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Stereochemical Aspects of Cholinesterase Catalysis

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Proceeding from the stereochemical regularities of the nucleophilic substitution reaction at the carbonyl group and the assumption that the spatial structure of the active center of cholinesterases is complementary to the molecule of the ester substrates for these enzymes, some general features of the stereoselectivity phenomena in the reactions of acetylcholinesterase (EC 3.1.1.7) and butyrylcholinesterase (EC 3.1.1.8) with organophosphorus inhibitors are discussed. For these enzymes the models of the active center are proposed in terms of different binding sites and the catalytic center. On the basis of this model, the stereochemical pecularities and the physicochemical background of the stereoselectivity effects on enzyme inhibition, reactivation, and "aging" reactions can be understood. Knowledge of the absolute configuration of several chiral organophosphorus inhibitors also makes it possible to determine the absolute spatial arrangement of the hydrophobic binding sites on the active surface of cholinesterases. © 1984 Academic Press, Inc.

INTRODUCTION

The reaction of cholinesterases with ester substrates YCOOX and organophosphorus compounds of the general formula YZP(O)X can be described formally by a common three-step reaction scheme (*1–4*);

$$E + S \overset{K}{\rightleftharpoons} ES, \qquad [1]$$

$$ES \xrightarrow{k_2} EA + P_1, \qquad [2]$$

and

$$EA \xrightarrow{k_3} E + P_2, \qquad [3]$$

where ES is the noncovalent enzyme–ligand complex, and EA denotes the covalent intermediate. The bond-breaking steps [2] and [3] involve the nucleophilic displacement reactions at the carbonyl or phosphoryl groups, respectively. However, in spite of such an essentially similar feature of these reactions, the spontaneous decomposition of the covalent intermediate EA into the free enzyme (Reaction [3]) is very slow in the case of organophosphorus compounds (*1, 4*). On the basis of this fact, the latter reagents are ordinarily treated as "irreversible" inhibitors of cholinesterases. The remarkable difference in reactivity of the phosphoryl or phosphonyl groups in these successive catalytic steps [2] and [3] cannot be explained by a change in the reactivity (electrophilicity) of the phosphorus atom.

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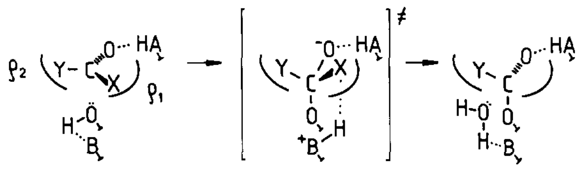
Most likely the slowness of the spontaneous recovery of the enzyme activity in the case of organophosphorus compounds is connected with some stereochemical requirements of the nucleophilic displacement reaction at the phosphorus atom, which differ from these of the carbonyl compounds (*5*). Proceedings from this idea, some aspects of the stereochemical background of acetylcholinesterase specificity in reaction with organophosphorus inhibitors have been discussed previously (*6*). In the present essay this approach is extended for butyrylcholinesterase, and a brief comparative review is given of the stereoselectivity of both types of cholinesterases in their reactions with chiral organophosphorus inhibitors in the binding and bond-breaking steps, as well as in the "aging" reaction of the inhibited enzyme. The latter reaction proceeds well in the case of phosphorylated or phosphonylated cholinesterases, and probably implies release of an alkoxy group from the inhibited enzyme (*7, 8*);

$$\mathrm{EA} \xrightarrow{k_a} \mathrm{EA}' + \mathrm{P}_3. \qquad [4]$$

The "dealkylated" enzyme–inhibitor intermediate EA′ cannot be further reactivated.

MODEL OF THE ACTIVE CENTER

It has been accepted in the following analysis that the active center of cholinesterases is complementary to the substrate molecule and, in the noncovalent binding step, the most favorable mutual localization of the reacting groups of the enzyme active center and the substrate molecule is achieved for the following reaction step. Proceeding from those general assumptions, the active center can be conventionally divided into two different binding sites accommodating the alcohol and acyl parts of the ester substrates YCOOX. These subsites will be denoted as ρ_1 and ρ_2 areas, respectively (see the schemes in Fig. 1). The "topography" of these sites has been thoroughly studied by means of quantitative structure–activity analysis, making use of the systematic reaction series of nonionic



I II III

FIG. 1. Schematic presentation of the active center of cholinesterases. The active center consists of two hydrophobic regions, ρ_1 and ρ_2, for binding the alkyl and acyl parts of ester substrate YCOOX, the serine residue activated by a basic group B (probably imidasole), and an acidic group AH. I, Noncovalent enzyme–inhibitor complex; II, the tetrahedral transition state of the enzyme acylation step; III, acyl–enzyme with the bound water molecule for the deacylation reaction.

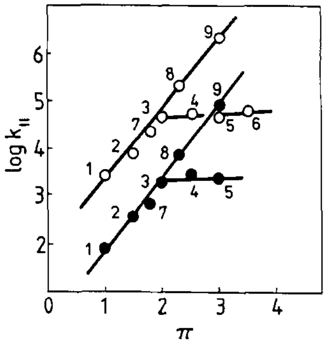


FIG. 2. Plot of log k_{II} vs π for the cholinesterase-catalyzed hydrolysis of alkyl acetates CH_3COOX, where X stands for C_2H_5 (**1**), C_3H_7 (**2**), C_4H_9 (**3**), C_5H_{11} (**4**), C_6H_{13} (**5**), C_7H_{15} (**6**), $CH_2CH(CH_3)_2$ (**7**), $CH_2CH_2CH(CH_3)_2$ (**8**), and $CH_2CH_2C(CH_3)_3$ (**9**). ○, Acetylcholinesterase; ●, butyrylcholinesterase; data taken from Refs. (*9*, *10*).

esters (*9*, *10*). This approach allows meaningful determination of all the specificity-determining factors governing the noncovalent and covalent steps of the enzyme-catalyzed hydrolysis. The complications arising from other approaches, like the "normalization" of the rate constants of the enzyme reaction for reactivity of these substrates in some nonenzymatic "model" reaction in order to characterize the binding effects in the enzyme catalysis, have been discussed elsewhere (*11*).

The binding properties of the ρ_I site can be characterized by using the second-order rate constants k_{II} of the enzyme-catalyzed hydrolysis of the acetic esters CH_3COOX, where X stands for normal and branched alkyl groups (*9*, *10*). This reaction series of substrates was specially designed in order to investigate hydrophobic effects. Therefore, the hydrophobicity of X was the only changing substituent parameter, and the log k_{II} vs π plots directly characterize the hydrophobic properties of the appropriate binding site. The hydrophobicity constants π were taken from Ref. (*12*) or calculated by the additive scheme, as only simple groups were involved in the analysis. The experimental data are summarized in Fig. 2. It can be seen that both types of cholinesterases reveal similar binding properties and can accommodate the normal or branched radicals if their length does not exceed that of the *n*-butyl group. Further increase in the length and hydrophobicity of X has no complementary effect, and most likely the remaining part of the substituents does not interact with the hydrophobic site of the enzyme molecule. At the same time the common linear relationship between log k_{II} and π involves several substrates with π values considerably greater than 2 on condition that the length of the substituent X does not exceed that of the *n*-butyl group. These data were taken as an indication of the limited size of the hydrophobic binding site ρ_I of both types of cholinesterases (*9*, *10*).

It should be noted that the same "topography" of the binding site for the leaving group can also be observed in the case of cationic substrates, which are derivatives of acetylcholine with a variable structure of the ammonium group. The kinetic data for two groups of esters, $CH_3COOC_2H_4N^+(CH_3)_nH_{3-n}$ (n = 1–3) and $CH_3COOC_2H_4N^+(CH_3)_2C_nC_{2n+1}$ (n = 1–5), were collected from different papers

(*13–15*), and were normalized in relation to acetylcholine. The steric and inductive effects do not change within the framework of these reaction series, and the similar localization of the cationic group in all the compounds should guarantee a similar location of the substrates relative to the "putative anionic site" of cholinesterases. Therefore, the $\Delta\log k_{\text{II}}$ vs $\Delta\pi$ plot for these "normalized" data should characterize the hydrophobic properties of the binding site. Figure 3 shows that a break occurs in this plot if the length of the substrate leaving group rises above four atoms, except the oxygen atom of the ester group. Thus, good agreement between the kinetic data for cationic and nonionic substrates can be seen. This points to a similar range of the corresponding hydrophobic sites. Moreover, the slopes of the initial linear part of the plots in Figs. 2 and 3 are also close. This linear part of the relationship can be described by a single-parameter equation (9);

$$\log k_{\text{II}} = \log k^{\circ}_{\text{II}} + \varphi\pi, \qquad [5]$$

where the π constant characterizes the hydrophobicity of the substituent, and φ is the intensity factor of the hydrophobic interaction. The validity of this equation means that the other reactivity-determining factors—the inductive effect, and the steric and resonance influences—remain negligible or have constant values for all the compounds included in the reaction series. The close φ values and the similar topography of the hydrophobic sites point to the conclusion that both the ionic and nonionic substrates interact with the same hydrophobic locus.

The binding properties of the ρ_2 locus have also been analyzed by means of the structure–activity relationships. But, in this case a meaningful and detailed analysis can only be made regarding butyrylcholinesterase (*16*), while a sharp decrease in the activity of the ester substrates with a variable structure of the acyl part in the acetylcholinesterase-catalyzed hydrolysis does not allow systematic kinetic analysis (*1*). Hence, the ρ_2 area of acetylcholinesterase is ordinarily considered to be limited in size, providing accommodation only for the acyl moiety (*17*).

The reaction series of *n*-butyl esters of various carboxylic acids, $YCOOC_4H_9$-*n*,

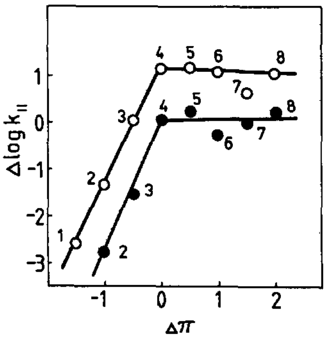


FIG. 3. Plot of Δ log k_{II} vs $\Delta\pi$ for the cholinesterase-catalyzed hydrolysis of acetylcholine (compound **4**) and its derivatives $CH_3COOC_2H_4N^+(CH_3)_nH_{3-n}$ (compounds **1–3**) and $CH_3COOC_2H_4N^+(CH_3)_2C_nH_{2n+1}$ ($n = 2 - 5$, compounds **5–8**). The kinetic constants k_{II} were normalized relative to the k_{II} value for butyrylcholinesterase reaction with acetylcholine. The hydrophobicity constants were normalized relative to the π constant for acetylcholine. ○, Acetylcholinesterase; ●, butyrylcholinesterase; data taken from Refs. (*13–15*).

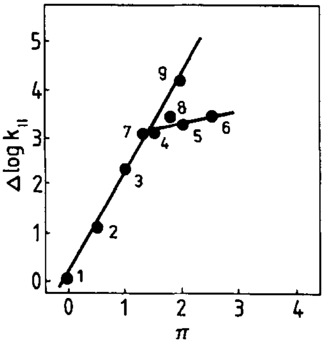


FIG. 4. Illustration of the hydrophobic properties of the binding site ρ_2 for the acyl part of esters $YCOOC_4H_9$ in their reaction with butyrylcholinesterase. The increments $\Delta \log k_{II}$ were calculated from Eq. [6]: $\Delta \log k_{II} = \log k_{II} - \log k_{II}^{\circ} - \delta^{\circ}E_s^{\circ} - \rho^*\sigma^*$. Substituents Y are H (1), CH_3 (2), C_2H_5 (3), C_3H_7 (4), C_4H_9 (5), C_5H_{11} (6), $CH(CH_3)_2$ (7), $CH_2CH(CH_3)_2$ (8), and $C(CH_3)_3$ (9); data taken from Ref. (*16*).

was used to survey the binding properties of the ρ_2 site of the butyrylcholinesterase active center (*16*). In this case, however, no simple relationship between $\log k_{II}$ and the π constants can be obtained, as the steric and inductive effects should be quantified for all the substrates involved in this analysis. This has been made by means of the following correlation equation;

$$\log k_{II} = \log k_{II}^{\circ} + \varphi\pi + \delta^{\circ}E_s^{\circ} + \rho^*\sigma^*. \qquad [6]$$

This equation is valid for all substrates, the acyl part of which can be accommodated in the hydrophobic binding site of the ρ_2 area. Furthermore, this equation was used to calculate the contributions of the hydrophobic influence, $\Delta \log k_{II} = \log k_{II}^{\circ} - \delta^{\circ}E_s^{\circ} - \rho^*\sigma^*$, with the aim to illustrate the topography of the ρ_2 site shown in Fig. 4. In the case of butyrylcholinesterase the hydrophobic properties of the ρ_2 and the ρ_1 sites are quite similar and can be characterized by the φ values 1.9 ± 0.3 (*16*) and 1.6 ± 0.1 (*10*), respectively. At the same time these sites reveal different lengths, and the ρ_2 area can accommodate the radicals Y, which are not longer than the *n*-propyl group. This means that the best fit between the substrate molecule and the active center can be obtained in the case of butyric esters. This obvious conclusion follows from even a simple comparison of the experimental data for butyrylcholinesterase substrates without any sophisticated analysis. However, the results given above clearly show that the real hydrophobic properties of the ρ_2 site are covered up by the steric effect as the intensity factor $\delta^{\circ} = 1.5 \pm 0.2$, calculated from Eq. [6] (*16*), is remarkably high for a nucleophilic displacement reaction at the carbonyl group, as follows from the extensive survey of the corresponding experimental data collected (*18*).

It should be noted that the steric effect must also be accounted for in discussing the substrate specificity of acetylcholinesterases. If the ρ_2 area is not hydrophobic in the case of this enzyme, the steric effect of the acyl part of the substrates is not compensated for and the activity of the butyric and propionic esters is weak in the acetylcholinesterase-catalyzed reactions. Some experimental data discussed below seem to support this idea.

In summary, the experimental data given in Figs. 2–4 clearly show that definite hydrophobic binding sites can be identified on the active surface of cholinesterases. The mutual spatial localization of these sites can be schematized proceeding from the stereochemical requirements of the enzyme acylation step. The most probably mechanism of this reaction involves an imidazole group activating the hydroxyl group of the serine residue located in the esteratic site of cholinesterases (*19*). This activated OH group initiates the hydrolytic mechanism by nucleophilic attack on the carbonyl group of the ester molecule. The preferable side direction of this nucleophilic attack on the ester group (*20*) allows the determination of the mutual spatial positioning of the binding sites ρ_1 and ρ_2 with respect to the serine residue, as shown schematically in Fig. 5. Besides the serine OH group and the imadazole residue B, an acidic functional group of pK_a approx 9.5 is revealed in the cholinesterase-catalyzed reactions (*19, 21*). In Fig. 1 this acidic group is denoted as AH. Although there is no generally recognized role known for this acidic group, it can be assumed that this residue is involved in H-bond formation with the carbonyl atom of the ester group of the substrate molecule. This H-bond seems to be important, providing electrophilic assistance on the catalytic step and at the same time helping to align the substrate molecule for the acylation of the serine residue in the active center. This acylation reaction probably proceeds via tetrahedral intermediate formation analogically with the nonenzymatic ester hydrolysis reaction (*20*). This intermediate, having a tetrahedral carbon atom in the reaction center, should also be accommodated inside of the enzyme active center. Hence, the enzyme structure should possess remarkable flexibility to meet the alteration of the valence angles arising from the change in the central carbon atom hybridization.

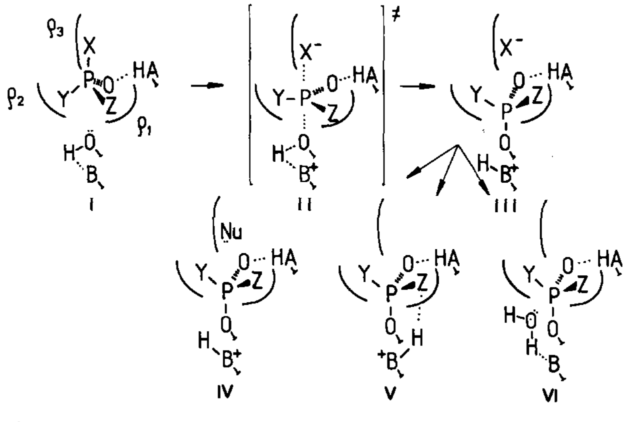


FIG. 5. Schematic representation of the active center of cholinesterases as revealed by the experiments with chiral organophosphorus inhibitors YZP(O)X. The additional hydrophobic binding site ρ_3 is necessary to accommodate the leaving group X of the inhibitor molecule. I, Noncovalent enzyme–inhibitor complex; II, the bipyramidal transition state if the enzyme phosphorylation or phosphonylation reaction; III, the covalent enzyme–inhibitor complex; IV, possible mechanism of the enzyme reactivation by an external nucleophile (Nu); V, the acid-catalyzed "aging" reaction resulting from the $S_N1(C)$ displacement at the group Z; VI, the base-catalyzed "aging" reaction occurring through the $S_N2(P)$ mechanism at the phosphorus atom, with participation of the water molecule bound in the active center of cholinesterases.

It should be noted that the mutual arrangement of the functional groups of the active center and the binding sites ρ_1 and ρ_2 is also consistent with the stereochemical requirements of the enzyme deacylation step. In this reaction the water molecule bound inside the active center can be activated by the same basic group B and, at the same time, it has the axial positioning with respect to the planar acyl moiety of the covalent intermediate EA required for the nucleophilic displacement reaction. According to our present knowledge, the active centers of acetylcholinesterase and butyrylcholinesterase seem to have similar chemical compositions, and thus the chemical mechanism of the covalent steps is assumed to be the same in the case of both enzymes.

REACTION WITH ORGANOPHOSPHORUS INHIBITORS

The nucleophilic displacement reaction at the tetrahedral phosphorus atom proceeds through an intermediate or transition state, in which the central phosphorus atom is coordinated with five groups (*5*). This complex probably adopts the trigonal bipyramidal conformation with axial positioning of the leaving and entering groups. Therefore, the attacking nucleophile should be positioned on the side opposite to the leaving group X of the organophosphate molecule YZP(O)X. To meet this requirement in the active center of cholinesterases the substituents Y and Z should be located in the ρ_1 and ρ_2 areas, as shown in Fig. 5, while the leaving group X of the inhibitor molecule should occupy a separate area, further denoted as the ρ_3 site. According to such positioning the oxygen atom of the phosphoryl group can form a hydrogen bond with the functional group AH.

It is obvious from Fig. 5 that the "catalytic machinery" of the active center can participate in the enzyme reaction with organophosphorus compounds. This point of view is supported by the similar pH dependence of the cholinesterase-catalyzed reactions with ester substrates (*19, 21*) and organophosphorus inhibitors (*22, 23*). But, these similar features of the reaction mechanism can only be found in the acylation and phosphorylation or phosphonylation steps. Figure 5 shows that the catalytic group B cannot contribute to the enzyme dephosphorylation reaction owing to the above-mentioned stereochemical requirements of the nucleophilic displacement at the phosphorus atom. Therefore, organophosphorus compounds can be named "quasisubstrates" to emphasize the fact that only partial analogy exists between the mechanisms of cholinesterase reactions with organophosphates and ester substrates. These differences in the reaction mechanisms will be discussed in the next part of this paper.

The binding properties of the ρ_3 site may be analyzed, proceeding from the structure–activity relationships between the second-order rate constants of cholinesterase inhibition by *O,O*-diethyl-alkylthiophosphates $(C_2H_5O)_2P(O)SC_nH_{2n+1}$ (*24, 25*). Figure 6 reveals that the variable alkyl radicals of these inhibitors are located in hydrophobic sites. As hydrophobic influence is the only variable structural factor, the linear parts of the log k_i vs π plots in Fig. 6 can also be described by the same correlation equation as is used for ester substrates:

$$\log k_i = \log k_i^\circ + \varphi\pi. \qquad [7]$$

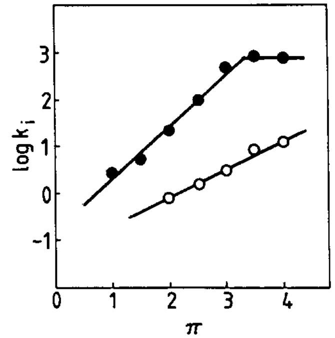


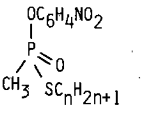
FIG. 6. Plot of log k_i vs π for acetylcholinesterase (○) and butyrylcholinesterase (●) inhibition with *O,O*-diethylalkylthiophosphates $(C_2H_5O)_2P(O)SC_nH_{2n+1}$, where *n* changes from 2 (π = 1) to 8 (π = 4). Data taken from Refs. (*24, 25*).

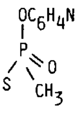
The plots in Fig. 6 show that the ρ_3 areas accommodate long alkyl radicals and thus differ essentially from the ρ_1 and ρ_2 sites. Moreover, the intensity factors of the hydrophobic effect of ρ_3 are equal to 0.6 ± 0.1 (*24*) and 1.0 ± 0.1 (*25*) in the cases of acetylcholinesterase and butyrylcholinesterase, respectively, and thereby point to considerably weaker hydrophobic properties of these sites in comparison with those of the ρ_1 and ρ_2 areas.

It should be noted that a similar "topography" of the ρ_3 sites can be found when analyzing the log k_i vs π plots for the inhibitors $(C_2H_5O)(CH_3)P(O)SC_nH_{2n+1}$. The rate constants for the latter compounds can be obtained from the extensive study reviewed in Ref. (*26*).

All the data discussed point to the fact that the leaving parts of ester substrates and organophosphorus inhibitors are located in different hydrophobic areas, supporting the ideas on the basis of which the model of the active center has been designed.

The binding sites for the radicals Y and Z of the inhibitors YZP(O)X can be investigated separately by means of the stereoisomers of the chiral organophosphorus compounds with variable alkoxy or alkyl groups Y and Z. The log k_i vs π plots for both the L and D series of the chiral inhibitors (*27*)





are shown in Fig. 7. In these compounds the *p*-nitrophenyl group is the leaving part in the reaction with cholinesterases. In the case of butyrylcholinesterase the variable alkyl group of both L and D isomers is bound in hydrophobic sites which can accommodate *n*-butyl and *n*-propyl radicals, respectively. Consequently, these sites are the ρ_1 and ρ_2 loci, as is implied by the schemes shown in Fig. 5. Likewise, it can be seen from Fig. 7 that the alkyl radicals of the L inhibitors are also located in the ρ_1 locus of acetylcholinesterase. This means that the active centers of both types of cholinesterases have similar spatial arrangement of the hydrophobic binding sites. This allows us to assume that, in the case of acetyl-

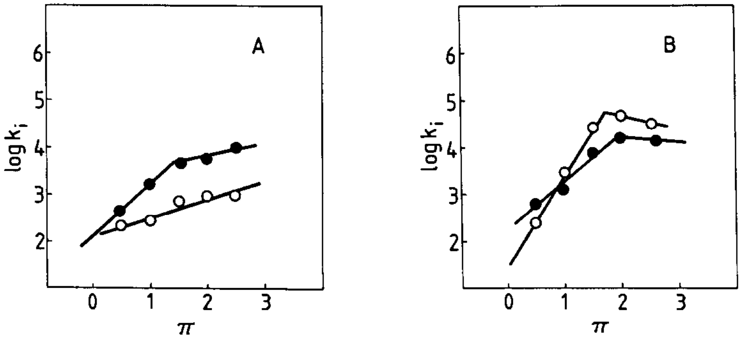
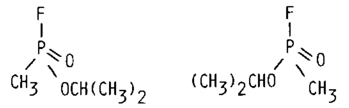


FIG. 7. Plot of log k_i vs π for acetylcholinesterase (○) and butyrylcholinesterase (●) inhibition with the dextrorotatory (A) and levorotatory (B) forms of inhibitors $(C_nH_{2n+1}S)(CH_3)P(O)OC_6H_4NO_2$-*p*, where $n = 1 - 5$. Data taken from Ref. (*27*).

cholinesterase, the structure–activity relationship for the D isomers describes the binding properties of the ρ_2 site. It is evident from Fig. 7 that nothing hinders the binding of quite long and bulky alkyl radicals in this site. Moreover, this site even reveals weak hydrophobicity. Thus, the outstanding specificity of acetylcholinesterase toward acetic esters can be well explained by the steric effect as proposed above. This means that the negative value of the steric effect $\delta^\circ E_s^\circ$ in Eq. [6] is not compensated for by the hydrophobic interaction between the acyl part of substrates and the ρ_2 site.

Establishment of the binding properties of the ρ_1 and ρ_2 sites is important for understanding the structural background of the stereoselectivity effects in the reaction of cholinesterases with organophosphorus inhibitors. As the ρ_1 and ρ_2 sites are both hydrophobic and have rather similar binding properties in the case of butyrylcholinesterase, this enzyme cannot sensitively differentiate the shape of groups Y and Z of the inhibitors, and thus reveals only moderate stereoselectivity in reactions with chiral organophosphorus compounds (*27–31*). However, the remarkably different binding properties of the ρ_1 and ρ_2 sites of acetylcholinesterase give reason for expecting sensitive recognition of the chiral organophosphates by this enzyme. Therefore, in case groups Y and Z are of sufficiently different bulkyness, acetylcholinesterase will react preferentially with one of the enantiomers (*27–31*). If the absolute configuration of the enantiomeric inhibitor is known, the spatial structure of the enzyme active center can be established in terms of the three binding sites ρ_1, ρ_2, and ρ_3.

The enantiomeric forms have been separated, and the absolute configuration of the substituents around the central phosphorus atom has been established, for instance, in the case of isopropyl-methylphosphonofluoridate (*31*):



S-(P) *R*-(P) [9]

The L enantiomer of this compound has the *S* configuration and reacts with acetylcholinesterase more than 4200 times faster than the D enantiomer having the *R* configuration. The difference between the activity of these enantiomeric molecules is easy to explain by the different binding properties of the ρ_1 and ρ_2 sites. In the case of the levorotatory sample the hydrophobic isopropyl radical evidently interacts with the hydrophobic binding site in the ρ_1 locus, as this bulky radical of the dextrorotatory *R* enantiomer is located in the ρ_2 area, providing only weak, if any, hydrophobic stabilization of the enzyme–inhibitor complex. Proceeding from those experimental data, the mutual localization of the ρ_1 and ρ_2 sites was established and used in drawing up the schemes in Figs. 1 and 5. The same model can be applied for butyrylcholinesterase, as follows from the results and discussion above, although there is only a small difference in the k_i values for the inhibition of this enzyme by the enantiomeric forms of the inhibitor [9] (*31*).

It should be noted that the preferential inhibition of acetylcholinesterase by one of the enantiomeric forms of the chiral compound YZP(O)X is valid for a wide range of leaving groups X, although these groups vary greatly in their chemical structure and in their interactions with the enzyme active center (*31*). Therefore, acetylcholinesterase can be proposed as a tool for determining the absolute configuration of the enantiomeric organophosphorus compounds. Of course, this "reversible" analysis is valid only if the compound has an asymmetric phosphorus atom, and not in such cases where the chirality center is located on one of its substituents.

It has been shown above that the stereoselective recognition of the enantiomeric forms of organophosphorus inhibitors first occurs at the noncovalent binding step, which is governed by hydrophobic interaction between the ligand molecule and the binding sites of the enzyme active center. However, the hydrophobic interaction has also been shown to play an important role at the bond-breaking steps of the cholinesterase-catalyzed reactions (*9, 10, 16, 25*). That means that additional stabilization of the transition state of the cholinesterase-catalyzed reactions is possible. For instance, the rate constants of the enzyme acylation step show a clear dependence upon hydrophobicity of the substituents in the substrate molecules (*9, 10, 16*). That phenomenon increases approx twofold the contribution to the hydrophobic influence on the overall catalytic process characterized by the second-order rate constants k_{II} (or k_i). On the level of these constants, this "double effect" of the hydrophobic interaction results in the extra high values of the intensity factors φ in the corresponding correlation equations (see Refs. (*9, 10, 16, 25, 32*)). Although the physicochemical implications of this phenomenon are not clear (*25, 32*), it has been shown that this "double effect" of hydrophobicity is only characteristic for certain binding sites (*16*). For instance, this phenomenon is revealed in the case of the ρ_1 and ρ_2 sites of butyrylcholinesterase (*10, 16*) and the ρ_1 site of acetylcholinesterase (*9*). On the other hand, the ρ_3 sites of both enzymes (*25*) and very probably also the ρ_2 site of acetylcholinesterase do not possess the ability to stabilize the activated complex of the catalytic step. Therefore, the stereoselectivity connected with the asymmetric properties of the hydrophobic binding sites can also be expected to reveal itself at the covalent step of the enzyme inhibition reaction. Most likely these effects on the binding and catalytic stages are similar and, consequently, the twofold amplification of the enzyme stereoselectivity should be observed in the case of the second-order rate constants of the inhibition reaction. Unfortunately, no systematic data can be found to check this hypothesis, but comparison of the k_2 and K values for a few pairs of enantiomeric inhibitors seems to agree with this conclusion (*33*).

Returning to the mechanism of the enzyme phosphorylation or phosphonylation reactions, it should be emphasized that there exists no facilitated proton transfer to the leaving group of the organophosphorus inhibitor. Therefore, the anion is released as the primary product of the nucleophilic displacement reaction. This conclusion agrees well with its extra-high sensitivity to the inductive influence of the leaving groups, as shown for both types of cholinesterases in their reaction with *O,O*-diethylthiophosphates (*24, 25*). The intensity factor of the inductive effect, denoted as the ρ^* constant in the following correlation equation,

$$\log k_i = \log k_i^\circ + \varphi\pi + \rho^*\sigma^*, \qquad [10]$$

exceeds by more than two times the ρ^* for the alkaline hydrolysis of these organophosphates (*39*). Similar conclusions as to the extra-high sensitivity of the enzyme phosphorylation or phosphonylation reactions to the inductive effect of the inhibitor leaving group can be made on the basis of other experimental data (*35, 36*), which also allow the estimation of the ρ^* or ρ° constants, making use of the corresponding LFE relationships. All these data point to an extensive cleavage of the covalent bond between the leaving group and the phosphorus atom without any compensation for the polarity of the transition state of this reaction by a proton-donating group of the protein molecule.

REACTIVATION AND "AGING" OF CHOLINESTERASES

The covalent intermediate EA formed in the reaction between cholinesterases and organophosphorus inhibitors can be further hydrolyzed to yield the free enzyme (*1*). In this spontaneous reaction of reactivation the enzyme molecule is the leaving group at the phosphorus atom. According to the stereochemical requirements of this reaction, the attacking water molecule should be located on the side opposite to the enzyme–phosphorus bond. This situation is schematized in Fig. 5 where the entering nucleophile is denoted as Nu. It follows from these schemes that, owing to the external localization of the attacking nucleophile, the reactivation process cannot be assisted by the functional groups of the esteratic site, which are responsible for the activation of the nucleophilic component of the active center on the acylation and deacylation steps. The same nucleophilic residue (serine OH group) and the assisting groups are probably involved in the enzyme reaction with organophosphates (Fig. 5). Therefore, the slowness of the spontaneous recovery of the inhibited enzyme can simply be explained by the stereochemical requirements of the mechanism of the nucleophilic displacement reaction at the tetrahedral phosphorus atom.

The proposed external localization of the nucleophilic reagent Nu agrees with the well-known fact that the activity of the inhibited cholinesterases can be restored by different nucleophilic compounds added into the reaction medium. This possibility has been used to design effective antidotes against organophosphorus inhibitors (*1, 4*). Many of these compounds are related to hydroxylamine (oximes, hydroxamic acids, etc.), and they also react easily with other simple organophosphorus compounds, for instance, with tetraethyl pyrophosphate, showing rates about the same as in the case of inhibited cholinesterases (*37–39*). Such a comparison shows that the enzyme should be a reasonably good leaving group in the nucleophilic displacement reaction. The $S_N2(P)$ mechanism of the reactivation of acetylcholinesterase is also supported by the straight line obtained between the log k_r and σ° values for the congeneric series of *O*-arylmethylphosphonylated acetylcholinesterase, yielding $\rho^\circ = 1.56$ (*40*). This value is comparable with $\rho^\circ = 1.0$ for the spontaneous hydrolysis of the corresponding phosphonochloridates in water (*40*).

It should be emphasized, however, that the reactivation process cannot be entirely treated as a common nucleophilic displacement reaction. First, the effectiveness of the external reactivators can be further increased by their noncovalent binding to the active center of cholinesterases. For this reason the most powerful reactivators contain, besides their nucleophilic moiety, some hydrophobic radicals and an ammonium group to facilitate their interaction with the enzyme molecule (*1, 4*). In principle, the hydrophobic site ρ_3 seems to be a suitable binding place for the reactivator molecule, as this area is free due to the absence of the inhibitor's leaving group in the covalent intermediate EA. Moreover, in this position there can be no stereochemical complications for the following nucleophilic displacement reaction leading to the cleavage of the phosphorus–enzyme covalent bond and the recovery of the free enzyme.

Second, the reactivation process obeys a bell-shaped pH dependence, revealing a basic group of pK_a 6.2–7.2 and an acidic group of pK_a 9.0–9.8 (*40–44*). As such dissociation equilibria can also be seen in the case of spontaneous reactivation, the corresponding functional groups should belong to the enzyme molecule. It is noteworthy that similar ionogenic groups appear in other reactions of cholinesterases with the participation of the "catalytic machinery" of the active center.

It can be seen from Figs. 1 and 5 that the acidic group AH, which is involved in the enzyme-catalyzed hydrolysis of esters as well as in the enzyme inhibition step, can well participate in the reactivation reaction. The inability of the basic group B to directly assist the reactivation process has been shown above. But, in the case of butyrylcholinesterase, this basic group is also responsible for a conformation change in the active center of this enzyme (*22*), yielding the form of the protein which is unable to bind any types of ligands, including such purely hydrophobic compounds as napthalene (*45*). Therefore, it can be assumed that the same conformational transition also affects the reactivation reaction, for instance, by hindering the admittance of the nucleophilic reagent to the phosphoryl or phosphonyl group. This explanation can be extended to acetylcholinesterase, although no experimental data are available about the pH dependence of the binding equilibrium of nonionic ligands in the case of this enzyme.

Third, the reactivation process is a stereoselective reaction, as follows from the kinetic data on the recovery of *O*-cyclohexylmethylphosphonylated butyrylcholinesterase (*29*). Although no systematic kinetic analysis of this phenomenon has been made, it can be assumed that the asymmetric binding properties of the ρ_1 and ρ_2 areas also govern the reactivation step. This may be possible owing to the hydrophobic effect on the rate constant k_3 of the reaction scheme [3], as has been established for the enzyme acylation step. Due to the sharp difference between the binding properties of the ρ_1 and ρ_2 sites it can be presumed that acetylcholinesterase reveals much greater stereoselectivity in this process. To sum up, it should be mentioned that all of the points referred to above are, to a great extent, hypothetical, and specially designed experiments are necessary to obtain more detailed information about these features of cholinesterases. Unfortunately, meaningful kinetic analysis of the reactivation process is complicated by the "aging" reaction of the inhibited enzyme.

The "aging" reaction of the phosphorylated or phosphonylated cholinesterases can also be treated as the spontaneous hydrolysis of the enzyme–inhibitor covalent complex. But, in this reaction, one of the substituents Y or Z is the leaving group (*7, 8*). The dealkylated complex formed by this reaction cannot be further reactivated by water or even by other more potent nucleophilic reagents. This has been explained by the negative charge of the phosphate or phosphonate moiety formed (*46*) and by a conformational change in the active center of acetylcholinesterase resulting from the dealkylation reaction (*47*). The "aging" reaction seems to occur through two different mechanisms depending, first and foremost, on the chemical nature of the organophosphorus inhibitor. In the first case the dealkylation reaction depends on the degree of the protonation of a single acidic group of pK_a between 5 and 7 (*48, 49, 50, 55*). Therefore, the rate constant k_a of the reaction scheme [4] increases in acidic medium, pointing to the participation of an undissociated acidic group of the enzyme molecule in this reaction. The possible mechanism of this reaction involves the formation of a hydrogen bond with the oxygen atom of the leaving alkoxy group, facilitating its fission by formation of a carbonium ion (*48, 50*). In principle, it may also be assumed that the catalytically important acidic group is the imidazolium ion BH^+. This hypothetical case is shown in Fig. 5.

The second mechanism of the "aging" reaction seems to arise from the nucleophilic attack on the phosphorus atom, and occurs fast if the radical released from the inhibited enzyme is a good leaving group (*40, 41, 50, 51*). In the case of *O*-aryl-methylphosphonylated acetylcholinesterase, a good correlation between the log k_a and σ° constants support the idea of the displacement mechanism (*40*). It has been shown that this type of "aging" reaction is assisted by a basic and an acidic functional group, having pK_a's of 6.2–7.5 and 9.0–9.8, respectively (*40, 41, 52*). Therefore, the rate of this "aging" reaction shows the common bell-shaped pH dependence, as can be found in other cholinesterase-catalyzed reactions. This nucleophilic displacement mechanism is characteristic of *O*-aryl-substituted phosphonylacetylcholinesterase, and can also be assumed in the case of *O*-cyclohexylmethylphosphonylated enzyme (*48*); in that of butyrylcholinesterase this displacement mechanism seems to be even more common (*48*).

A striking resemblance can be seen in the stereoselectivity of acetylcholinesterase in the inhibition and "aging" reactions (*48, 53*). Both of these processes occur fast in the case of inhibitors of the *R* configuration of substituents around the phosphorus atom. Inhibitors of the *S*-(P) configuration inhibit the enzyme slowly, for the stereochemical reasons discussed above. But, the covalent intermediate slowly formed in this reaction shows no measurable "aging" at all (*53*). Thus, the stereochemical factors governing the "aging" reaction seem to be absolutely strict and probably point to some pecularities of the reaction mechanism. It is noteworthy that exactly the same conclusions can be drawn for butyrylcholinesterase, although this enzyme shows much weaker or even practically no stereoselectivity in the inhibition reaction (*53*). On the strength of the stereoselectivity data on the acetylcholinesterase inhibition and the "aging" reaction in the case of several *O*-alkyl-methylphosphonates (*53*), it can be concluded that the dealkylation occurs in the ρ_1 area.

In accordance with the first mechanism of the "aging" reaction, the displacement occurs virtually at the carbon atom of the alkoxy group (*48, 54, 55*). As formation of the carbonium ion is assumed in this reaction, the direction of the nucleophilic attack on the α-carbon atom cannot be discussed as proceeding from the model of the enzyme active center built up on the basis of the stereochemical regularities of the nucleophilic displacement reaction at the phosphorus atom. However, the strict stereoselectivity of this reaction can be explained by the fixed localization of the acidic group participating in the dealkylation reaction. The scanty data available in the literature do not allow any more detailed discussion of this route of the "aging" process.

The second mechanism of the "aging" reaction assumes nucleophilic displacement at the phosphorus atom. Thus, the stereochemical viewpoints applied above can also be used to analyze the possible mechanism of this reaction. Localization of the leaving *O*-aryl or *O*-alkyl group in the ρ_1 site means that an external water molecule cannot be used in the "aging" reaction of the second type. But, this reaction can be initiated by the internal water molecule located inside the enzyme active center, and used to hydrolyze the acyl–enzyme in the case of cholinesterase-catalyzed hydrolysis of ester substrates. This deacylation step is figuratively illustrated by Fig. 1. This scheme shows that there is no steric hindrance to the use of this water molecule as the nucleophilic component in the second type of "aging" reaction. Both catalytic groups of the enzyme, B and AH, can assist in this reaction, showing the common bell-shaped pH dependence. Absolute stereoselectivity is also guaranteed by this reaction mechanism thanks to the fixed position of the water molecule in the enzyme active center.

THE ANIONIC SITE OF CHOLINESTERASES

Proceeding from the molecular structure of the choline esters $YCOOCH_2CH_2N^+(CH_3)_3$, the active center of cholinesterases has been assumed to involve the anionic binding site, which is responsible for interaction with the cationic group of these specific substrates (*19, 56, 57*). This anionic site is believed to be a carboxylic group of pK_a 4.0–4.5, found from the pH dependence of acetylcholinesterase inhibition by betaine (*58*), and pK_a 4.5–5.0, calculated from the pH dependence of butyrylcholinesterase reaction with cationic organophosphorus inhibitors (*59*) and cationic substrates (*21*). For the latter enzyme it has been shown that this ionogenic group of the active center is not revealed in the case of nonionic reagents, which supports the view that it belongs to the anionic binding site (*59*). Therefore, there is every reason to raise the question about the localization of the anionic site in the above-discussed model of the active center.

For some decades it has been generally accepted that, owing to the rigid complementarity of the substrate molecule and the active center of cholinesterases, the anionic point must be in the same area in which the leaving part of the ester substrate is located (*19, 56, 57*). According to the proposed model, this means that the anionic point cooperates with the ρ_1 site. This concept of an anionic charge fixed on the active surface of the enzyme active center has been found expedient in explaining several features of cholinesterase specificity. Moreover, proceeding from this concept and the idea that the ester substrates and organophosphorus inhibitors interact in a similar manner with the active center of cholinesterases, many organophosphorus compounds have been designed with cationic groups in their leaving part (*26, 60–65*). In spite of the fact that the leaving parts of substrates and organophosphorus inhibitors are actually located in different areas, many highly active inhibitors have been obtained on the basis of the formal analogy between the molecular structure of esters and organophosphorus compounds. Consequently, those data seem to contradict the classical view on the structure of the active center of cholinesterases containing a fixed anionic group in the vicinity of the binding site for the substrate leaving group.

Lately, a systematic investigation has been undertaken with the purpose of quantifying the effect of the anionic site in the cholinesterase-catalyzed reactions by means of structure–activity relationships (*10, 25, 66, 67*). In this approach all other specificity-determining factors have been taken into account by using correlation equations, as the electronic effects and hydrophobicity can also be changed by the introduction of the onium atom into the reagent molecule. Any additional effects arising from the appearance of the cationic group in the molecule of ligand have been defined as the effects of the anionic site. Due to this "anionic site effect," the experimental data on the cationic substrates and inhibitors should deviate from the correlation equations obtained for the subseries of the nonionic congeneric compounds. These deviations have been denoted by an extra term, Θ, introduced into the corresponding correlation equations. For instance, Eqs. [6] and [10] for the second-order rate constants can be modified to

$$\log k_{\text{II}} = \log k_{\text{II}}^{\circ} + \varphi\pi + \delta^{\circ}E_s^{\circ} + \rho^*\sigma^* + \Theta \qquad [11]$$

and

$$\log k_i = \log k_i^{\circ} + \varphi\pi + \rho^*\sigma^* + \Theta. \qquad [12]$$

As the regularities of the variation of the Θ values depending upon the structure of the ligand molecule cannot be predicted, the numerical values of Θ were calculated separately for each compound, making use of the intensity factors φ, δ°, and ρ^*, and the constants k° for the corresponding reaction series, and the substituent constants π, σ^*, and E_s° for the particular cationic groups

$$\Theta = \log k_{II} - \log k_{II}^{\circ} - \varphi\pi - \delta^{\circ}E_s^{\circ} - \rho^*\sigma^*, \qquad [13]$$

and

$$\Theta = \log k_i - \log k_i^{\circ} - \varphi\pi - \rho^*\sigma^*. \qquad [14]$$

It is clear that this approach used in characterizing the influence of the anionic site is independent of any presumptions about the mechanism of the "anionic site effect," and is not connected with any model of the active center of cholinesterases.

The reaction series of ester substrates and *O*,*O*-diethylthiophosphates with variably structured leaving parts that were used in this analysis are $CH_3COO(CH_2)_nN^+(CH_3)_3$, $n = 1 - 3$; $CH_3COO(CH_2)_nS^+(CH_3)C_2H_5$, $n = 1 - 4$; and $(C_2H_5O)_2P(O)S(CH_2)_nS^+(CH_3)C_2H_5$, $n = 1 - 6$. These reaction series have a polymethylene chain of variable length intervening between the cationic group and the reaction center. All these reaction series were specially designed with the hope of localizing the "anionic point" on the active surface of cholinesterases. The results obtained for these enzymes are summarized in the form of Θ vs *n* plots in Fig. 8. It can be seen that, in the case of all compounds at $n \geq 2$, Θ remains constant irrespective of the length of the polymethylene chain. The chemical nature of the onium atom also has no effect on the Θ values. But, the magnitude of parameter Θ is different for substrates and inhibitors and has different values for these reagents in the case of different cholinesterases.

It follows from Fig. 8 that the anionic site has a remarkable effect on the activity of the cationic compounds. In the case of ester substrates, the second-order rate constants are increased by this effect by more than nine orders of magnitude. Such contributions cannot be predicted by a simple comparison of the kinetic constants for structural analogs of cationic and nonionic compounds. In this connection, doubts have been expressed concerning the occurrence of the anionic site in the active center of cholinesterases (*28, 68, 69*).

It can be assumed that the elimination of the anionic site by its selective labeling

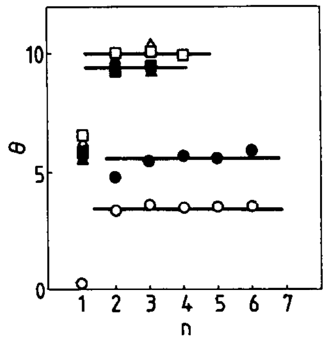


FIG. 8. Plot of Θ vs *n* for acetylcholinesterase (open symbols) and butyrylcholinesterase (closed symbols) reactions with esters $CH_3COO(CH_2)_nN^+(CH_3)_3$ ($n = 1 - 3$; △, ▲), $CH_3COO(CH_2)_nS^+(CH_3)C_2H_5$ ($n = 1 - 4$; □, ■), and inhibitors $(C_2H_5O)_2P(O)S(CH_2)_nS^+(CH_3)C_2H_5$ ($n = 1 - 6$; ○, ●). Data taken from Refs. (*10, 25, 65, 67*).

should lead to a decrease in the activity of the cationic reagents. The extent of the decrease in the rate constants is predicted by the Θ values. It is noteworthy that the second-order rate constants for acetylcholinesterase reaction with cationic inhibitor $(C_2H_5O)_2P(O)SC_2H_4N^+(CH_3)_2H$ decreases approx 1000-fold after treatment of the enzyme with aziridinium ions (*70*), which are the anionic-site-directed alkylating labels of cholinesterases. This result is in good agreement with the Θ value 3.5, calculated by means of Eq. [14] for a similar inhibitor $(C_2H_5O)_2P(O)SC_2H_4S^+(CH_3)C_2H_5$ in reaction with acetylcholinesterase (*66*). For all the other reaction series surveyed in Fig. 8 the predicted decrease in the rate constants is too large to be measured experimentally, and thus the conclusion that cholinesterases lose their hydrolytic activity with respect to cationic substrates after treatment with the aziridinium inhibitor agrees well with the results referred to in the literature (*70, 71*).

Among the regularities revealed by means of the quantitative structure–activity relationships, the most striking points seem to be the clear dependence of the θ values upon the hydrophobic properties of their respective binding sites on the one hand, and the permanence of the Θ values irrespective of the structure of the cationic group and the length of the polymethylene chain intervening between the onium atom and the reaction center on the other hand. All those results cannot be explained by the simple model which assumes the fixed positioning of the "anionic point" on the active surface of cholinesterases, and restricts the corresponding effect simply to the electrostatic attraction between the anionic and cationic groups of the enzyme and the reagent molecules. Moreover, the anionic site cannot even be located in one of the areas ρ_1 or ρ_3, as the "inverse" substrates with the trimethylammonium group in their acyl part, probably located in the ρ_2 area, were also effective in reaction with butyrylcholinesterase (*72*).

On the strength of all these facts, it has been suggested that the observed effect of the anionic site obtained from the extrathermodynamic analysis described above reflects the interplay of the solvation and ion-pair formation phenomena, while the electrostatic contribution accounts for only a minor part of the Θ value. The anionic group responsible for the formation of the crucial ion pair at the noncovalent binding step [1] should belong to the enzyme molecule, but it can be a part of a flexible polypeptide chain which allows its "diving" together with the cationic ligand into the hydrophobic "phase" of the enzyme active center irrespective of the structure of the ligand and the partial hydrophobic region. This situation is schematically illustrated in Fig. 9. A more detailed discussion of this hypothesis and some other problems connected with the putative role of the

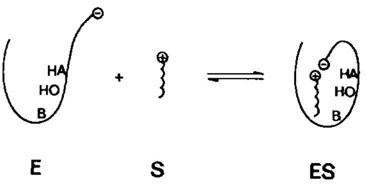


FIG. 9. Schematic illustration of the model of the "nonfixed anionic site" of cholinesterases. E, Free enzyme; ES, noncovalent enzyme–substrate complex.

anionic site in the cholinesterase-catalyzed reactions can be found elsewhere (*73*).

To sum up, it seems to be certain that the anionic site cannot be fixed at a single point of the active surface of cholinesterases, and thus cannot be shown in the model discussed above. Most probably completely new ideas on the structure of function of the anionic site are necessary to understand the physicochemical background of its influence on the cholinesterase-catalyzed reactions.

CONCLUSIONS

Although stereoselective molecular recognition in enzymatic catalysis is a very complex and still relatively poorly understood phenomenon, thorough analysis of the structure–activity relationships, together with a knowledge of the chemical mechanism of the bond-breaking steps, have allowed us to create a model of the active center of cholinesterases. For this purpose only the most typical features of the substrate specificity and stereoselectivity of acetylcholinesterase and butyrylcholinesterase were analyzed, without any attention being paid to many details characterizing the enzymes of particular tissues or organisms. Therefore, the proposed model characterizes only the most outstanding features of the substrate specificity of two different types of cholinesterases in terms of different binding sites and their mutual spatial localization. Proceeding from this model, the reactions of these enzymes with chiral organophosphorus inhibitors can also be specified. We hope that the considerations put forward may help to design further investigations into the mechanism of cholinesterase catalysis.

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