChE.

In the class of quaternary compounds (B) the substituents in the benzene ring in comparison with the nonsubstituted compound (B-1) highly potentiate anticholinesterase activity (4—15 times). The p-derivatives (B-5, B-6 and B-7) are weaker inhibitors than the m-derivatives (B-2, B-3 and B-4). In the class of tertiary compounds the substituents in the benzene ring potentiate the inhibition rate only 1,4 to 3 times. None the less the m-methyl (A-2) and m-methoxy (A-3) derivatives inhibit SChE at a lower rate than the nonsubstituted analogue (A-1). This could be attributed to the high activation energy (> 14 kcal) of these two compounds (see Table 1) which covers the difference in preexponential factor* (about 3,4 units). The p-derivatives (A-5, A-6 and A-7) are stronger inhibitors than the m-derivatives (A-2, A-3 and A-4), the case being opposite with the corresponding quaternary derivatives (B). An explanation of this phenomenon has not yet been found.

The k_i values of compounds B-1 to B-7 are higher than the k_i of a similar compound O-ethyl-S-[(N-dimethylamino)ethyl]-methylthiophosphonate (12), $k_i = 1{,}1 \cdot 10^5$ M^{-1} min^{-1}, or its methosulphate $k_i = 0{,}7 \cdot 10^5$ M^{-1} min^{-1}, indicating a positive effect of the aryl radical in the acyl part of phosphorylthiocholines. The same degree of inhibition in tertiary and quaternary aliphatic phosphorylthiocholines in contrast to the aromatic ones could be explained by the fact that in very dilute solutions at pH 7 —, tertiary compounds are nearly 100% protonated, and quaternization cannot produce any additional charge. The aromatic ring bound to the N-atom does not allow protonation at physiological pH (13), so compound A-1 is a weaker inhibitor than the corresponding aliphatic phosphorylthiocholines.

The methosulphates of 0,0 diethyl[(N-dimethyl-N-arylammonium)ethyl] thiophosphates which differ from compounds B-1 to B-7 only in being phosphates instead of phosphonates, have k_i 100 times higher (13). This is in accordance with the fact that phosphates are stronger inhibitors of SChE than phosphonates, the latter being stronger inhibitors of AChE (8).

The high anticholinesterase activity of aromatic phosphorylthiocholines can be attributed also to hydrophobic aryl radical in the thioethyl-

* The preexponential factor (A) is composed of two components: number of collisions of reactant molecules and the steric factor (A = PZ). Supposing that at the same temperature, independently of the inhibitor change, the number of collisions remains the same the preexponential factor becomes higher owing to the steric factor (16).

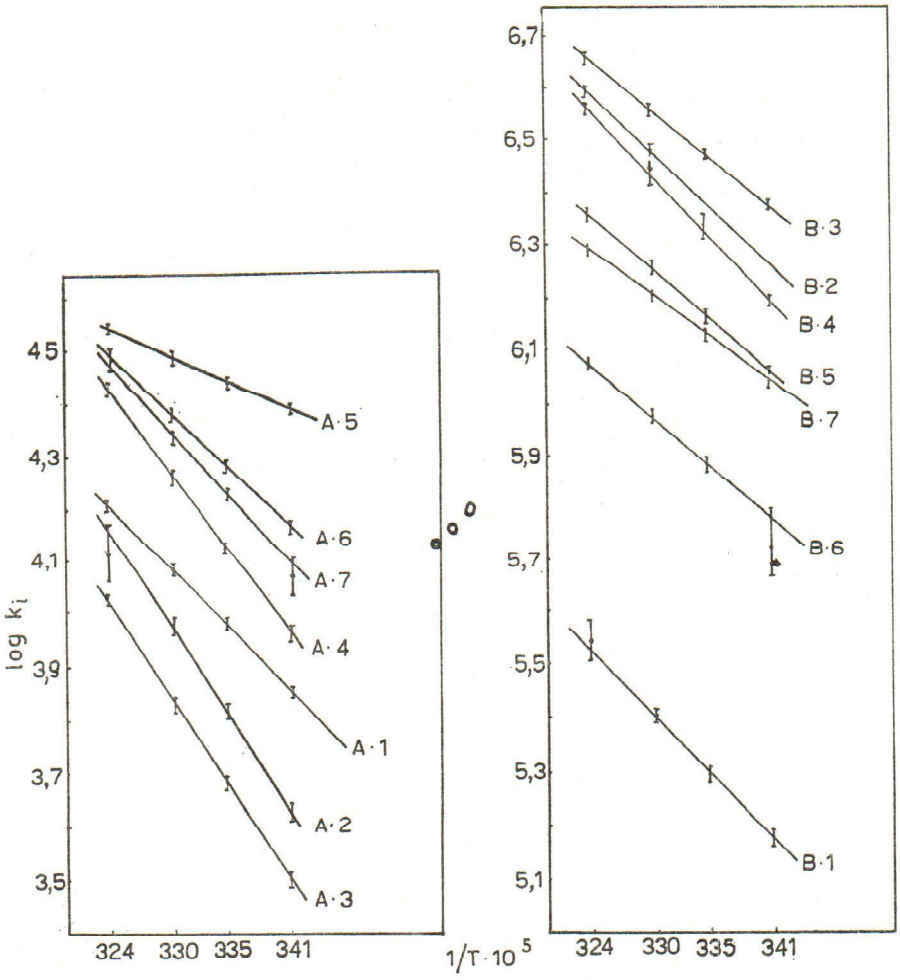


Fig. 1. *Temperature dependence of the second order inhibition rate constant of purified SChE*

ammonium group, which together with the positively charged nitrogen atom betters their orientation and adsorption on the active surface of the enzyme.

The correlation between k_i and Hammet's sigma constants (14) is presented graphically (Figure 2). No correlation between k_i and the polar effect of the substituent in benzene ring (kind and position) was observed. It can be assumed that in this type of compounds the steric factor compared with the polar one is predominant (15), and results in dissipation of points.

The activation energy (E) of 14 tested compounds corresponds to the values which are known for many other chemical reactions (see Table 1). The same activation energy was observed only in nonsubstituted compounds (A-1 and B-1). In other cases, with the exception of p-methyl

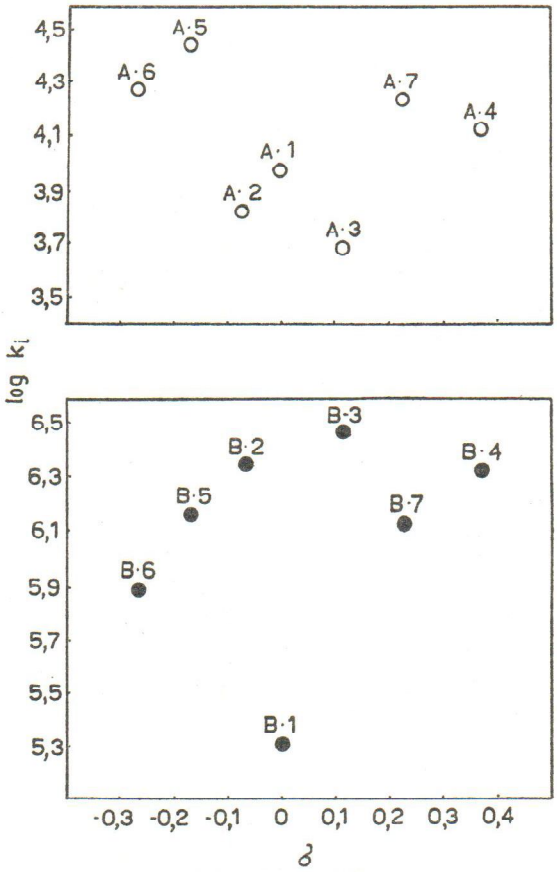


Fig. 2 *Anticholinesterase activity (k_i) and Hammet's sigma constants (δ) of the substituents in the benzene ring*

substituted compounds (A-5 and B-5) the energetic barrier of inhibition is higher in the group of tertiary compounds (A). This could be explained by the fact that the cationic center in the molecule (B), owing to the inductive effect of the quaternized N atom, is linked to the anionic site of the enzyme, this bettering the orientation of the compound to the active surface of SChE, and inducing the lowering of the energetic barrier in reaction with SChE.

All tertiary compounds with the substituents in p-position (A-5, A-6 and A-7) have higher energetic barries than their m-analogues (A-2, A-3 and A-4). In the group of quaternary compounds (B) this is not so clear. At the same time p-compounds have a lower preexponential factor, but differences are too small to permit any definite conclusion.

To determine more precisely if and how the kind and position of the ring substituent could influence the energetic barrier, which ultimately represents the energy of breaking P-S bond, we tested the correlation between activation energy and preexponential factor.

The correlation observed in the tertiary group of compounds (A) between E and log A, shows that the thioethylarylamino group, with its steric properties defined by the benzene ring and the kind and place of ring substituents highly influences the interaction between the inhibitor phosphoryl group and the nucleophyllic center of SChE (Figure 3). This steric influence which is in tight connection with the conditions of hydrophobic adsorption on the active surface of the enzyme is greater in m-substituted compounds. Although a correlation exists between E and log A in the class of quaternary compounds (B), dissipation of points shows a lower steric influence on the ring substituents in the reaction between the inhibitor and the enzyme. In the class of bifunctional inhibitors (B) the orientation towards the catalytic center of SChE is im-

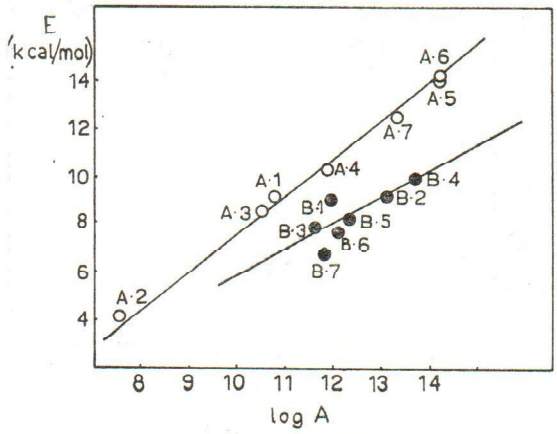


Fig. 3. *The relationship between activation energy and preexponential factor*

proved, while the hydrophobic properties of the acyl part of the inhibitor as well as steric factors influence to a lesser extent the interaction between the inhibitor and the enzyme.

Our results are in agreement with the results of *Godovikov* (13), who showed that the influence of benzene ring substituents is more important in tertiary, than in analogous quaternary groups of inhibitors. Although the results of k_i, E and log A are not entirely identical, they show that quaternization is of major importance in activation of this type of SChE inhibitors. As regards the steric factor, the position of benzene ring substituents is more important than the kind of substituent.

ACKNOWLEDGEMENT

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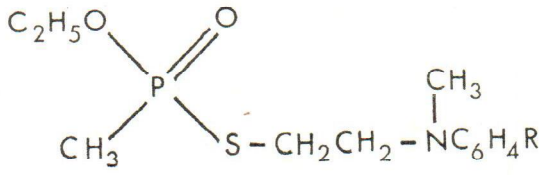
Sažetak

AROMATIČNI FOSFORILTIOHOLINI. III. KINETIKA INHIBICIJE PREČIŠĆENE SERUMSKE KOLINESTERAZE KONJA

Ispitana je kinetika i termodinamika inhibicije pročišćene serumske kolinesteraze (SChE) konja sa sedam O-etil-S[(2-N-metil-N-arilamino)etil]metiltiofosfonata (A) i sedam O-etil-S-[(2-N,N'-dimetil-arilamonij)etil]metiltiofosfonat metosulfata opće formule



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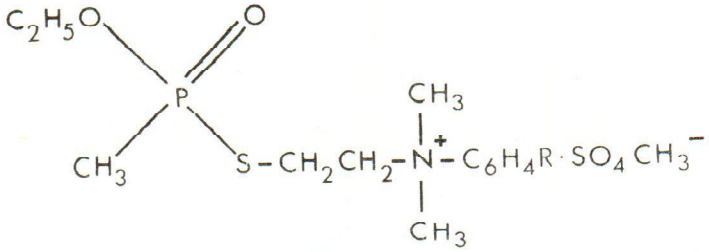

B

gdje je R = H, —CH_3. —OCH_3 i Cl u *m* i *p* položaju.

Konstante brzine inhibicije za spojeve A kreću se od $0{,}056 \cdot 10^5$ do $0{,}284 \cdot 10^5$ $M^{-1} \cdot min^{-1}$, a za spojeve B od $1{,}95 \cdot 10^5$ do $29{,}4 \cdot 10^5$ $M^{-1} \cdot min^{-1}$. Energije aktivacije iznose za spojeve A od 4,2 do 14,38 kcal/mol, a za spojeve B od 6,87 do 10,06 kcal/mol. Predeksponencijalni faktor kreće se za grupu A od 7,53 do 14,22, a za grupu B od 11,16 do 13,7.

Osim utjecaja što ga imaju kvaternizacija dušika i prisutnost benzenskog prstena na antikolinesterazna svojstva inhibitora, konstatiran je i utjecaj položaja supstituenta na sterne osobine tioetilarilamino i tioetilarilamonij skupine u molekuli inhibitora.

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