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Kinetic Analysis of Butyrylcholinesterase Inhibition with *N,N*-Dimethyl-2-Phenylaziridinium Ion

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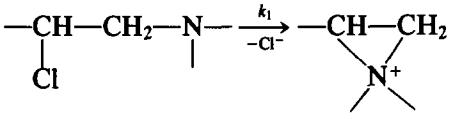
The kinetics of the butyrylcholinesterase interaction with *N,N*-dimethyl-2-phenylaziridinium ion, which probably alkylates the anionic site of the enzyme, was investigated at pH 7.5 and 25°C. It was found that the main product of the spontaneous hydrolysis of the aziridinium compound in water, *N,N*-dimethyl-2-hydroxy-2-phenylethylamine, binds effectively in the enzyme active center and protects it against alkylation. This binding equilibrium as well as the kinetics of the spontaneous decomposition of the aziridinium inhibitor were taken into consideration in calculating the rate and equilibrium constants for the enzyme alkylation reaction. The kinetics of the formation of the aziridinium compound and of the spontaneous hydrolysis reaction were investigated separately at pH 5.8–7.8, and the rate constants obtained from these experiments agree with the corresponding data calculated from the enzyme inhibition kinetics.

INTRODUCTION

In water 2-halogenoamines form aziridinium ions which are relatively stable and decompose at a moderate speed, yielding highly reactive carbonium ions. These carbonium ions react with available nucleophiles, and the main product in dilute water solution is the appropriate 2-hydroxyethylamine (*1*):



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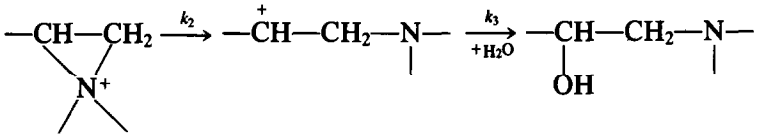

[2]

Like many other ammonium compounds, aziridinium ions can be bound reversibly by the active centers of cholinesterases. In this case the ensuing formation of the carbonium ion can lead to the alkylation of some nucleophilic group in the protein molecule. As ammonium compounds interact with the "anionic site" of cholinesterases (*2*), the enzyme alkylation probably occurs in the same locus (*3*).

The overall kinetics of the enzyme alkylation reaction is, however, complicated

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by several side reactions. In addition to the enzyme alkylation process [3], the spontaneous hydrolysis of the inhibitor [4] should be taken into consideration. The alcohol product of this latter reaction can also interact reversibly with the enzyme and thus influence the alkylation reaction. Altogether, this can be summarized by the following reaction scheme:

$$E + I \overset{K_i}{\rightleftharpoons} EI \overset{k_a}{\rightarrow} EI', \qquad [3]$$

$$I \overset{k}{\rightarrow} I^*, \qquad [4]$$

$$E + I^* \overset{K_i^*}{\rightleftharpoons} EI^*, \qquad [5]$$

where E is the free enzyme, I the alkylating agent, EI and EI' the reversible enzyme–aziridinium complex and the alkylated enzyme, I^* the alcohol product of the aziridinium hydrolysis, and EI^* the reversible complex of the latter compound with the enzyme.

In a previous attempt at the kinetic analysis of the butyrylcholinesterase reaction with *N,N*-dimethyl-2-phenylaziridinium and *N,N*-dimethyl-2-p-nitrophenylaziridinium ions, only the first reaction path [3] was taken into consideration (*4*), although even simple comparison of the available rate constants k for the spontaneous solvolysis reaction (*5, 6*) and the incubation times used in these experiments clearly show that at least half of the aziridinium compound is hydrolyzed during the kinetic runs. Also, in that earlier study (*4*) no attention was paid to the possibility of the inhibitory effect of the alcohol product of the spontaneous hydrolysis of the aziridinium compound on the alkylation reaction (see Eq. [5]).

In the present study the kinetics of the butyrylcholinesterase alkylation with the *N,N*-dimethyl-2-phenylaziridinium ion was analyzed proceeding from the complex reaction scheme [3]–[5]. Under the conditions where $[E]_0 \ll [I]_0$ the rate equation for this scheme can be integrated:

$$\ln \frac{[E]_0}{[E]_0 - [EI']} = \frac{k_a}{k(1 - K_i/K_i^*)} \ln \frac{K_i/[I]_0 + 1}{K_i/[I]_0 + K_i/K_i^* + (1 - K_i/K_i^*)e^{-kt}}, \qquad [6]$$

and the constants K_i, k_a, k, and K_i^* were determined by using the nonlinear least-squares regression method.

EXPERIMENTAL

Chemicals

Horse serum butyrylcholinesterase was purchased from the Mechnikov Institute of Sera and Vaccine, Moscow (5 μkat/mg protein). The stock solution of this enzyme was prepared in 0.15 *M* KCl or in phosphate buffer (pH 7.5, $I = 0.15$) and stored at +4°C. Under such conditions the enzyme solution can be kept several weeks without any change in activity. The operational normality of this solution was determined by the method of Berry (*7*) using *O,O*-diethyl-*p*-nitrophenyl phosphate as the active site titrant. The experimental procedure and the chemicals used for titration have been described earlier (*8*).

N,N-Dimethyl-2-chloro-2-phenylethylamine hydrochloride was prepared from $SOCl_2$ and *N,N*-dimethyl-2-hydroxy-2-phenylethylamine (*1*). The latter compound was in turn synthesized from styrene oxide (EGA Chemie) and dimethylamine in benzene (*9*). Instead of long refluxing at the boiling temperature the reaction was carried out under heating in closed ampoules at 120°C for 2 to 3 hr (95% yield, bp 117–118°C/12 mm; n_d^{20} 1.5204; d_4^{20} 1.0028. Lit. bp 136–140°C/18 mm (*1*)).

N,N-Dimethyl-2-chloro-2-phenylethylamine hydrochloride was recrystallized from the absolute ethanol–ether and the absolute methanol–ether mixtures to mp 204–205°C (decomp.) (66% yield, found: C, 54.43%; H, 7.00%; N, 6.22%. Calcd for $C_{10}H_{15}NCl$: C, 54.56%; H, 6.87%; N, 6.36%). Lit. mp 205–207°C (decomp.) from methanol–ethanol mixture (*1*) and 202–204°C (decomp.) from *i*-propanol–ether mixture (*10*). The stock solutions of $C_6H_5CHClCH_2N(CH_3)_2 \cdot HCl$ were prepared before use in absolute methanol.

N,N,N-Trimethyl-2-hydroxy-2-phenylethylammonium iodide was obtained from the appropriate alcohol and methyl iodide and recrystallized from ethanol–ether mixture (mp 220–223°C (decomp.), lit. mp 221–223°C (decomp.) (*1*)).

Acetylthiocholine iodide and acetylcholine iodide of analytical grade, 5,5′-dithiobis(2-nitrobenzoic acid) of chemical grade, and other chemicals of higher available purity were purchased from Reachim, USSR. The solvents used for synthesis and kinetic experiments were purified and dried as described in (*11*). Quarz-bidestilled water was used in all experiments.

Kinetics

The formation of *N,N*-dimethyl-2-phenylaziridinium was followed at different pH values on a Radiometer pH-stat (TTT/1, SBR/2, ABU/1) by titration of the acid liberated (0.15 *M* KCl, 25°C) or on a Perkin–Elmer 402 recording spectrophotometer by registrating the increase in the optical density at 269.5 nm (phosphate buffer, $I = 0.15$, 25°C). The reaction was followed up to 90–95% decomposition. The further solvolysis of the aziridinium ion was too slow to interfere with these kinetic runs. The concentration of methanol in the reaction mixtures was 1–1.5 v/v% for the pH-stat method and 0.1–0.2 v/v% for the spectrophotometric method.

The spontaneous hydrolysis of *N,N*-dimethyl-2-phenylaziridinium was followed spectrophotometrically by a decrease in the optical density at 269.5 nm in phosphate buffer of various pH containing 0.8 v/v% methanol. The reactions were monitored for 4 to 5 half-periods and the pH of the reaction mixture was measured before and after the hydrolysis and no change was found.

The initial solution of the aziridinium compound in these and all the following experiments was prepared through the hydrolysis of the 2-halogenoamine salt immediately before use in the reaction medium.

Reversible inhibition of the butyrylcholinesterase-catalyzed hydrolysis of acetylcholine was studied titrimetrically on the pH-stat (0.15 *M* KCl, pH 7.5, 25°C). The K_m and k_{cat} values were determined from the Linewevear–Burk plots at different inhibitor concentrations and the inhibition constants were calculated from the K_m^i/K_m and k_{cat}^i/k_{cat} versus $[I]$ plots. In the case of *N,N*-dimethyl-2-

phenylaziridinium the substrate and inhibitor were added simultaneously and practically no irreversible inhibition of the enzyme occurred during the time interval used for monitoring the initial rate of the substrate hydrolysis.

Irreversible inhibition (alkylation) of butyrylcholinesterase was performed in phosphate buffer containing 0.1–0.3 μM enzyme and 0.1–0.77 mM aziridinium inhibitor. At appropriate time intervals 10 µl of this reaction mixture was diluted into 3.0 ml of the enzyme assay buffer containing 2 mM acetylthiocholine and 0.3 mM 5,5′-dithiobis(2-nitrobenzoic acid). The residual activity of butyrylcholinesterase was measured spectrophotometrically as described by Ellmann *et al.* (*12*). The 300-fold dilution of the reaction mixture and the large excess of the substrate exclude reversible inhibition of the enzyme by the aziridinium ion or by some other amines in the assay buffer. Thus the observed rate of substrate hydrolysis is proportional to the concentration of the active enzyme in the reaction medium and

$$[E]_0/([E]_0 - [EI']) = v_0/v, \qquad [7]$$

where v_0 and v designate the initial rate of substrate hydrolysis and the rate at time interval t, respectively. For each kinetic curve 20–30 v_t values were measured for 10 to 11 hr.

Data Treatment

The first-order rate constants were calculated by the differential method of Rudakov (*13*) on an Iskra 1250 computer. For calculating the K_i values from the initial rates (Table 2) a linear least-squares regression program was used on the same computer.

The experimental data for the alkylation reaction were analyzed by a nonlinear least-squares regression program making use of the function.

$$Y_{ij} = X_0 \ln \frac{X_1/A_i + 1}{X_1/A_i + X_2 + (1 - X_2)X_3^{A_j}}, \qquad [8]$$

represented in coordinates Y_{ij} versus A_i and A_j. This function corresponds to Eq. [6] if

TABLE 1

RESULTS OF THE KINETIC ANALYSIS OF BUTYRYLCHOLINESTERASE INHIBITION WITH *N,N*-DIMETHYL-2-PHENYLAZIRIDINIUM[a]

$[I]_0$ (mM)	K_i (mM)	$10^4 k_a$ (sec^{-1})	K_i^* (mM)	$10^4 k$ (sec^{-1})
0.104	0.10 ± 0.04	1.5 ± 0.3	0.35 ± 0.28	0.893 ± 0.002
0.128	0.102 ± 0.002	2.1 ± 0.2	0.36 ± 0.06	1.000 ± 0.005
0.244	0.100 ± 0.003	2.1 ± 0.2	0.33 ± 0.07	1.000 ± 0.007
0.447	0.099 ± 0.003	2.0 ± 0.2	0.32 ± 0.06	0.980 ± 0.005
0.766	0.104 ± 0.002	2.1 ± 0.7	0.35 ± 0.03	1.365 ± 0.01
0.104 – 0.766	0.099 ± 0.003	2.01 ± 0.09	0.34 ± 0.03	1.02 ± 0.01

[a] pH 7.5, 25°C, phosphate buffer, $I = 0.15$.

$$Y_{ij} = \ln \frac{[E]_0}{[E]_0 - [EI']},$$

$$X_0 = \frac{k_a}{(1 - K_i/K_i^*)k}, \qquad X_1 = K_i, \qquad X_2 = \frac{K_i}{K_i^*}, \qquad X_3 = e^{-k},$$

and

$$A_i = [I]_0, \qquad A_j = t.$$

The best values for the constants X_0, X_1, X_2, and X_3 were obtained from the computer fit and were further used to calculate the constants K_i, k_a, and k and their standard errors (Table 1). A Nairi 3-2 computer was used for this nonlinear regression analysis.

RESULTS

Nonenzymatic Reactions

The kinetic data obtained for the formation and spontaneous hydrolysis reactions of *N,N*-dimethyl-2-phenylaziridinium over the pH interval 5.8–7.8 are given in Figs. 1 and 2. For the first reaction the data can be described by (Fig. 1)

$$\log k_1 = \log K_a + \log k_1^{\lim} - \log([H^+] + K_a), \qquad [9]$$

where K_a is the dissociation constant for the protonated amine (see Eq. [1]). The equation above corresponds to the reaction mechanism where only the free amine is reactive. The pK_a for *N,N*-dimethyl-2-phenyl-2-chloroethylamine estimated from these kinetic data is approx 7.7. At pH 7.5, $k_1 = (6.52 \pm 0.64)\ 10^{-2}\ sec^{-1}$. Thus preincubation of the present 2-halogenoamine in the reaction medium of pH 7.5 for 1.5 to 2 min is sufficient to attain complete formation of the aziridinium compound.

Spontaneous decomposition of the aziridinium ion is a relatively slow process

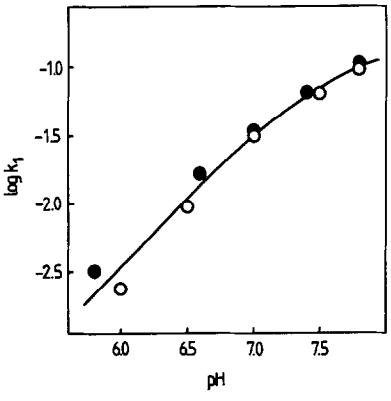

FIG. 1. Formation of *N,N*-dimethyl-2-phenylaziridinium from *N,N*-dimethyl-2-chloro-2-phenylethylamine, measured by the spectrophotometric (○) and pH-stat (●) methods.

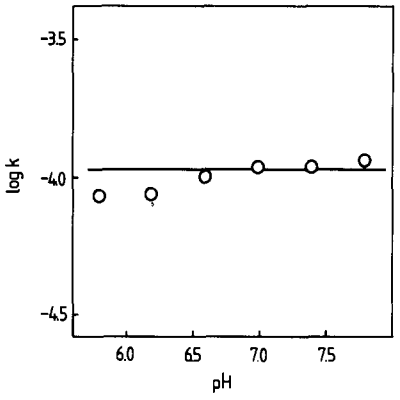

FIG. 2. Spontaneous hydrolysis of *N,N*-dimethyl-2-phenylaziridinium in phosphate buffer, $I = 0.15$, 25°C.

compared with its formation reaction. The apparent rate constant k of the hydrolysis reaction [4] is independent of the pH (Fig. 2), which agrees with the proposed $S_{N}1$ mechanism for this process (see Eq. [2]); although in the presence of very strong nucleophiles incursion of the $S_{N}2$ mechanism is also possible (*1*). At pH 7.5, $k = (1.02 \pm 0.09) \times 10^{-4}$ sec^{-1}, which agrees with earlier k values 1.23×10^{-4} sec^{-1} at pH 7.4 and 25°C (*5*), 1.27×10^{-4} sec^{-1} at pH 7.5 and 31°C (*1*) and $k = 7.5 \times 10^{-5}$ sec^{-1} calculated from the acetylcholinesterase inhibition data (*6*).

Butyrylcholinesterase Alkylation

Butyrylcholinesterase incubation with 0.1 to 0.77 m*M* solution of *N,N*-dimethyl-2-phenylaziridinium results in a progressive and irreversible decrease in the enzyme activity in reaction with acetylthiocholine (see Fig. 3). In spite of the large excess of the aziridinium ion, however, the inhibition was not complete and depends on the initial concentration of the inhibitor (Fig. 4). The maximal level of inhibition can be reached at $[I]_0 > 0.3$ m*M*.

In Fig. 3 it is shown that additional amounts of the aziridinium inhibitor lead to a

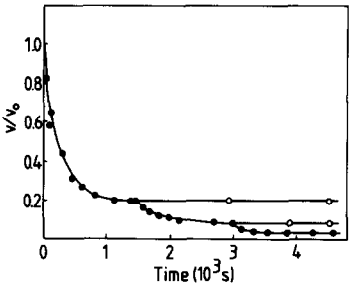

FIG. 3. Irreversible inhibition of the butyrylcholinesterase-catalyzed acetylthiocholine hydrolysis by *N,N*-dimethyl-2-phenylaziridinium ($[I]_0 = 0.15$ m*M*). Additional amounts of the inhibitor were added at time intervals of 250 and 500 min.

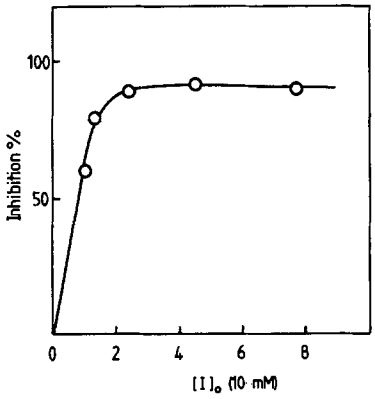

FIG. 4. Residual activity of butyrylcholinesterase in reaction with acetylthiocholine after alkylation at different initial concentrations of *N,N*-dimethyl-2-phenylaziridinium.

further decrease in the enzyme activity, and finally practically complete inactivation can be achieved.

The K_m value for acetylthiocholine hydrolysis was determined for several partially inactivated enzyme preparations, and the results obtained coincide well with the respective value for the unmodified enzyme preparation. Thus it seems reasonable to suppose that the observed progressive decrease in the rate of the acetylthiocholine hydrolysis is caused by a decrease in the concentration of the catalytically active enzyme and not by any change in the catalytic activity of the enzyme. The following treatment of the experimental data is based on that assumption, and the results obtained below agree with it.

Using the condition given by Eq. [7] the kinetic curves of butyrylcholinesterase inhibition were analyzed in coordinates $\ln(v_0/v)$ versus t (see Fig. 5). First, the K_i, K_i^*, k_a, and k values were calculated separately for each kinetic curve at different initial concentrations of the aziridinium inhibitor. That these results are practically independent of the inhibitor concentration is shown in Table 1. Secondly, all those data were used together as a single "data bank," and the results obtained

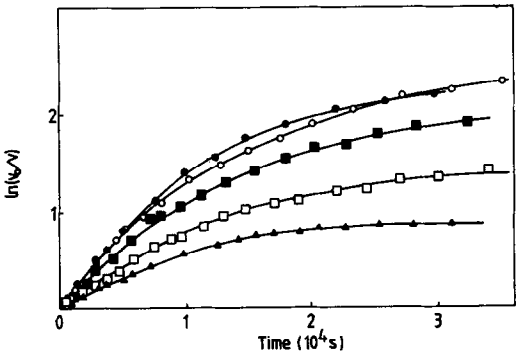

FIG. 5. Butyrylcholinesterase alkylation with *N,N*-dimethyl-2-phenylaziridinium at initial concentrations of 0.1 (▲), 0.13 (□), 0.25 (■), 0.45 (○), and 0.77 (●) m*M*.

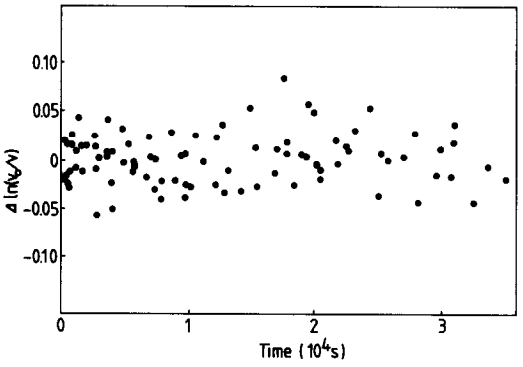

FIG. 6. Deviation of the experimental data from the theoretical $\ln(v_0/v)$ values, calculated from Eq. [6] and the data in Table 1.

are listed at the bottom of Table 1. The "quality" of the data fitting is illustrated in Fig. 6, where the deviations of the experimental $\ln(v_0/v)$ values from Eq. [6] are given.

Reversible Inhibition of Butyrylcholinesterase

The reversible inhibition of the acetylcholine hydrolysis reaction was studied as described above, and the inhibition constants corresponding to the competitive and noncompetitive inhibition types are listed in Table 2.

DISCUSSION

The present results clearly show that the constants K_i and K_i^* of the reaction scheme [3]–[5] have close values, and thus the binding of the alcohol product of the spontaneous hydrolysis of the aziridinium ion must be taken into consideration in the kinetic analysis of the alkylation reaction. The binding of this alcohol

TABLE 2

REVERSIBLE INHIBITION OF BUTYRYLCHOLINESTERASE-CATALYZED HYDROLYSIS OF ACETYLCHOLINE[a]

Inhibitor	K_i (mM)	
	Competitive type	Noncompetitive type
N,N-Dimethyl-2-phenylaziridinium	0.12 ± 0.02	0.46 ± 0.03
N,N-Dimethyl-2-hydroxy-2-phenylethylamine	—	0.35 ± 0.03
N,N,N-Trimethyl-2-hydroxy-2-phenylethylammonium iodide	0.11 ± 0.03	—

[a] pH 7.5, 25°C, 0.15 *M* KCl.

protects butyrylcholinesterase against further alkylation, which seems to be the main reason why residual activity of the enzyme can be found after treatment with the aziridinium compound. As this residual activity levels off at $[I]_0 > 0.3$ mM, completely alkylated butyrylcholinesterase samples can be prepared only after repeated treatment with the alkylating inhibitor or an experimental procedure for the separation of the modified and unmodified enzyme molecules should be elaborated.

The results of the data fitting in Fig. 6 show that Eq. [6] describes well the experimental kinetic curves of the enzyme alkylation reaction, since no systematic trends of the deviation of the experimental points from the calculated kinetic curves could be observed during the time interval used for the kinetic measurements. Moreover, the rate constant k calculated from the enzyme inhibition experiments coincides well with the respective value obtained directly from aziridinium ion solvolysis kinetics. Thus it can be maintained that the reaction scheme [3]–[5] adequately describes the enzyme alkylation process. This reaction sequence assumes the strictly stoichiometric 1 : 1 ratio between the reacting enzyme and the inhibitor molecules, although different nucleophilic groups of the protein can evidently be alkylated by the highly reactive carbonium ion. Therefore, the present results point to the possibility that these side reactions have no remarkable influence on the catalytic properties of butyrylcholinesterase, and the alkylation of a single group is crucial for the loss of the catalytic activity of the enzyme in reaction with cationic substrates.

Some earlier data (*4*) have revealed that the alkylated butyrylcholinesterase remains active for nonionic substrates. In this aspect close similarity between the properties of alkylated acetylcholinesterase and butyrylcholinesterase can be seen, as the first enzyme also retains its activity against nonionic reagents (*3, 4, 14, 15*). Thus it can be concluded that the esteratic site of cholinesterases is not involved in the alkylation reaction, and most probably modification of the anionic site occurs, which is important in the noncovalent binding step of the cationic substrates (*16*).

The results given in Table 2 show that the alcohol product of the aziridinium ion solvolysis also effectively inhibits the butyrylcholinesterase-catalyzed hydrolysis of acetylcholine. In this reaction, however, this alcohol, *N,N*-dimethyl-2-hydroxy-2-phenylethylamine, was a noncompetitive inhibitor, while in the enzyme alkylation process a competitive mechanism is suggested by the results described above (Eqs. [3]–[5]). At the same time the methylation of this *N,N*-dimethylalkylamine derivative implies the competitive inhibition type in the substrate reaction. The reasons for such a sharp change in the apparent inhibition mechanism due to quaternization cannot be explained only on the basis of the present results.

The solvolysis reaction of aziridinium compounds has been found to be faster in acetone–water mixtures than in pure water (*1*). According to the Hughes–Ingold theory of solvent action, such an increase in the S_N1 reaction rate at a change from a more polar to a less polar solvent indicates a dispersal of the ionic charges in the transition state (*17*). If the same S_N1 mechanism can be applied to the enzyme alkylation reaction, the different values of the rate constants k and k_a of the reaction scheme [3]–[5] also point to the apolar (hydrophobic) nature of the reac-

tion medium in which the alkylation reaction occurs. This conclusion agrees with other data on the binding of several alkylammonium compounds as reversible inhibitors of cholinesterases in the hydrophobic region of the active center of these enzymes (*2*).

It has been proved that the active centers of both acetylcholinesterase and butyrylcholinesterase also reveal hydrophobic properties in reactions with substrates (*16*). Therefore, the specific cationic substrates and inhibitors of these enzymes should also be transferred into the "hydrophobic phase" of the active center. The transformation of a cationic particle from water into any hydrophobic phase is, however, strongly hampered by the antihydrophobic (hydrophilic) nature of the ionic part of this molecule. In the case of cholinesterases this effect of antihydrophobicity of the ionic substituent is compensated for by the enzyme anionic site (*16*). As alkylation of some functional group of the anionic site makes this compensation effect impossible, the modified cholinesterases lose their ability to interact with any cationic reagent, although the catalytic groups of the enzyme remain unchanged. Thus the aziridinium-type alkylating reagents that are specifically directed to the anionic site of cholinesterases are valuable tools for special investigation into the role of the anionic and catalytic subsites of the active center.

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