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Cholinesterases: structure of the active site and mechanism of the effect of cholinergic receptor blockers on the rate of interaction with ligands

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Contents

I. Introduction 713
II. Structure of the cholinesterase active site 714
III. Mechanism of the interaction of acetylcholinesterase with organophosphorus and N-heterocyclic inhibitors 717
IV. Activators of the acetylcholinesterase interaction with organophosphorus inhibitors 720

Abstract. Modern views on the structure of cholinesterase active sites and the mechanism of their interaction with organophosphorus inhibitors are considered. The attention is focused on the mechanism of the effect of cholinergic receptor blockers, acetylcholine antagonists, on the rate of interaction of acetylcholine esterase with organophosphorus inhibitors. The bibliography includes 79 references.

I. Introduction

Since the discovery of acetylcholinesterase (AChE), the enzyme that catalyzes the hydrolysis of acetylcholine (ACh),[1] an enormous number of papers dealing with its properties have been published. This keen attention of researchers working in different areas — biochemists, physiologists, pharmacologists, physicians, ecologists, analytical chemists, and so on — to AChE is related to the key role of this enzyme (apart from cholinergic receptors) in the transmission of nervous impulses in the cholinergic synapses. The considerable interest in the properties of AChE is indicated by the regularly held International Congresses on Cholinesterase (for example, the materials of the IX Congress were published in the journal *Chemico-Biological Interactions*[2]). This is due to the fact that the knowledge of the AChE active site structure and the interaction mechanism with various ligands (substrates, inhibitors) opens up prospects for successful solution of many problems faced by modern science such as specification of the mechanism of nervous impulse transfer along neural tissues; development of more efficient drugs for treating victims of organophosphorus chemical agents; development of drugs against Alzheimer's diseases; development of highly sensitive means and technological devices for the ecological monitoring of environmental objects, *etc*.

More than 90 sorts of cholinesterases from 65 various sources (vertebrates, insects, mollusks) have now been isolated.

The interest in the structure of cholinesterases (ChE) and the mechanism of their irreversible inhibition is related to the great attention devoted in recent years to detoxification of organophosphorus compounds (OPC) by biological systems. It is well known that OPC are widely used in agriculture as insecticides, fungicides and herbicides. Although their use is strictly regulated by State Standards (GOSTs); however, owing to the ability of OPC to be accumulated in soil and in waste water, insecticides were found in many foodstuffs in amounts higher than the maximum permissible concentrations (MPC). This served as the grounds for European Union's decision to restrict the production of insecticides.

The greatest number of publications concerning cholinesterases is devoted to the effect of various factors on the rates of their irreversible inhibition by organophosphorus compounds, which is related to chemical weapons (CW) destruction by the countries that signed the Convention on the Prohibition of the Development, Production, Stockpiling and Use of Chemical Weapons and on their Destruction (Chemical Weapons Convention).[3] Clause 10 of Article IV of the Convention ratified by Russia on November 5, 1997 (Federal Law No. 138-F3) states that Each State Party, during transportation, sampling, storage and destruction of chemical weapons, shall assign the highest priority to ensuring the safety of people and to protecting the environment. Therefore, at the CW destruction facilities, a system of measures is implemented aimed at monitoring the contents of toxic compounds both at the facility site and outside the site over the area of the facility possible impact.

Monitoring of the contents of organophosphorus chemical agents distinguished by high toxicity and cumulative effect, which calls for development of highly sensitive methods for quantification of these compounds, is most

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complicated. The maximum permissible concentration of OPC in air is $10^{-7}-10^{-8}$ mg m^{-3} (see Refs 3 and 4). None of known methods provides detecting of OPC in that low amounts without preconcentration. The enzymatic method of OPC determination is most sensitive and specific.

The rate of AChE-catalyzed hydrolysis of acetylcholine is high, approaching the limiting value that is possible for an enzyme–substrate system; this was used to develop a sensitive and specific enzymatic method for quantification of anticholinesterase compounds and to develop a procedure on this basis for monitoring environmental objects for the OPC content.

For increasing the sensitivity of enzymatic OPC determination, it is necessary to know the structure of the AChE active site and the mechanism of effect of cholinergic receptor blockers† on the rate of AChE interaction with organophosphorus inhibitors. In this review, the attention is focused on the mechanism of acceleration of AChE interaction with organophosphorus inhibitors in the presence of cholinergic receptor blockers.

II. Structure of the cholinesterase active site

According to the Enzyme Nomenclature Rules published in 1973 by the IUPAC Commission on the Biochemical Nomenclature, cholinesterases refer to the class of hydrolases.[5] Cholinesterases that mainly hydrolyze acetylcholine have been termed acetylcholine-acetyl hydrolases in the enzyme nomenclature (EC 3.1.1.7); however, their trivial name, *viz*., acetylcholinesterases, is used more often in the scientific literature. The hydrolases that mainly hydrolyze choline esters of other carboxylic acids (propionylcholine, butyrylcholine, and so on) are called acylcholine-acylhydrolases (EC 3.1.1.8) according to the enzyme nomenclature; their trivial name is cholinesterases.

There are three sorts of ChE in a mammalian body differing by their functions and substrate specificity: synaptic AChE (EC 3.1.1.7), erythrocyte AChE (EC 3.1.1.7) and serum ChE (EC 3.1.1.8). The synaptic AChE (AChE **1**) terminates the nerve impulse transmission from the nerve cell presynaptic membrane to postsynaptic membrane through the synaptic gap (or from the nerve cell membrane to skeletal muscles) through fast hydrolysis of acetylcholine. The functions of erythrocyte cholinesterase (AChE **2**) and blood serum cholinesterase (butyrylcholinesterase, BChE) is not entirely clear. Since all three sorts of ChE have similar active site structures and specific substrates and since they all are targeted by the same toxic compounds, it is commonly accepted that BChE protects AChE from inactivation by anticholinesterase substances (in particular, it hydrolyzes butyrylcholine, which is AChE inhibitor, at a higher rate).

Cholinesterases AChE **1** and AChE **2** have similar structures. A correlation between the activity of AChE **2** and the activity of AChE **1** in the synapses of nerve impulse transmission from the nerve cell membrane to skeletal muscles was found.[6,7] The established correlation can be used in the clinical practice for considering the efficiency of AChE reactivators (for example, oximes). This is an important therapeutic task.

The information on the amino acid sequence and three-dimensional structure of many enzymes, including cholinesterase, can be found in databases, which are considered in detail in a review.[8]

Catalytic hydrolysis of the substrates occurs in the cholinesterase active site. It was shown by X-ray diffraction analysis that the active sites of acetylcholinesterases isolated from a number of natural sources are located on the inner surface of the enzyme globule in a 20 Å deep slit, which gets narrower towards the bottom. The active site is lined inside by amino acid residues with hydrophobic groups (tyrosine, phenylalanine, *etc*.). The catalytic hydrolysis of the substrate occurs with the catalytic triad Ser203-Glu334-His447 (the numbering of amino acid residues corresponds to the mammalian acetylcholinesterase), the oxygen atom of the Ser203 hydroxyl is spaced by 4 Å from the active site bottom.[9]

Figure 1 shows the scheme of cholinesterase active site. The catalytic triad Ser203-Glu334-His447 is surrounded by polypeptide chain regions that are important for the enzyme catalytic activity. These include the acylation region or the 'acyl pocket' (the key amino acid residues of this region are Phe295, Phe297, Phe338), the choline binding region or the 'anionic well' (amino acid residues Gly121, Gly122, Ala204), and the Me_3N^+ binding region (amino acid residues Trp86 and Tyr337).[10,11]

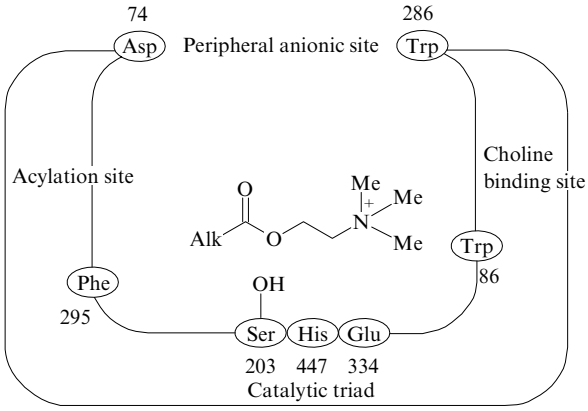


Figure 1. Scheme of cholinesterase active site.

Some ChE inhibitors (competitive inhibitors) are known to bind to its active site, whereas the other can affect the enzyme activity being associated with its allosteric‡ site located at the active site gorge.[11]

Crystallographic studies showed that the active site gorge of free AChE is closed; however, it opens upon interaction with ligands.[11] The shape of the active site gorge is determined by four key amino acid residues of the PAS, Tyr72, Leu76, Tyr337 and Tyr341 (the amino acid numbering corresponds to the mammalian AChE).[11]

The structure and different functions of the PAS and the prospects of development of new, more efficient AChE inhibitors and therapeutic means based on them for treating malignant tumours and Alzheimer's disease are discussed in the review.[12]

† Cholinergic receptor blockers that are acetylcholine antagonists activate acetylcholinesterase.

‡ The allosteric site is also referred to as the peripheral anionic site (PAS).

The PAS contributes to the enzyme catalytic activity owing to its short-term selective binding to the substrate as the latter moves towards the acylation site where ACh undergoes enzymatic hydrolysis involving the catalytic triad Ser203-Glu334-His447.[10,11] The binding rate of the substrate to the PAS is very high: almost every collision is effective.[13]

The enzymatic hydrolysis of ACh in the enzyme active site at relatively low ACh concentrations can be represented by the following transformations:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{H_2O,\, k_2} ES' + p' \xrightarrow{k_3} E + p'', \qquad (1)$$

where E is cholinesterase, S is substrate, ES is the Michaelis complex, ES′ is the acetylated cholinesterase, p′ is choline, p″ is acetic acid.[14]

The mechanism of enzymatic hydrolysis of acetylcholine in the active site of cholinesterase according to Eqn (1) is shown in Fig. 2.[14]

As follows from Fig. 2 *b*, the formation of the Michaelis complex is caused by binding of acetylcholine to functional groups of the amino acid residues of the catalytic site through Coulomb and hydrophobic interactions and through H-bonding involving the ester and the carbonyl oxygen atoms of ACh.[14] The benefit in the activation energy for the formation of acetylated enzyme (Fig. 2 *c*) is gained due to the conformational changes of the enzyme catalytic site.

The mechanism proposed in Fig. 2 does not answer the question of how a 1000-fold increase in the rate of acetylcholine hydrolysis rate is attained in the presence of acetylcholinesterase.[9,15] A multitude of studies tried to find the answer.

High rate of ACh hydrolysis by acetylcholinesterase is attained owing to high nucleophilicity of the Ser203 oxygen atom in the catalytic triad. The increase in the nucleophilicity of the serine residue is a function of the catalytic triad. ^{1}H NMR studies demonstrated that the nucleophilicity of Ser203 oxygen increases due to proton transfer from the hydroxy group of Ser203 to the imidazole nitrogen atom of His447. Simultaneously, the proton located at the second nitrogen atom of the imidazole ring goes to the carboxy group of Glu334. Using ^{1}H NMR and X-ray diffraction analysis and AChE **2** as an example, the presence of strong van der Waals interactions between the amino acid residues His447, Glu334 and Ser203 of the catalytic triad was shown.[15–17] In particular, it was found that the length of the O···H—N hydrogen bond between Glu334 and His447 is 2.62 ± 0.02 Å. It is hydrogen bonding that ensures the high nucleophilicity of the AChE catalytic triad, Ser203 residue participating in the enzymatic hydrolysis of the substrate.[17]

From analysis of the dependence of the AChE enzymatic activity on the spatial structure of the substrate, it was concluded that the rate of hydrolysis depends on the compactness of the substrate 'cationic head'.[9,18] Presumably, the active site of AChE has an 'anionic well', which is localized at the site of acylcholine sorption and whose size strictly corresponds to the size of the oriented sorbed acylcholine. The oriented sorption of acylcholine derivatives in this 'anionic well' induces a change in the spatial

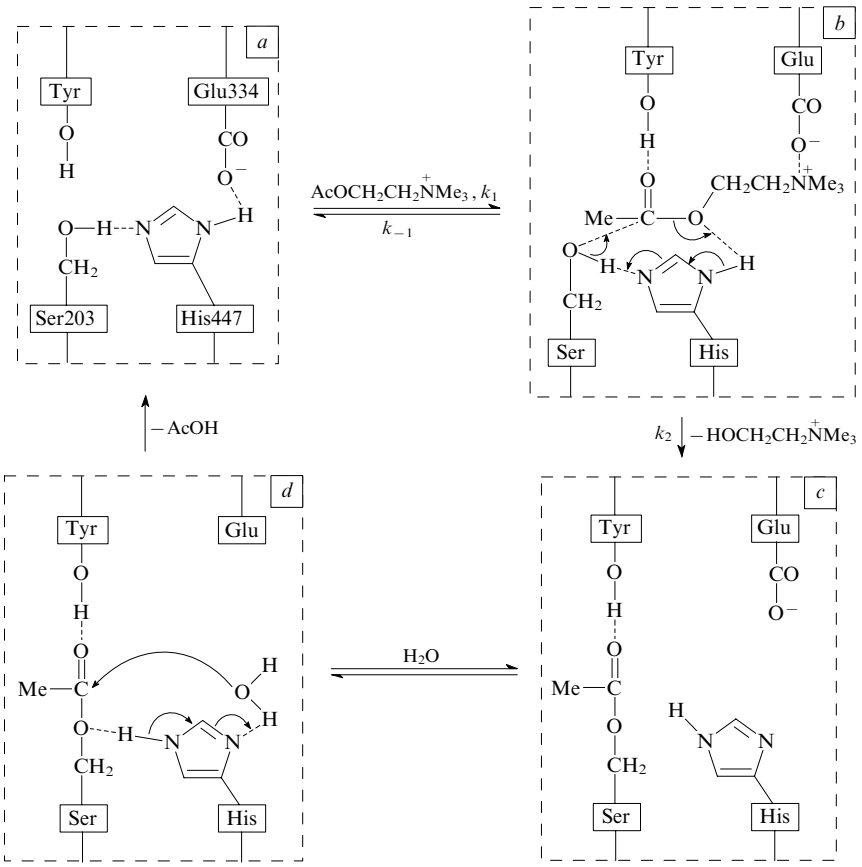


Figure 2. Mechanism of enzymatic hydrolysis of acetylcholine in the acetylcholinesterase active site.[14]

structure of the substrate as acylated enzyme is formed and a decrease in the activation energy of the hydrolysis. The obtained data indicate that a change in the substrate configuration entails a change in the spatial configuration of the enzyme active site, resulting in 'extrusion' of the substrate hydrolysis products (either 'push-pull' or 'brake principle' mechanism is implemented).[9, 14, 18]

The role of hydrophobic regions of the active site in the selective ligand binding is confirmed by site-directed mutagenesis, *i.e.*, replacement of one or more amino acid residues in the ChE active site. Thus the replacement of six aromatic amino acid residues (Tyr72Asn/Tyr124Gln/Trp286Ala/Phe295Leu/Phe297Val/Tyr337Ala) near His447 residue of the human BChE (BChE **1**) active site afforded a 'hexamutant', which proved to be inactive with respect to the natural substrate of native BChE — butyrylcholine (BCh).[19]

The data on participation of the indole fragment of the Trp84 residue [amino acid numbering corresponds to the AChE isolated from the electric ray *Torpedo californica* (AChE **3**)] located in the AChE active site in the enzymatic hydrolysis of ACh have been obtained by the isotope technique and X-ray diffraction analysis.[20] Instead of acetylcholine, model compounds were used as substrates, in particular, edrophonium chloride [ethyl(3-hydroxyphenyl)-dimethylammonium chloride], tacrine (1,2,3,4-tetrahydro-9-aminoacridine) and decamethonium bromide [trimethyl(10-trimethylammoniodecyl)ammonium bromide]. These studies used the tritium-labelled reversible AChE inhibitor, 4-(*N*,*N*-dimethylamino)phenyldiazonium tetrafluoroborate ([^{3}H]DDF). The use of the inhibitor [^{3}H]DDF provided knowledge about the role of amino acid residues of the AChE active site in the ACh binding. The compound [^{3}H]DDF is a reversible AChE inhibitor in the dark but upon photoactivation it covalently binds to the catalytic site. The molecule [^{3}H]DDF is inserted in the active site and completely blocks the enzymatic activity of AChE.[21] Previously, it was shown that tetramethylammonium bromide can completely protect AChE **3** from binding to [^{3}H]DDF upon photoactivation, which is indicative of a specific action of the latter on AChE.[22]

A crystallographic study of the structure of tacrine complex with AChE **3** showed that tacrine is located between the benzene ring of Phe300 and the indole ring of Trp84; this provides the conclusion that the indole fragment of Trp84 participates in binding of the quaternary ammonium group of acetylcholine.[20] These data are consistent with the results of earlier studies on identification of the Trp residue in the AChE active site.[21, 22]

The participation of the Trp84 residue in the enzymatic hydrolysis of acetylcholine was also confirmed by a fluorescence probe, tacrine being chosen as this probe as a model compound for acetylcholine. Spectroscopic studies have shown that the addition of cholinesterase of the horse blood serum (BChE **2**) to a solution of tacrine results in fluorescence quenching of the latter. It was established that the degree of tacrine fluorescence quenching depends on the activity of BChE **2**.[23] A mechanism of fluorescence quenching was proposed and studied, considering the formation of a non-fluorescent charge transfer complex involving tacrine in the excited state and the tryptophane indole ring of the PAS of the BChE **2** active site.[23]

An important role in the high enzymatic activity of acetylcholinesterase is played by water molecules located in the enzyme active site and in its vicinity. It was found by X-ray diffraction analysis that the molecule AChE **3** and its complexes with inhibitors have 45 regions containing 36 to 41 water molecules.[24] About half of these regions are arranged in the area adjacent to the active site, about 20 water molecules being within the active site (the number of water molecules in the active site varies from 16 to 22); these are bound to the active site amino acid residues by weak hydrogen bonds. After the ligand has been fixed in the AChE active site, some of the water molecules move to other positions and some remain in the positions they occupied in the native enzyme. The latter molecules may serve as a lubricant ensuring large-amplitude oscillations of the polypeptide loops that form the active site. These oscillations evidently facilitate migration of the substrate, hydrolysis products and water molecules towards and away from the catalytic site of the enzyme. Being weakly coordinated, the water molecules located in the enzyme active site are readily displaced to other positions by the incoming substrate molecules.[24]

An X-ray diffraction study of AChE **3** clearly demonstrated the presence of a hydrogen bond between the water oxygen atom and the nitrogen atom of Trp279. According to X-ray diffraction data of the AChE **3** complex with the chemical warfare agent VX, a considerable number of water molecules are located in the enzyme active site near the VX molecule in the region adjacent to the choline binding site (Trp84 and Trp279 positions).[24]

The relatively loose packing of water molecules in the vicinity of the enzyme active site located on the bottom of a narrow slit leaves numerous small voids, which may provide more efficient filling of this slit by the substrate (inhibitor); this may be the key driving force for substrate (inhibitor) fixing in the active site.[24]

Computer simulation of water molecule dynamics in the AChE active site extended the views on the influence of water molecules on the ligand (substrate, inhibitor) behaviour in the active site.[25–28] It was found that water molecules enter and leave the active site through the 'main entrance' (peripheral site) on average every 200 ps.[25] There are seven such entrances (seven local positions of water clusters): main, two front, two back and two side entrances. The functions of the second front entrance and the two back entrances remain obscure; however, they are known to be sometimes open fairly wide so that water molecules are free to move through them.

Of the 20 water molecules located in the active site of AChE **3**, 18 molecules were detected by X-ray diffraction.[24] All water molecules can be conventionally divided into two types, mobile and low-mobile ones. The water molecules located at the centre of the slit, near the main entrance of the active site and near the amino acid residues Ser203 and Glu202 are disordered; they readily move, have short residence times at particular positions and do not participate in hydrogen bonding. Conversely, the water molecules located at the choline binding site and in the 'acyl pocket' are ordered and low-mobile. These differences are attributable to the fact that the amino acid residues of the acylation site and the sodium ion present in the choline binding site substantially reduce the mobility of the water clusters located at these sites due to hydrogen bonding.

The magnitude and frequency of oscillations of water clusters control the size of the ligand as it enters the active site. Bear in mind that less structured mobile water clusters located at the binding site of the natural substrate form the majority and ensure the substrate migration towards the catalytic triad, *i.e.*, they facilitate the substrate diffusion.

The migration of water clusters and microvoids that accompanies the entry of the substrate into the slit can enhance its sorption. This is especially true for the large area (void) located near Ser203.[28]

Note that the effect of water clusters on the sorption of the ligand can be fully estimated only by comparison of the ligand behaviour in the absence and in the presence of water, which now cannot be experimentally implemented.[29]

The studies mentioned above explain the mechanism of acceleration of acetylation of the Ser203 residue of the catalytic triad with the substrate but do not disclose the acceleration mechanism of deacetylation of the acetylated AChE or the mechanism of AChE inhibition by high concentrations of acetylcholine. This issue was considered in a number of publications.[30–32]

The kinetic studies of the enzymatic hydrolysis of a mixture of acetylthiocholine (ATCh) (the closest analogue of acetylcholine) with carbamoylcholine by acetylcholinesterase confirmed the importance of the enzyme PAS in the activation of enzymatic hydrolysis of acetylcholine. In this experiment, ATCh served as the activating agent. It was shown that ATCh activates the acylation of the Ser203 residue with carbamoylcholine but does not participate in the activation of deacylation; thus inhibition of AChE can be attributed to high concentrations of the specific substrate, *viz*., acetylcholine.[30–32]

The two-step enzymatic hydrolysis of acetylcholine occurs at a very high rate:[32] the rate constants for enzyme acetylation (k_2) and deacetylation (k_3) are approximately 10^4 s^{-1}. A study[33] is devoted to the mechanism of acceleration of deacetylation during the enzymatic hydrolysis of acetylcholine with acetylcholinesterase AChE **4** isolated from *Drosophila*. The enzymatic hydrolysis of acetylcholine involving this enzyme occurs at a high rate over a broad range of substrate concentrations (2 μmol litre^{-1} to 300 mmol litre^{-1}); however, the enzyme activity sharply decreases at higher concentrations of acetylcholine. For interpreting this fact, the authors proposed a model according to which the process activation at rather low substrate concentrations is a result of acceleration of the deacetylation of the acetylated enzyme. The authors verified this hypothesis by using methylcarbamoylcholinesterase (which they regarded as an equivalent of the acetylated form of acetylcholinesterase) as the enzyme and 4-oxo-*N*,*N*,*N*-trimethylpentylammonium iodide as the non-hydrolyzable substrate analogue. With the use of this compound, the rate of decarbamoylation of methylcarbamoylcholinesterase increased by a factor of 2.2. Presumably, the substrate molecule attached to the activation site (K_d = 130 ± 47 μmol litre^{-1}) would accelerate the enzyme deacylation. These data are consistent with the data for enzymatic hydrolysis of the substrate. The position of the activation site in the enzyme was determined using site-directed mutagenesis. It was detected at the enzyme active site gorge. Thus, substrate binding in the activation site increases the enzyme deacylation rate; however, a substrate molecule located at the active site gorge retards the entrance of a second substrate molecule; this results in enzyme inhibition at high substrate concentrations.[33]

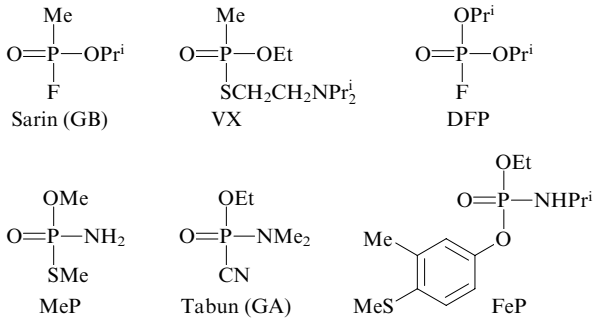
It follows from analysis of published data that high rate of the enzymatic hydrolysis of the specific substrate, ACh, is ensured by a number of factors related to the unique structure of the acetylcholinesterase active site.

III. Mechanism of the interaction of acetylcholinesterase with organophosphorus and N-heterocyclic inhibitors

The cholinesterase inhibition by organophosphorus inhibitors (OPI) — phosphorus esters — is based on phosphorylation of the hydroxy group of the Ser203 amino acid residue of the catalytic triad of the enzyme active site according to the nucleophilic substitution mechanism at phosphorus.[10,14] The OPI interaction with the cholinesterase active site comprises two steps. The first step affords a reversible ChE–OPI complex, which is converted in the second step into an irreversible complex ('ageing' of phosphorylated ChE, *i.e*., transition to the form incapable of activation) upon elimination of the OAlk group.

Using human AChE (AChE **2**) as an example, it was shown by ^{1}H NMR spectroscopy that the reaction of AChE **2** with OPI involves a trigonal bipyramidal transition state, which is stabilized through the formation of a short hydrogen bond between the hydrogen atom at imidazole nitrogen of His447 and the oxygen atom of a non-protonated Glu334 situated nearby.[16] The authors believe that this hydrogen bonding between His447 and Glu334 residues of the catalytic triad is significant for inhibition of the enzymatic activity of AChE **2**.[16]

An important role in the irreversible inhibition of AChE by organophosphorus inhibitors is played by ageing of phosphorylated AChE, which is caused by hydrolysis of the P–OAlk ester bond. A publication[10] is devoted to the ageing mechanism of AChE complexes with organophosphorus inhibitors in relation to the mouse *Mus musculus* AChE (AChE **5**). In this study, the structures of AChE **5** complexes with isopropyl methylphosphonofluoridate (sarin, GB), *O*-ethyl *S*-[2-(diisopropylamino)ethyl] methylphosphonothioate (VX), diisopropyl phosphorofluoridate (DFP), *O*,*S*-dimethyl phosphoramidothioate (methamidophos, MeP), ethyl *N*,*N*-dimethylphosphoramidocyanidate (tabun, GA) and ethyl [3-methyl-4-(methylthio)phenyl] *N*-isopropylphosphoramidate (phenamiphos, FeP) before and after ageing were studied by spectroscopy and X-ray diffraction.



The studied complexes can be divided into four groups. The first group includes AChE **5** complexes with MeP and sarin where the structure of the catalytic site before and after ageing is close to the catalytic site structure of uninhibited native AChE **5** (Fig. 3 *a*–*c*). The complex of AChE **5** with VX corresponds to the second group. The structure of the catalytic site in the phosphorylated complex before ageing differs from that in native AChE **5**, whereas after ageing, the catalytic site of the complex is similar to

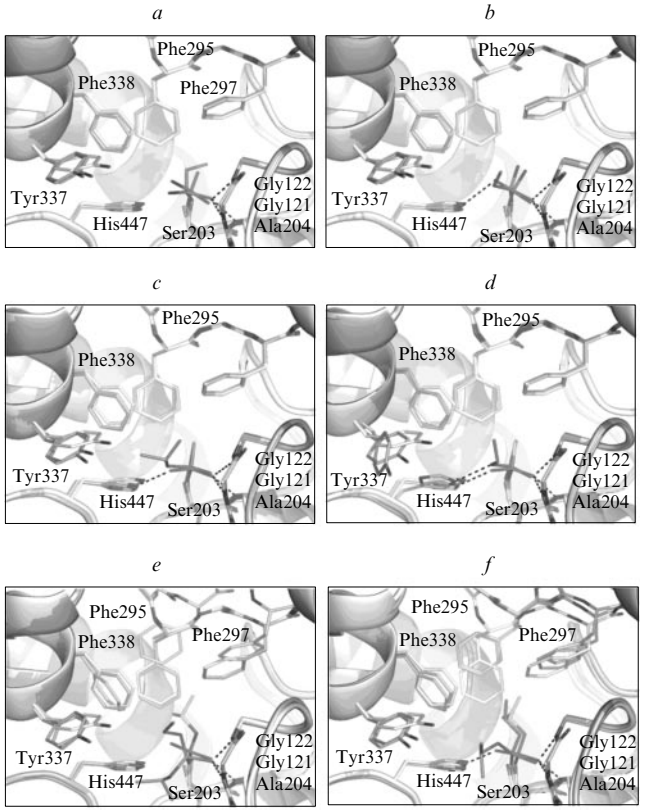


Figure 3. Crystal structures of AChE **5** complexes with (*R*)-methamidophos (*a*), (*S*)-methamidophos (*b*), sarin (*c*), VX (*d*), phenamiphos (*e*) and isopropyl isopropylphosphonofluoridate (*f*).[10]

that of native AChE **5** (Fig. 3*d*). The third group includes complex AChE **5** with FeP. Here, the structure of the catalytic site before ageing is identical to the structure of this site in uninhibited AChE **5**; however, after ageing, the catalytic site structure changes (Fig. 3*e*). Finally in the AChE **5**–DFP complex, the catalytic site structure before and after ageing differs from that in native AChE **5** (Fig. 3*f*). The structure of the rat AChE complex with tabun described previously [34] refers to the second group.

The electron density maps of AChE **5** complexes with MeP before and after ageing obtained with 2.74–2.50 Å resolution can be used to represent the structure of the catalytic site of uninhibited AChE **5**.

All of the studied crystal structures of the complexes showed high electron density in the vicinity of Ser203, indicating the presence of a covalent bond between the Ser203 oxygen atom and the OPI phosphorus atom.

The above four groups of complexes have different ageing periods. For example, AChE **2** complexes with DFP and sarin have a short ageing half-period (about 3 h); the ageing half-periods of AChE **2** complexes with MeP, tabun and VX are about 10, 19 and 37 h, respectively; and the complex of AChE **2** with FeP has a relatively long ageing half-period, 130 h.[10]

The studied complexes also differ in their stability against reactivation. The complexes of AChE with tabun, FeP and DFP are stable against reactivation,[35] whereas the complex with MeP is readily reactivated by most oximes. The complexes with VX and sarin refer to an intermediate group; they can be reactivated by some but not by all oximes.[36] The differences in the ageing rate and stability against reactivation depend on both the structure of the complex and the structure of the reactivating agent.[36, 37]

The key specific feature of AChE **5** complexes with the OPI studied is the formation of three hydrogen bonds involving the phosphoryl oxygen atom and functional groups of the amino acid residues belonging to the 'anionic well', which ensure the occurrence of the OPI molecules within the catalytic site of the enzyme. This feature accounts for the stereochemical course of inhibition and reactivation, as was shown for methylphosphonates.[38–43] Strong interaction between the phosphoryl oxygen atom and the amino acid residues of the 'anionic well' prevents motion of substituents at the phosphorus atom in the complex during the reaction. Presumably, large substituents in the OPI molecule can be located only around the 'acyl pocket' of the enzyme.

These investigations brought the authors to conclusion that the ageing of phenamiphos-phosphorylated AChE **5** follows a nucleophilic substitution mechanism at phosphorus and is accompanied by considerable shortening of the 'acyl pocket' loop of the AChE **5** catalytic site caused by elimination of the relatively large aroxyl substituent of the OPI (see. Fig. 3*e*).[10]

The X-ray diffraction analysis of AChE complexes with inhibitors in combinations with kinetic studies of ageing of these complexes suggested two ageing mechanisms, in particular, nucleophilic substitution at phosphorus and dealkylation through the intermediate formation of a carbocation.[10]

Using conformational analysis by molecular mechanics method, it was shown that the inhibiting action of OPI depends on the accessibility of the phosphorus atom for the nucleophilic attack from the side opposite to the leaving group position.[9] Hence, ageing by the former mechanism takes place when the phosphorus atom is sterically accessible.[44]

The ageing of AChE phosphorylated by soman, *i.e.*, pinacolyl methylphosphonofluoridate, follows a different pathway, which implies the formation of a carbocation stabilized through interaction with the indole ring of Trp86. This is related to the fact that the pinacolyl group creates steric hindrance for nucleophilic reagent approach to the phosphorus atom.[44] Fast ageing of soman-inhibited AChE ($t_{1/2}$ = 0.1 h) is caused by high rate of elimination of the pinacolyl group.[10] The pinacolyl group that leaves during ageing of soman-phosphorylated AChE is an analogue of the choline group in acetylcholine.

The organophosphorus inhibitors with smaller substituents (compared to the pinacolyl group in soman) studied in another work [10] are located further from Trp86; therefore, in the case of MeP, sarin, VX and DFP, the formation of a carbocation is unlikely.[10, 11]

Thus, the mechanism of ageing of sarin-, MeP-, VX-, DFP- and FeP-phosphorylated AChE includes the nucleophilic attack of a water molecule on the phosphorus atom to give a trigonal-bipyramidal transition state. The authors arrived at the conclusion that the acyl pocket loop changes the rate of ageing, thus being favourable for conformational transformations of ligands at the phosphorus atom.[10, 11]

The reactivation of phosphorylated acetylcholinesterase by oxime and spontaneous reactivation also involve the formation of a trigonal-bipyramidal transition state, the acyl pocket loop performing a similar function in this process.[10]

The key cause of the irreversible inhibition of AChE by organophosphorus inhibitors is the exceptionally slow dephosphorylation brought about by steric factors. The AChE catalytic site is optimized for deacetylation of the acetylated serine residue of the catalytic triad rather than for dephosphorylation. The transition state of deacetylation differs from the trigonal-bipyramidal transition state of the nucleophilic substitution at phosphorus. The other reason is fast ageing of the phosphorylated enzyme.[10]

Site-directed mutagenesis with replacement of one or two amino acid residues in the polypeptide chain of BChE **1** was used to determine the amino acid residues of the catalytic site responsible for the irreversible inhibition of cholinesterase. For this purpose, three mutants were obtained: G117H, E197Q and G117H/E197Q.[45,46] Computer simulation demonstrated that placing His near the 'anionic well' in the BChE **1** active site (replacement of Gly117 by His117) should affect considerably both the rate of its inhibition by OPI and its enzymatic activity. Indeed, the G117H mutant was inhibited at a lower rate by a number of OPI including sarin and VX and even catalyzed their hydrolysis.[45]

In order to confirm the important role of histidine in the decrease in the inhibition rate, the G117K mutant was obtained. Table 1 summarizes the values of dissociation constants (K_d) of complexes formed by BChE **1** and its G117H and G117K mutants with sarin and VX and the values of rate constants for the inhibition of these enzymes.[45] It follows from the data presented in Table 1 that the rate constants for inhibition of the G117H mutant by sarin and VX, respectively, are four and three orders of magnitude lower than that of native BChE **1**. The role of histidine in the stability of the G117H mutant against inhibition by sarin is evidenced by rather close k_i values for the G117K mutant and native BChE **1**.

Table 1. Dissociation constants (K_d) of complexes of BChE **1** and its mutants G117H and G117K with organophosphorus inhibitors and rate constants for inhibition (k_i) of these enzymes.[45]

Inhibitor	Enzyme	Inhibitor concentration /μmol litre^{-1}	K_d /μmol litre^{-1}	k_i /litre mol^{-1} s^{-1}
Sarin	BChE **1**	0.05 – 0.13		22000 ± 400
	G117H	1.25 – 200	110 ± 10	2.7 (2.4 – 3.4)
	G117K	1.6 – 50	13 ± 2	3400 (800 – 3500)
VX	BChE **1**	0.05 – 0.13		30400 ± 2800
	G117H	40 – 150	50 ± 9	25 (20 – 35)
	G117K	20 – 101	300 ± 100	50 (30 – 100)

In order to identify the amino acid residue responsible for the high rate of soman dealkylation, computer simulation of the BChE **1** active site phosphorylated by this inhibitor was carried out.[46] The G117H, E197Q and G117H/E197Q mutants were prepared by site-directed mutagenesis. All mutants were stable against soman inhibition, and, according to computer simulation data, the last-mentioned enzyme exhibited simultaneously two programmed functions (synergism), in particular, it combined very low rate of ageing typical of the phosphorylated mutant E197Q with high dephosphorylation rate characteristic of G117H.[46]

It was concluded[46] that the introduction of two amino acid residues, His and Gln, in the BChE **1** active site transformed this enzyme to an esterase capable of catalyzing hydrolysis of a strong irreversible inhibitor such as soman. The rate constants for soman inhibition of BChE **1** and its mutants are summarized in Table 2. As can be seen from data presented in Table 2, the rate constant for soman inhibition of the G117H/E197Q mutant is 3000 times lower than that of native BChE **1**.

Table 2. Comparison of the rate constants for inhibition of BChE **1** and its mutants by soman P_R,C_R-stereoisomer.[46]

Enzyme	Soman concentration /μmol litre^{-1}	k_i, /litre μmol^{-1} s^{-1}
BChE	0.04 – 0.2	6 ± 2
E197Q	1 – 10	0.35 ± 0.05
G117H	8 – 80	0.033 ± 0.004
G117H/E197Q	8 – 200	0.0021 ± 0.0002

Note. The kinetic parameters for enzymes in solutions were determined using the Ellman procedure at pH 7.5 and 25 °C.

The esterase G117H/E197Q catalyzed hydrolysis of sarin, VX and all stereoisomers of soman. The phosphorylation and dephosphorylation reaction were stereospecific, dephosphorylation being the limiting step. The spontaneous reactivation (without using oximes) of the sarin-inhibited Gl17H/E197Q mutant showed that the destruction of the 'anionic well' in the catalytic site of BChE **1** directly affects the ageing and the enzymatic activity of the phosphorylated enzyme.

Cholinesterases obtained in a number of works[45,46] by site-directed mutagenesis of BChE **1** can be used, together with organophosphate hydrolases, in chemical weapon destruction techniques.[47]

The reactions of OPI with cholinesterases can be represented as follows:

$$E + I \underset{k^i_{-1}}{\overset{k^i_1}{\rightleftharpoons}} EI \xrightarrow{k^i_2} EI' + p' \xrightarrow{H_2O,\, k_3} EI'' + p'', \qquad (2)$$

where I is an organophosphorus inhibitor, EI is the noncovalent cholinesterase – inhibitor complex, EI′ is phosphorylated cholinesterase, p′ is the leaving group, EI″ is the phosphorylated cholinesterase after ageing, p″ is the ageing reaction product, k^i_1, k^i_{-1}, and k^i_2 are the rate constants of the intermediate steps of the inhibition of cholinesterase, k_3 is the rate constant for ageing of phosphorylated cholinesterase.[14] The rate constant for the inhibition of cholinesterase (k_i) depends on the rate constants for the intermediate steps outlined in (2).

Figure 4 shows the mechanism of reactions of OPI with cholinesterase.[14] The formation of the cholinesterase – inhibitor complex (Fig. 4 *b*) upon attack of the oxygen atom of the Ser203 hydroxy group on the phosphorus atom of the organophosphorus inhibitor is accompanied by conformational changes at the cholinesterase active site, resulting in the formation of a trigonal-bipyramidal transition state.[14] The step of formation of the phosphorylated enzyme is shown in Fig. 4 *c*.

A publication devoted to theoretical investigations of the reaction of OPI with cholinesterases[48] proposes new methods for simulation of these reactions.

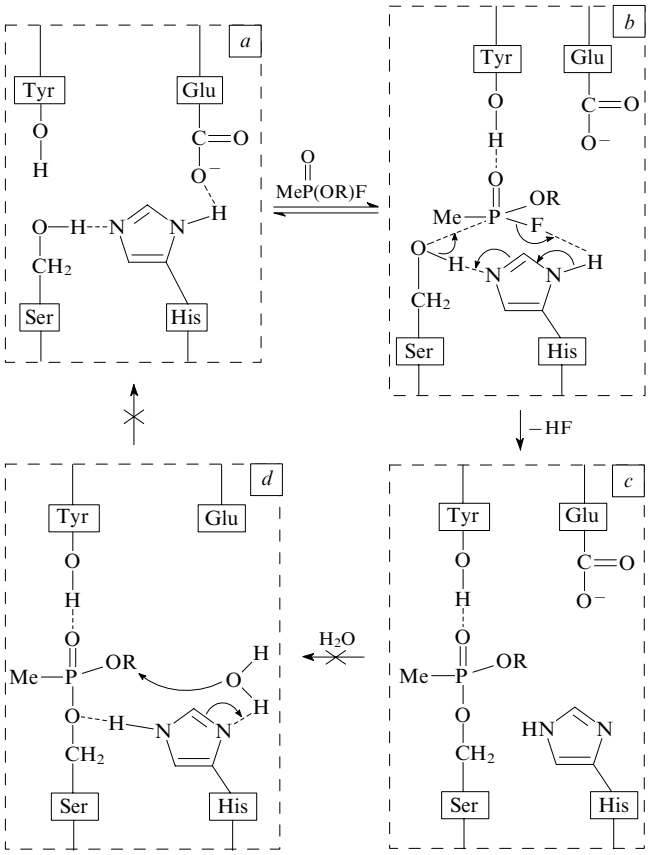
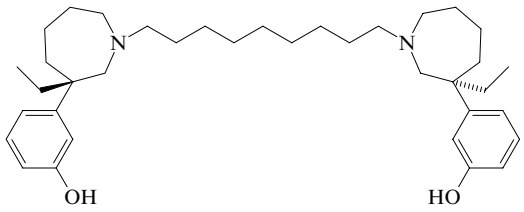


Figure 4. Mechanism of OPI interaction with cholinesterase.[14] (*a*) Functional groups incorporated in the cholinesterase active site; (*b*) formation of the non-covalent enzyme–inhibitor complex; (*c*) formation of phosphorylated enzyme, (*d*) dephosphorylation (does not occur).

Thus, the most pronounced contribution to the irreversible inhibition of cholinesterase by organophosphorus compounds is made by the following factors: high rate of enzyme inhibition, high rate of ageing and low rate of dephosphorylation of the phosphorylated enzyme.[10]

The irreversible AChE inhibition by a new class of inhibitors, 6-methyluracil, alloxazine and xanthine derivatives containing *N*-(ω-tetraalkylammonioalkyl) groups in the pyrimidine ring follows a mechanism other than the classical phosphorylation. Inhibitors of this class are devoid of the acylation function. It was suggested that they react with amino acid residues located at the active site gorge.[49] These inhibitors are highly active and selective with respect to mammalian AChE. The results of studies of the anticholinesterase activities of such inhibitors have been reported.[49,50] Of the compounds studied, the highest irreversible AChE inhibition rate was found for the compounds containing two ethyl groups and a substituted phenyl fragment in the tetraalkylammonium group at one or both nitrogen atoms of the pyrimidine ring. These compounds behave as typical irreversible inhibitors of AChE **2**: the rate constants for the inhibition vary from 7.6×10^8 to 3.5×10^9 litre mol^{-1} min^{-1}. Simultaneously, they are reversible BChE inhibitors.

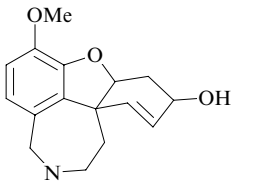
The bis-(−)-*nor*-meptazinol derivative in which two meptazinol fragments are connected by a nonamethylene spacer also inhibits AChE by a different mechanism.[51]



Bis-(−)-*nor*-meptazinol derivative

This compound inhibits at one stroke two ligand binding sites in the enzyme. One meptazinol fragment is linked to the His440 residue in the 'anionic well' thus destroying the catalytic triad. The oxygen atom of the phenolic substituent in meptazinol forms a hydrogen bond with His440 (2.55 Å), which is hydrogen-bonded in the native enzyme with the oxygen atom of serine of the catalytic triad. The second meptazinol fragment is linked to the P-region at the active site gorge.[51]

A study of the crystal structure of galantamine complex with the active region of AChE **3** demonstrated that this alkaloid also inhibits AChE.[52]



(−)-Galantamine

(−)-Galantamine interacts with the Trp84 amino acid residue in the choline binding region and with Phe288, Phe290 residues positioned in the acyl pocket. The galantamine interaction with Trp84 does not occur through its tertiary amino group but rather through stacking of the double bond of the cyclohexene ring with the indole ring of Trp84, the tertiary amino group being involved in the hydrogen bond (3.4 Å) with the Asp72 residue. The hydroxy group of galantamine forms a strong H-bond (2.7 Å) with the Glu199 residue.

IV. Activators of the acetylcholinesterase interaction with organophosphorus inhibitors

A series of allosteric inhibitors of the acetylcholinesterase PAS that activate ligand interaction with the catalytic site of the AChE active site are known.[11,53–56] The participation of the tryptophan indole groups of the PAS in the binding of allosteric inhibitors was proved using ethidium bromide [3,8-diamino-5-ethyl-6-phenylphenanthridinium bromide (BEt)].[57,58] The latter reversibly inhibits BChE (K_d = 52 μmol litre^{-1}). Ethidium bromide shows intense fluorescence in the red spectral region, which is far removed from the fluorescence regions of tryptophan and other aromatic amino acids and, hence, it can be used as a fluorescence probe.

Spectroscopic investigations of ethidium bromide in phosphate buffer (pH 8) showed that in the presence of BChE **2**, a new strong absorption band (λ_{max} = 410 nm) appears in the spectrum of this compound at shorter-wavelengths than the absorption band of free ethidium bromide, which is at 470 nm (Fig. 5).[57]

Excitation of ethidium bromide at 470 nm gives rise to a fluorescence band at about 610 nm (Fig. 6, spectrum *1*). In

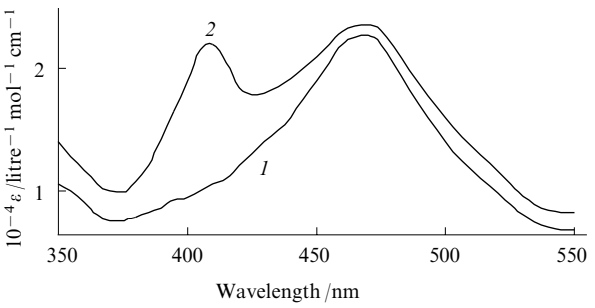


Figure 5. Absorption spectra of ethidium bromide (*1*) and its complex with BChE (*2*).[57]
ε_H is the extinction coefficient. Concentration of BEt, 7.5 μmol litre^{-1}; enzyme activity, 0.25 U ml^{-1}.

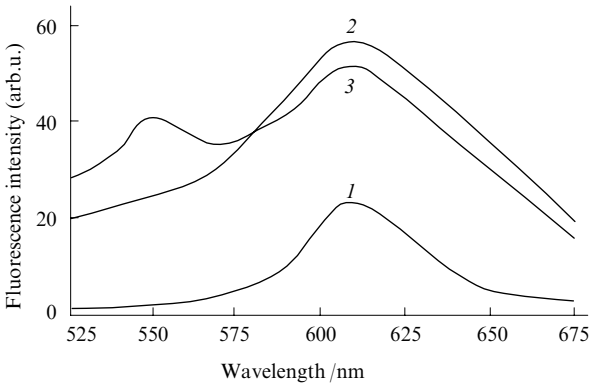


Figure 6. Fluorescence spectra of BEt (*1*) and its complex with BChE (*2*, *3*).[57]
The activity of BChE **2** is 0.36 (*2*) and 0.50 U ml^{-1} (*3*); BEt concentration, 0.6 μmol litre^{-1}.

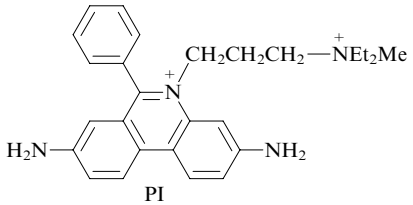
the presence of BChE **2**, the intensity of BEt fluorescence increases without shift of the maximum (Fig. 6, spectrum *2*). For example, the relative fluorescence quantum yield of BEt at 610 nm increased 2.5-fold upon the addition of BChE **2** with activity of 0.36 U ml^{-1}. The addition of butyrylcholinesterase with activity of 0.50 U ml^{-1} to ethidium bromide gave rise to a new fluorescence band at 550 nm (Fig. 6, spectrum *3*). The intensity of the new fluorescence band increased proportionally to the increase in the enzyme activity. Similar results were obtained when studying the effect of bovine AChE and squid ganglia propionylcholinesterase on the BEt fluorescence. However, the addition of eel AChE to ethidium bromide did not produce a new fluorescence band.[58]

New absorption and fluorescence bands were assigned to BChE **2** complexes with ethidium bromide formed upon ethidium bromide binding to the tryptophan residue. The increase in the BEt fluorescence intensity at 610 nm in the presence of the studied enzymes can be attributed to the resonance energy transfer from the excited enzyme–inhibitor complex to the free BEt molecule. In this case, the main conditions required for resonance energy transfer are met, in particular, substantial overlap of the fluorescence spectrum of the donor (enzyme complex with BEt; see Fig. 6, spectrum *3*) with the absorption spectrum of the acceptor (free BEt, λ_{max} = 470 nm, see Fig. 5, spectrum *1*); short distance between the donor and the acceptor [less than the Förster radius (20–50 Å)] because BEt is a reversible inhibitor of these enzymes and the enzyme–inhibitor system occurs in dynamic equilibrium. This conclusion relies on the structural data obtained for tacrine, which is a reversible fluorophore inhibitor structurally similar to ethidium bromide. The shortest distance between tacrine and the tryptophan indole ring in the PAS of the AChE active site is known to be less than 5 Å.[59–61] Thus, the results of spectral studies lead to conclusion that ethidium bromide binds to the indole fragment of tryptophan in the PAS.

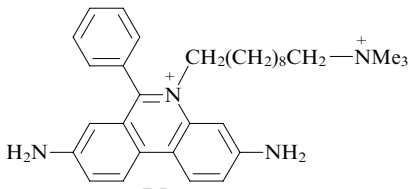
The role of the PAS in the activation of ligand interaction with the catalytic side of the AChE active site is due to specific features of its structure. The PAS is located near the active site gorge of the enzyme and represents a 'pocket' for binding ligands with a cationic group.[62] By kinetic measurements and computer simulation, it was found that the ligands that specifically bind to the serine residue of the active site catalytic triad interact first of all with the amino acid residues of the PAS. It was shown for AChE **2** as an example that binding to the PAS Asp74 residue bearing a negative charge is the first step in the enzymatic hydrolysis of the ATCh substrate.[63] Some amino acid residues of the PAS are linked to the surface loops of the polypeptide chain, thus ensuring their high conformational flexibility (allosteric modulation).

A study[11] is devoted to the mechanism of allosteric modulation of the catalytic site of the AChE active site during substrate hydrolysis and interaction with inhibitors.

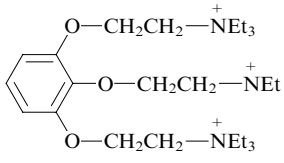
Spectrophotometric investigations and X-ray diffraction analysis of complexes of dimer AChE **5** with propidium iodide (PI), decidium iodide (DI) and gallamine (GAL), which are allosteric enzyme inhibitors, made it possible to specify the structure of the PAS of AChE **5** and of adjacent fragments involved in the regulation of AChE interaction with substrates that acylate the serine residue of the catalytic triad in the enzyme active site.[11]



PI



DI



GAL

The polycyclic compounds DI and PI show absorption and fluorescence spectra in the region other than the protein absorption region. Using this feature, it was possible not only to analyze the AChE **5** complexes with DI and PI in solutions and optimize the population of the PAS during

complexation but also to choose those complexes that showed the highest PAS population.

Since the decidium and propidium iodide molecules have similar structures, the crystal structures of their complexes with AChE also bear resemblance.[11] In the complex of DI with AChE **5**, two similarly oriented DI molecules are linked to two PAS of the AChE dimer. The DI molecule is linked to the Trp286 residue of the enzyme PAS through the π–π-interaction of the slightly bent phenanthridinium ring of DI with the indole ring of Trp286 provided by their nearly parallel arrangement (the phenanthridinium ring is located at a 3.7 Å distance from the Trp286 indole ring). The electron density maps of the DI complex of AChE **5** demonstrate electron delocalization between the Trp286 indole ring and the phenanthridinium ring of DI, which suggests the formation of a charge transfer complex between the electron-deficient phenanthridinium and electron-saturated indole residue of Trp286. A hydrogen bond is formed between the nitrogen atom of the phenanthridinium ring and the imidazole substituent of His287. The alkyl chain by which the trimethylammonium cation is linked to the phenanthridinium ring is involved in numerous van der Waals contacts with Leu289 and Glu292 amino acid residues of the adjacent chain, thus providing considerable contribution of this region of the loop to DI binding to the PAS. One solvent molecule and one chloride ion are arranged at a distance of ~3.7 Å from two phenanthridinium benzene rings opposite to the Trp286 indole ring (without a bound DI molecule, the chloride ion is located above the Trp286 indole ring). Their arrangement accounts for the fact that the ligand binding by the PAS is hampered.

It follows from the electron density distribution map for the PI–AChE **5** complex that the PAS region accommodates two PI molecules in different conformations differing by the arrangements of phenyl and methyldiethylammonioalkyl substituents. In one conformer, the alkyl chain is arranged along the PAS surface of AChE **5**, the $MeEt_2N^+$ group being linked to the imidazole ring of the His287 residue, whereas the phenyl substituent is directed towards the Gly342 and Glu292 residues of the adjacent chains, which form the hydrophobic surface pocket.

In the other conformer, the $MeEt_2N^+$ group is located between the benzene ring of PI and the functional groups of His287 and Ser293; this gives rise to a loop similar to that observed in the AChE **5** complex with DI.

Of the three allosteric inhibitors studied, the most pronounced effect on the conformation of the enzyme PAS is exerted by gallamine whose structure differs substantially from structures of the AChE complexes with DI and PI.

The benzene ring of GAL (which is smaller than the phenanthridinium ring) occupies a central position at the enzyme active site gorge. It is equally distant from the Trp286 indole ring situated on one side of the gorge and the Tyr341 phenol ring, which is on the other side. These two amino acid residues participate in the van der Waals contacts with GAL. Also, additional contacts occur between the GAL molecule and the Tyr72 and Tyr124 residues of the adjacent chains.

Two of the three alkyl chains (terminal and median ones) in the GAL molecule are disordered, while the third chain (pointing to His287) is partially visible. One ether oxygen atom of gallamine participates in binding of the iodide ion. The iodide ion is located between this oxygen and Phe295 nitrogen where a solvent molecule resides in the native enzyme. Results of electrostatic calculations suggest that the iodide ion present in the AChE active site would correct the enzyme functioning.[11]

Fixing of a GAL molecule on the PAS affects dramatically the stability of the upper parts of two polypeptide loops, Val340–Gly342 and Asp74–Pro78, located at the active site gorge. Low stability of these two loops is probably associated with relatively large oscillations of the alkyl chains of GAL. It is significant that the polypeptide chains adjacent to these loops can acquire different conformation, thus becoming flexible. This apparently leads to some expansion of the active site gorge and facilitates the ligand access. This unique structure distinguishes the AChE **5** complex with GAL from the complexes with DI or PI or from native AChE **5**.

A comparative analysis of the structures of AChE **5** and its three complexes with the ligands DI, PI and GAL attests to substantial mobility of the polypeptide chains adjacent to the PAS, which include Tyr72, Leu76, Tyr337 and Tyr341, *i.e.*, four key amino acid residues circumscribing the active site gorge. In particular, the phenol ring of the Tyr337 residue located near the active site gorge can deflect to a distance of up to 2 Å. This migration is sufficient for expanding the active site gorge and facilitating the ligand access to the serine residue of the catalytic triad. The functional groups of Leu76 and Gly79 are also mobile in the presence of bound GAL and deflect to a distance of up to 0.75 Å. Thus, joint motion of the upper part of the large ω-loop Cys69–Cys96 and the loop Val340–Gly342 incorporating the Tyr341 residue can considerably expand the active site gorge.

The results of crystal structure studies of complexes of AChE **5** with DI, PI and GAL disclose the mechanism of influence of allosteric inhibitors of the enzyme PAS on the rate of reaction of AChE with substrates that acylate the serine residue of the catalytic triad.

The presented data are consistent with the data on the effect of allosteric inhibitors on the conformational changes in the PAS obtained by means of fluorescence probing.[64–66]

Using acrylodan (fluorescent probe), which reacts selectively with the Cys69–Cys96 residues of the ω-loop of AChE **5** on its external side, it was shown that association of reversible ligands with the PAS causes the conformational changes in this loop, namely, displacement of the ω-loop from the hydrophobic to more hydrophilic area.[64] These changes induce a change in the fluorescence of acrylodan covalently linked to the enzyme PAS.[64–66] The conformational changes induced by the inhibitor interaction with the enzyme active site and PAS attest to flexibility of the ω-loop.[65] Moreover, phosphorylation of Ser of the catalytic triad brings about specific changes in the fluorescence spectrum of acrylodan, which can be used to detect organophosphorus inhibitors.

Thus, a ligand bound to the enzyme PAS causes serious conformational changes in the enzyme ω-loop.[64–66]

Examples of the effect of allosteric inhibitors on the phosphorylation rate of the serine residue of the catalytic triad by organophosphorus compounds are given below. In particular, it was established that the rate of reaction of AChE **2** with OPC increases in the presence of the allosteric reversible inhibitor PI.[53,54] As OPC, fluorogenic compounds were used, namely, 1-methyl-7-[(diethoxyphosphoryl)oxy]quinolinium iodide (DEPQ) and 4-methyl-7-[(methylethoxyphosphoryl)oxy]coumarin (EMPC).

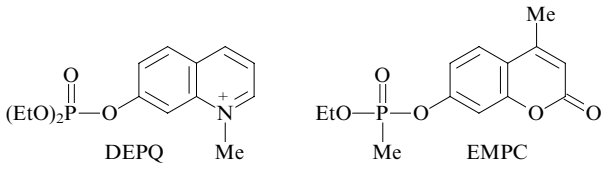


Table 3 presents the rate constants for phosphorylation of AChE **2** with the inhibitors EMPC and DEPQ in the absence and in the presence of PI.[53] It can be seen from these data that in the presence of PI, the rate constant for the inhibition of AChE **2** with neutral EMPC increases sixfold, while the rate of inhibition by positively charged DEPQ slightly decreases.

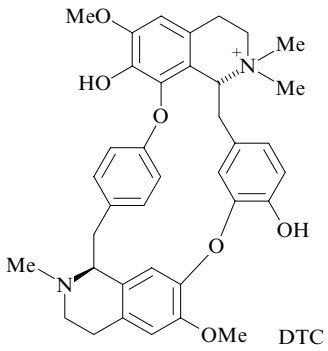
Table 3. Rate constants for inhibition of AChE **2** by fluorogenic inhibitors EMPC and DEPQ in the presence and in the absence of propidium.[53]

Inhibitor	k_1 /min^{-1} (see [a])	
	in the absence of PI	in the presence of PI
EMPC	149 ± 9	900 ± 500
DEPQ	1600 ± 300	1100 ± 500

[a] The rate constants for inhibition were determined from the rate of growth of the fluorescence intensity of the reaction solution upon release of fluorophore, a product of inhibitor cleavage.

Presumably, an increase in the phosphorylation rate of the serine residue of the acetylcholinesterase catalytic site by EMPC is a result of the molecular strain caused by the close arrangement of the PI and EMPC molecules in the ternary complex.[53] The lack of acceleration of the inhibition of AChE **2** by DEPQ whose molecule bears a positive charge was attributed to the electrostatic repulsion of the DEPQ molecules and propidium, which also bears a positive charge.[53] The modest effect of increase in the inhibition rate is consistent with the proposed steric model for the AChE blockage.[54, 67]

More pronounced effect on the reaction rate of AChE with OPI is exerted by cholinergic receptor blockers, acetylcholine antagonists (below referred to as activators), in particular, d-tubocurarine chloride (DTC) and gallamine.[55, 56] These compounds are reversible AChE inhibitors, which selectively bind to the PAS of the AChE active site.[55, 56, 68]



The effect of a number of activators on the rate of AChE **5** inhibition by paraoxone is shown in Fig. 7.[56] It follows from the presented data that d-tubocurarine has the most pronounced impact on the inhibition rate of AChE **5** by paraoxone. In the presence of d-tubocurarine chloride and gallamine, the reaction rate of AChE **5** with paraoxone increases over a broad range of activator concentrations to reach a maximum at 1 mmol litre^{-1}. In the activator concentration range from 0.001 to 1 mmol litre^{-1}, the effects of d-tubocurarine chloride and gallamine are more pronounced than the effects of atropine and propidium.

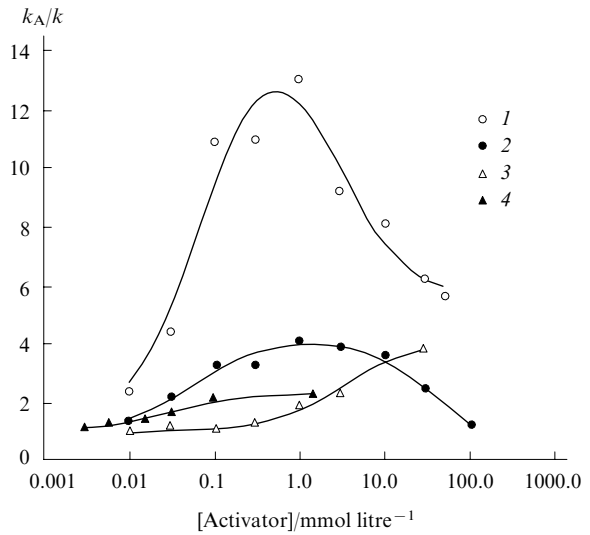


Figure 7. Ratio of the rate constants for inhibition of AChE **5** by paraoxone in the presence (k_A) and in the absence (k) of activator *vs.* concentration of activator: d-tubocurarine (*1*), gallamine (*2*), atropine (*3*) and propidium (*4*).[56]

A study of the effect of the above activators on the rate of inhibition of AChE **5** by a number of OPI demonstrated that the rate of reaction of AChE **5** with neutral OPI increases, whereas the rate of reaction with OPI containing a cationic group [for example, *O*-alkyl *S*-2-(*N*,*N*,*N*-trimethylammonioethyl) methylphosphonothioate] decreases (Refs 55 and 56)

A similar increase in the rate of phosphorylation of AChE **2** in the presence of d-tubocurarine was observed in another study.[69] Table 4 gives the detection limits for some OPI, including 2,2-dichlorovinyl dimethyl phosphate (dichlorvos), diethyl *p*-nitrophenyl phosphate (paraoxone), dimethyl (1-hydroxy-2,2,2-trichloroethyl)phosphonate (chlorophos), by enzymatic technique in the presence and in the absence of d-tubocurarine. One can see that d-tubocurarine provides a decrease in the OPI detection limit by two orders of magnitude. The results of studies lead to tentative inference concerning the general character of the effect of d-tubocurarine on the rate of irreversible inhibition of AChE **2** and AChE **5**.

Table 4. Determination limits of some insecticides in the presence and in the absence of d-tubocurarine.[69]

Insexticide	Detection limit of OPI /mg ml^{-1}	
	in the presence of DTC	in the absence of DTC
Paraoxone	$(2.6 \pm 0.4) \times 10^{-10}$	$(1.1 \pm 0.6) \times 10^{-8}$
Dichlorvos	$(4.6 \pm 0.3) \times 10^{-8}$	$(2.5 \pm 0.3) \times 10^{-6}$
Chlorophos	$(3.8 \pm 0.5) \times 10^{-7}$	$(1.8 \pm 0.2) \times 10^{-5}$

The acceleration of AChE phosphorylation by neutral OPI in the presence of activators was interpreted by hypothesizing that binding of the activator to the PAS induces a conformational change in the AChE catalytic site, which in turn increases the rate of phosphorylation of Ser203.[56] A conformational change is indicated, in particular, by the orientation of the leaving group of OPI favourable for its hydrolysis. Thus, this hypothesis envisages the conformational interaction between the PAS and the catalytic site of the AChE **5** active site.[56]

A certain contribution to the acceleration of the phosphorylation of AChE **5** by neutral OPI in the presence of activators is evidently made by their binding to the PAS, resulting in expansion of the active site gorge and facilitating the ligand access to the serine residue of the catalytic triad.[11]

More pronounced effect of gallamine, compared to propidium, on the rate of reaction of AChE with OPI can also be attributed to greater influence of GAL on the PAS conformation and, hence, more ready accessibility of the Ser residue of the catalytic triad for the ligand.[11]

The publications[70,71] are devoted to kinetic studies of the high efficiency of the impact of d-tubocurarine on the irreversible inhibition of AChE **4**. According to one study,[70] the enzymatic hydrolysis of ACh starts with its interaction with the negatively charged Asp413 residue of the PAS. A mutation of the enzyme resulting in charge migration away from the active site gorge prevents the activation.[70] Figure 8 shows the dependence of the rate constant for hydrolysis (k) of acetylthiocholine by native AChE **4** (*a*) and its D413G/F414L mutant (*b*) on the ATCh concentration in the presence and in the absence of d-tubocurarine. It can be seen from these data that both in the absence and in the presence of d-tubocurarine, the rate of substrate hydrolysis by the mutant is much higher than by the native AChE **4**. This contradiction was attributed to less efficient inhibition of the enzyme by d-tubocurarine owing to the shorter residence time of the substrate in the enzyme PAS.[70]

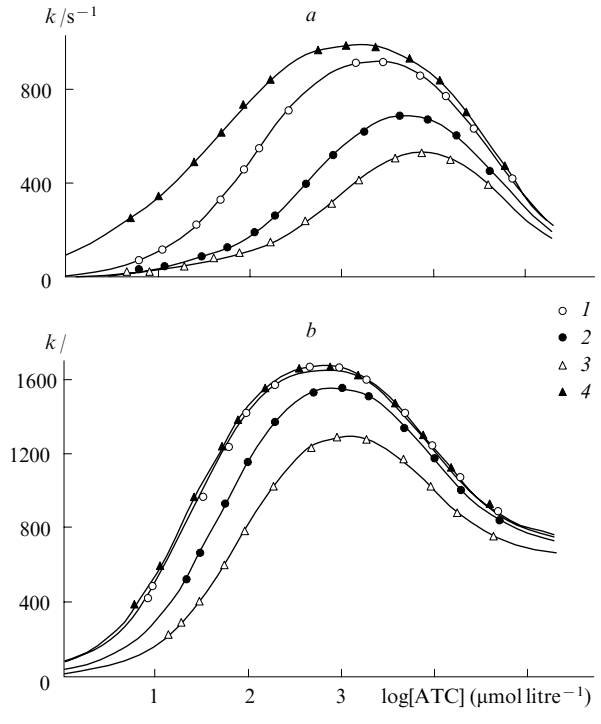
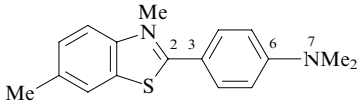


Figure 8. Rate constant for hydrolysis (k) of acetylthiocholine by native AChE **4** (*a*) and its mutant D413G/F414L (*b*) *vs.* ATCh concentration in the presence (curves *1*–*3*) and in the absence (curve *4*) of d-tubocurarine.[70]
d-Tubocurarine concentration, μmol litre^{-1}: (*a*) 0 (*4*), 2 (*1*), 20 (*2*), 100 (*3*); (*b*) 0 (*4*), 1 (*2*), 20 (*2*), 100 (*3*).

In a particular concentration range, d-tubocurarine behaves as an activator of irreversible inhibition of methanesulfonylation of mutant AChE **4** by methanesulfonyl fluoride with a small size but does not affect the rate of methanesulfonylation of native AChE **5**.[71]

Considering the obtained results, the authors concluded that under certain conditions, d-tubocurarine can behave as an activator of the acylation of Ser of the catalytic triad.[71] The publication[72] evidences for acceleration of the acylation of Ser of the enzyme catalytic triad upon binding of an allosteric inhibitor (d-tubocurarine) to the enzyme PAS as a result of conformational interaction between the PAS and the acylation site.

Using recombinant human erythrocyte AChE (AChE **6**) as an example and thioflavine T as the fluorogenic probe, a more than 1000-fold increase in the probe fluorescence at 490 nm upon excitation at 448 nm in the presence of AChE was found. Thioflavine T is an allosteric fluorogenic inhibitor binding selectively to the PAS of AChE **6** (the dissociation constant of the inhibitor–enzyme complex is 0.89 μmol litre^{-1}).[71]



Thioflavine T

Thioflavine T belongs to the group of 'molecular rotors'.§ In a thioflavine T molecule, the benzothiazole and benzene rings tend to rotate independently relative to each other around the axis through the C(2), C(3), C(6) and N(7) atoms. The fluorescence intensity of the molecular rotors depends on the solvent polarity: as the solvent polarity increases, their fluorescence intensity grows, which is attributable to the decrease in the torsion mobility in high-polarity media upon solvation.[73] This was confirmed by the fluorescence spectra of thioflavine T in the presence of various solvents.

The increase in the thioflavine T fluorescence intensity upon binding to the PAS of the active site of AChE **6** can be attributed to the retardation of the rotation of the benzothiazole and benzene rings. Owing to the complementary matching of the thioflavine T functional groups to the functional groups of amino acid residues in the peripheral site of AChE **6**, the thioflavine T molecule becomes planar and a system of conjugated bonds is formed, resulting in increased fluorescence intensity. Figure 9 presents the variation of thioflavine T fluorescence intensity *versus* concentration at a constant activity of AChE **6**.[72] One can see that the fluorescence intensity of thioflavine T grows as the concentration increases up to 10 μmol litre^{-1}. Further increase in the concentration does not lead to increase in the fluorescence intensity; this is due to saturation of the binding sites of allosteric inhibitors on the PAS of AChE **6**.

§ Compounds classified as molecular rotors are able to execute unidirectional rotation upon energy supply.

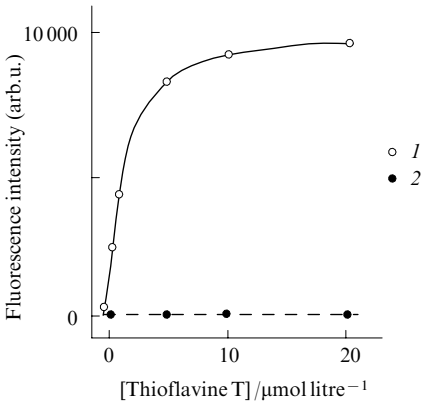


Figure 9. Fluorescence intensity *vs.* concentration for thioflavine T at as constant concentration of AChE **6** (0.87 μmol litre^{-1}).[72]
(*1*) Fluorescence intensity of thioflavine T in the presence of AChE, (*2*) fluorescence intensity of thioflavine T.

A similar growth of the thioflavine T fluorescence intensity in the presence of AChE **2** and squid ganglia propionylcholinesterase was observed by other researchers.[74–77]

The addition of (3-hydroxyphenyl)dimethylethylammonium chloride (edrophonium chloride, Ed) to the AChE **6** complex with thioflavine T induces a decrease in the fluorescence intensity of the complex. Edrophonium was found to selectively bind to the enzyme catalytic site to form a ternary complex.[72] Figure 10 shows the dependence of the fluorescence intensity of the AChE **6** complex with thioflavine T on the edrophonium chloride concentration.[56] A similar decrease in the fluorescence intensity of the complex of squid ganglia propionylcholinesterase with thioflavine T occurs also in the presence of paraoxone.[77]

A decrease in the fluorescence intensity of the complexes of AChE **6** and propionylcholinesterase with thioflavine T

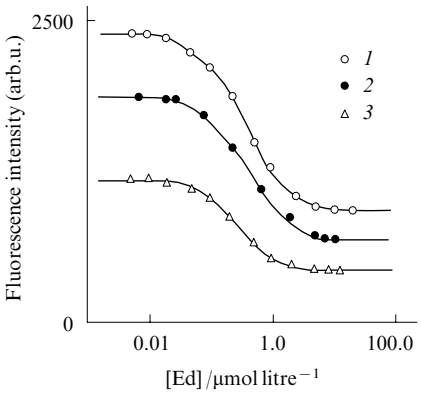


Figure 10. Fluorescence intensity of AChE **6** complex with thioflavine T *vs.* concentration of edrophonium chloride.[72]
Thioflavine T concentration/ μmol litre^{-1}: 10 (*1*), 3 (*2*) and 1 (*3*).

in the presence of Ed or paraoxone is probably due to an increase in the torsion mobility of the bound thioflavine T molecule, which can be attributed to a change in the local conformation of the PAS.[72, 77]

A change in the local conformation of the enzyme PAS also accounts for the results obtained in a study of interaction of thioflavine T with AChE **6** inhibited by organophosphorus insecticides, diethyl 3,5,6-trichloropyridin-2-yl phosphate and dichlorvos. For example, the fluorescence intensity of the thioflavine T complexes with AChE **6** inhibited by each of the above insecticides (in a 50 nmol litre^{-1} concentration) decreases by more than 50% relative to the fluorescence intensity of the thioflavine T complex with uninhibited AChE **6**.[78] This is caused by the fact that phosphorylation of AChE **6** changes the thioflavine T affinity to the PAS and changes K_d: the introduction of $(EtO)_2P(O)$ groups into Ser203 is accompanied by increase in the K_d value, while $(MeO)_2P(O)$ groups decrease K_d. These results provide the conclusion that these two inhibitors are not linked to the PAS, their observed effect on the thioflavine T affinity to the PAS being attributable to phosphorylation of the Ser203 residue.[78]

An increase in the phosphorylation rate of Ser of the catalytic triad in the presence of activator caused by conformational change of the AChE **5** catalytic site upon activator binding to the PAS of the enzyme,[56] which was proved by X-ray diffraction analysis of the thioflavine T complex with AChE **3**.[79]

Figure 11 presents the electron density maps of the thioflavine T complex with AChE **3** (Fig. 11 *a*) and the

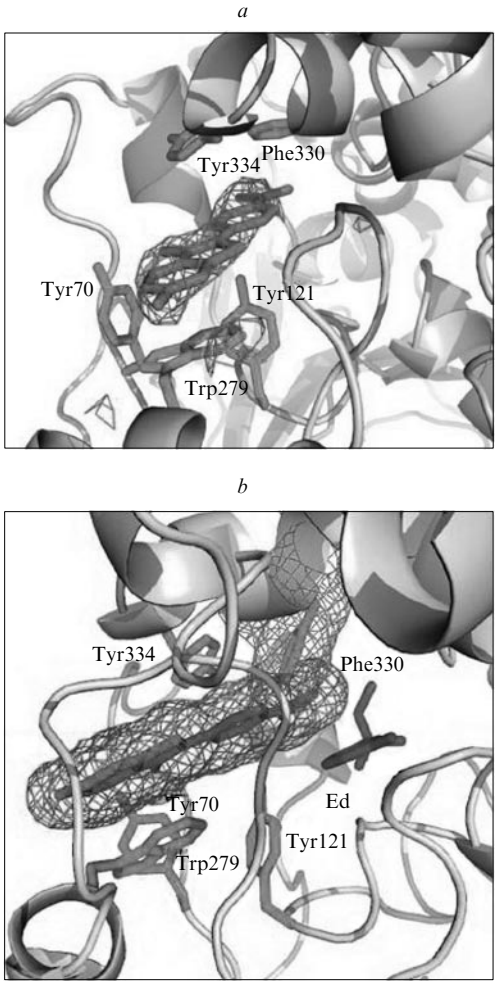


Figure 11. Electron density maps for thioflavine T complex with AChE **3** (*a*) and ternary complex thioflavine T – edrophonium chloride – AChE **3** (*b*).[79]

thioflavine T – edrophonium chloride – AChE **3** ternary complex (Fig. 11 *b*).[79]

It follows from Fig. 11 *a* that the thioflavine T molecule is planar. It is located within the PAS and participates in the stacking interactions with the aromatic substituents of the active site amino acid residues. The configuration of the amino acid residues of the polypeptide chain in the active site of the thioflavine T – AChE **3** complex corresponds to their configuration in free AChE **3**. The thioflavine T molecule occupies space within the active site, which has been occupied previously by four water molecules. The benzothiazole ring of thioflavine T is located opposite to the Trp279 residue, while the dimethylaminophenyl ring is nearly parallel to the phenyl group of Phe330 being spaced from it by a 3.5 Å distance. The dimethylaminophenyl ring of thioflavine T also interacts with the Tyr121 residue spaced by a 3.2 Å distance, and the benzothiazole ring interacts with Tyr70 and Tyr334, which are spaced from it by 3.4 and 3.7 Å distances, respectively. The planarity of thioflavine T is responsible for so high fluorescence intensity of its complex with AChE **3**.

Figure 11 *b* presents an electron density map for the ternary complex thioflavine T – edrophonium chloride – AChE **3**. If the conformation of the polypeptide chain region of the enzyme incorporating the Phe330 residue is similar in the thioflavine T – AChE **3** complex to this conformation of this region in native AChE **3** and the Phe330 substituent is located parallel to the dimethylaminophenyl group of thioflavine T, then the benzene ring in Phe330 in the thioflavine T – edrophonium chloride – AChE **3** ternary complex is rotated through approximately 115° from its position in the native enzyme. It can be seen from Fig. 11 *b* that if the Phe330 residue in the ternary complex had the same conformation as in the free enzyme, this would bring about collision of the Phe330 benzene ring with the nearest thioflavine T ring provided that the latter occupies the same position as in the thioflavine T – AChE **3** complex. Therefore, the Phe330 benzene ring in the ternary complex is rotated. This disturbs the planarity of the aromatic rings in thioflavine T within the ternary complex and results in partial quenching of thioflavine T fluorescence. Hence, the key role in the fluorescence quenching of the thioflavine T – AChE **3** complex in the presence of edrophonium chloride binding to the catalytic site of the enzyme belongs to the Phe330 residue involved in the interaction with thioflavine T.[79]

X-Ray diffraction studies[79] conform the hypothesis of conformational interaction between AChE PAS and catalytic site in the presence of allosteric PAS inhibitor.

* * *

The knowledge of the effect of cholinergic receptor blockers, *i.e.*, acetylcholine antagonists, on the rate of phosphorylation of AChE by irreversible inhibitors is of conceptual and applied value. The conceptual value is related to specification of the AChE active site structure, elucidation of the conformational interaction between the peripheral and catalytic sites of the AChE active site and the mechanism of the effect of activators of phosphorylation of the Ser residue of the catalytic triad on the selectivity and rate of AChE interaction with inhibitors. The applied value is related to the prospects for development of more sensitive methods and technical means for environmental monitoring of organophosphorus toxicants.

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