EFFECT OF A HYDROPHOBIC INTERACTION IN THE REACTION OF S-(β-ALKYLTHIOETHYL) O-PROPYL METHYLPHOSPHONOTHIONATES AND THEIR METHYL METHOSULFATES WITH CHOLINESTERASES OF WARM-BLOODED ANIMALS

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The action of S-(β-alkylthioethyl) O-propyl methylphosphonates and their methyl methosulfates with different lengths of the S-alkyl radical on the enzymatic activity of acetylcholinesterase from human blood erythrocytes and butyrylcholinesterase from horse blood serum has been investigated. The existence of a hydrophobic interaction on the sorption of inhibitors in the active site of the enzymes has been established. For the enzymes investigated a definite dependence has been observed of the antienzymatic activity of the compounds on the nature in the region of the anionic point and indicates differences in their length and structure.

In agreement with the structure of the natural substrate (acetylcholine) –

$$-CH_3-\underset{\underset{O}{\|}}{C}-O-CH_2-CH_2-\overset{+}{N}(CH_3)_3\bar{I}$$

the active site of cholinesterase (CE) includes two main binding sites: an "esterase" site performing the cleavage of the ester bond, and an "anionic" site which is bound to the ammonium group of acetylcholine. In addition to this, a substantial role both in the performance of the catalysis itself and in determining the specificity of the action of the enzyme is played by hydrophobic regions of the active surface of cholinesterase located in the region of the anionic and esterase points. The presence of these sections in CE promotes the sorption of substrates or inhibitors on the active site of the enzyme through hydrophobic interaction with the corresponding radicals of their molecules. Information on the investigation of the hydrophobic regions in CE with the aid of low-molecular-weight ligands is given in [1-5]. In the majority of cases, organophosphorus compounds (OPCs) of various types differing in the fine details of the structure of the carbohydrate radicals were used for this type of investigations.

We give information on a study of the interaction of acetylcholinesterase (ACE) from human blood erythrocytes and of butyrylcholinesterase (BuCE) of horse blood serum with S-(β-alkylthioethyl) O-propyl methylphosphonothionates containing alkyl radicals of different lengths, which were synthesized by the following scheme:

$$[C_3H_7O(CH_3)P(O)S]Na + ClC_2H_4SR \rightarrow C_3H_7O(CH_3)P(O)S-C_2H_4SR \xrightarrow{(CH_3)_2SO_4} C_3H_7O(CH_3)P(O)S-C_2H_4-\underset{\underset{CH_3}{|}}{\overset{+}{S}}-R\cdot\bar{S}O_4CH_3,$$

where $R = C_2H_5$, C_3H_7, C_4H_9, C_5H_{11}, iso-C_4H_9.

The inhibition of cholinesterases under the action of OPCs consists of the covalent binding of a serine hydroxyl located at the esterase point of the active site of the enzyme [6] with the phosphorus atom present in the structure of the inhibitor.

According to modern ideas, the reaction of OPCs with CE takes place in at least two stages according to the following scheme:

$$E + I \underset{k_{-I}}{\overset{k_I}{\rightleftarrows}} EI \xrightarrow{k_{II}} EI',$$

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where E is the enzyme; EI is an intermediate noncovalent complex; EI' is the phosphorylated enzyme; and k_I, k_{-I}, and k_{II} are the constants of the corresponding reactions.

In the first stage, the oriented sorption of the inhibitor on the active surface of the enzyme takes place with the formation of a sorption enzyme–inhibitor complex (EI). In the second stage a covalently-bound phosphorylated enzyme (EI') is formed which is fairly strong and is scarcely hydrolyzed.

The activity of irreversible inhibitors is generally evaluated from the magnitude of k_{II}. This magnitude is practically independent of the time of incubation and is calculated from the equation

$$k_{II} = \frac{2.3}{t([I]_0 - [E]_0)} \cdot \lg \frac{[E]_0([I]_0 - x)}{[I]_0([E]_0 - x)}, \quad (1)$$

where $[E]_0$ and $[I]_0$ are the initial concentrations of the enzyme and of the inhibitor, respectively; and x is the decrease in the concentration of enzyme and inhibitor after time t.

If $[I]_0$ is many times greater than $[E]_0$, then in place of equation (1) we have

$$k_{II} = \frac{2.3}{t[I]} \cdot \lg \frac{[E]_0}{[E]_t} = \frac{2.3}{t[I]} \cdot \lg \frac{V_0}{V_t},$$

where V_0 is the rate of hydrolysis of the substrate under standard conditions before the addition of the inhibitor at an enzyme concentration of $[E]_0$; and V_t is the rate of hydrolysis of the substrate after the incubation of the enzyme with the inhibitor for t min at a residual concentration of the enzyme of $[E]_t$.

The activity of the inhibitor depends primarily on its phosphorylating capacity, i.e., on the magnitude of the effective positive charge on the phosphorus atom due to the P=O bond. In addition to the phosphorylating capacity, an important role is played by ion–ion and ion–dipole interactions. This is shown particularly clearly when the OPC molecule contains an ammonium or sulfonium grouping [4, 7]. The effect of a charge is explained by the fact that cation-containing effectors not only take part in a reaction with a serine hydroxyl but also promote Coulomb interaction with the anionic section of the enzyme, which, on the whole, substantially raises the rate of interaction of the inhibitor and the enzyme. The presence in the structure of the OPC of various hydrocarbon radicals is of no little importance; this also leads to a more rapid reaction through hydrophobic interaction. The results of the anticholinesterase action of the organophosphorus inhibitors that were synthesized and the evaluated second-order rate constants K_{II} are given in Table 1.

S-(β-Alkylthioethyl) O-propyl methylphosphonothionates, and also their methyl methosulfates are fairly strong inhibitors of the cholinesterases studied. The sulfonium derivatives interact with ACE and BuCE far more effectively than the corresponding sulfide analogues. This indicates a role of the anionic points of these enzymes.

With an increase in the length of the hydrocarbon radical, when it has the normal structure, in the sulfide derivatives a proportional rise in the antibutyrylcholinesterase activity is observed up to the pentyl derivative. In the case of ACE, the value of k_{II} rises only up to the compound with a butyl radical. A further increase in the length of the thioalkyl radical causes a fall in k_{II}. The rise in the values of k_{II} for BuCE is most probably due to an enhancement of the effect of the hydrophobic interaction between the analogous sections of the enzyme and the inhibitor. The results presented show that the hydrophobic regions of the anionic points of the esterases investigated are different: in ACE the sections are more limited than in BuCE.

To convince ourselves more strongly than the hydrophobic sections of these enzymes are located just at the anionic points, we studied the inhibiting properties of OPCs with positively charged sulfur atoms. As Table 1 shows, the cation-containing OPCs interacted with ACE more effectively than with BuCE, which indicates a greater degree of expression of the anionic point of ACE than of BuCE.

The maximum value of k_{II} in this series corresponds to a substance with a pentyl radical (for BuCE) or an ethyl radical (for ACE). With an increase in the number of carbon atoms in the alkylthio radicals the efficacy of the interaction between BuCE and the inhibitors in-

creases while, at the same time, beginning with the propyl derivative, these properties in relation to ACE are less pronounced than for the substance with an ethyl radical.

Of course, there is no doubt that the presence of sulfonium sulfur in the structure of an OPC results in ionic interaction with the anionic point of an enzyme. This interaction with the anionic point ensures the correct orientation of the inhibitor molecule, on the surface of the active site. Under these conditions, the hydrocarbon radical connected with the sulfonium sulfur will be sorbed on the hydrophobic sections of the anionic points of ACE and BuCE. In the reactions in which this series of compounds is bound with ACE and BuCE the same laws of anticholinesterase activity appear, in the main, as were characteristic of the substance with sulfide sulfur. This shows that the alkyl radicals (both in the sulfide and in the sulfonium compounds) are sorbed on identical sections, i.e., on the hydrophobic regions in the neighborhood of the ionic point of ACE.

With the aid of OPCs it is possible to establish not only the presence but also the configuration of the hydrophobic sections of ACE. In this connection we investigated the anticholinesterase activities of compounds in which the alkylthio radical contained an isopropyl group.

The sulfide derivative inactivated ACE and BuCE with the same force. Its antienzyme derivatives of this series containing sulfide sulfur. The cation-containing analogue was more than 30 times as effective in relation to ACE than to BuCE. However, its inhibiting properties were several times less than for sulfonium OPCs having alkyl radicals of normal structure, both for ACE and for BuCE.

Thus, the results obtained leave no doubt of the existence of hydrophobic regions near the anionic points of cholinesterases. There are grounds for assuming that the alkyl radicals of the inhibitors obtained are sorbed on the same hydrophobic regions by the anionic points of both enzymes, which were revealed previously [1] with the aid of OPCs having a different structure.

EXPERIMENTAL

Methods of Investigation. The catalytic properties of ACE (EC 3.1.1.7) and of BuCE (EC 3.1.1.8; were determined by Ellman's method [9] from the rate of hydrolysis of acetylthiocholine (for ACE) and of butyrylthiocholine (for BuCE). The reaction was performed in a thermostated cell at 25°C and pH 7.5. The colored hydrolysis products were recorded with the aid of a type KFK-2 UKhL 4,2 photoelectric calorimeter at a wavelength of 400 nm.

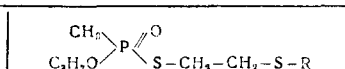
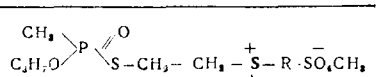
Enzymes. Purified preparations of ACE (activity 3.5 units/mg) and of BuCE (activity 9.6 units/mg) were produced by the Perm Scientific Research Institute of Vaccines and Sera.

Inhibitors. For an experiment, an S-(β-alkylthioethyl) O-propyl methylphosphonothionate or its methyl methosulfate was dissolved in ethanol, and the solution was then diluted with distilled water to the required concentration. To find k_{II}, the residual activities of the enzyme after incubation with the inhibitor for 1, 2, 3, 5, 8, and 10 min were determined.

CONCLUSIONS

The antienzyme properties of S-(β-alkylthioethyl) O-propyl methylphosphonothioates and their methyl methosulfates in reactions with human blood erythroocyte acetylcholinesterase

TABLE 1. Rate Constants (k_{II}) of the Inhibition of Cholinesterase by S-(β-Alkylthioethyl) O-Propyl Methylphosphonothionates and their Methyl Methosulfates

R				
	k_{II}, $M^{-1} \cdot min^{-1}$			
	ACE	BuCE	ACE	BuCE
C_2H_5	$1,4 \cdot 10^5$	$5,7 \cdot 10^4$	$8,3 \cdot 10^7$	$1,5 \cdot 10^6$
C_3H_7	$3,8 \cdot 10^5$	$2,2 \cdot 10^5$	$1,0 \cdot 10^7$	$1,6 \cdot 10^5$
C_4H_9	$3,0 \cdot 10^5$	$2,6 \cdot 10^5$	$1,8 \cdot 10^7$	$2,0 \cdot 10^6$
C_5H_{11}	$2,3 \cdot 10^5$	$3,0 \cdot 10^5$	$2,7 \cdot 10^7$	$2,7 \cdot 10^6$
iso-C_4H_9	$6,8 \cdot 10^5$	$8,5 \cdot 10^5$	$3,6 \cdot 10^6$	$1,0 \cdot 10^5$

and horse blood serum butyrylcholinesterase have been investigated.

It has been established that a change in the structure of the organophosphorus compounds has a definite effect on the hydrophobic interaction of the enzymes with the inhibitor.

The effect of such interaction is connected with the presence of hydrophobic regions in the neighborhood of the anionic points of the cholinesterases, the structures of which differ in ACE and BuCE.

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COVALENT IMMOBILIZATION OF HEPARIN ON A COLLAGEN FILM

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Four procedures for the covalent immobilization of heparin (Hp) on a collagen film (CF) have been investigated. In three of them (methods (I-A, B, C), the CF was first treated with epichlorohydrin and ammonia and the Hp was added with the aid of CMBC (method I-A), by reductive amination in the presence of NaCH CN (method I-B), and with the aid of CMEC after succinylation (method I-C). In the fourth procedure (method II), the CF was activated by treatment with alkali and the Hp was added with the aid of CMEC. It was shown that the maximum amount of Hp was immobilized by method II.

At the present time, heparin-containing materials are being investigated intensly [1]. The ionic and covalent immobilization of heparin on various supports is being studied most widely. Synthetic or natural polymers are used as supports for the covalent immobilization of heparin [2]. Over a number of years, we have been investigating the covalent immobilization of heparin on collagen — a natural fibrillar protein which is free from a number of the unfavorable properties of synthetic polymers.

It is known that heparin — a natural sulfated acidic polysaccharide — is an antigoagulant with a broad action spectrum and a regulator of many biochemical and physiological processes taking place in the organism. Heparin fixed to a polymer retains its anticoagulant properties.

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