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LEAVING GROUP EFFECTS IN BUTYRYLCHOLINESTERASE REACTION WITH ORGANOPHOSPHORUS INHIBITORS

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Summary

The kinetic constants k_2, K_Q and the second-order rate constant k_i of butyrylcholinesterase (acylcholine acylhydrolase, EC 3.1.1.8) inhibition by organophosphorus compounds $(C_2H_5O)_2P(O)SX$, with both ionic and non-ionic substituents X, were determined at 25°C and pH 7.5 in 0.15 M KCl. The data were analysed in terms of structure-activity relationships and the roles of the leaving group inductive effect and hydrophobicity in the enzyme specificity were established. This made possible calculations of the actual contribution of the substituent ionic charge in the effectiveness of butyrylcholinesterase action.

On the basis of the structure-activity relationships for butyrylcholinesterase and acetylcholinesterase the specificities of the enzymes are compared and some common features are discussed.

Introduction

The high specificity of cholinesterases for reagents with cationic substituents has led to the concept of an anionic site in the active center [1]. However, until recently there were no meaningful data on the contribution of the anionic site in cholinesterase catalysis, since the calculation calls for preliminary quantification of the other specificity-determining factors. Our previous papers have dealt with this complete structure-activity analysis for acetylcholinesterase reactions with both acetic esters [2,3] and organophosphorus inhibitors $(C_2H_5O)_2P(O)SX$ [4,5]. The present study is concerned with butyrylcholinesterase (acylcholine acylhydrolase, EC 3.1.1.8) specificity in reaction with the same series of organophosphorus inhibitors, where the leaving group includes both ionic and non-ionic substituents X (see Table I).

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The kinetic data were analysed according to the two-step reaction scheme [6]:

$$E + Q \overset{K_Q}{\rightleftharpoons} EQ, \quad (1)$$

$$EQ \overset{k_2}{\rightarrow} EQ' + P, \quad (2)$$

where E is enzyme; Q, organophosphorus inhibitor (quasisubstrate); EQ, the non-covalent enzyme · inhibitor complex: EQ', the phosphorylated enzyme; P, the reaction product. Under the pseudo-first-order conditions, where $[E] << [Q]$, the apparent inhibition rate constant k_I depends upon the inhibitor concentration:

$$k_I = \frac{k_2[Q]}{K_Q + [Q]}, \quad (3)$$

which allows evaluation of both constants k_2 and K_Q [6,7]. If $[Q] << K_Q$, the second order rate constant $k_i = k_2/K_Q$ may be directly obtained from k_I vs. [Q] plots as Eqn. 3 is reduced to:

$$k_I = k_i[Q]. \quad (4)$$

Materials and Methods

Chemicals

Butyrylcholinesterase from horse serum was purchased from Mechnikov Institute of Sera and Vaccine, Moscow, U.S.S.R. Enzyme stock solutions with concentration of approx. $5 \cdot 10^{-6}$ M were made in 0.15 M KCl and could be kept for 4 weeks at 4°C without marked decrease in activity. The molarity was determined by titration with organophosphorus inhibitors 9 and 15 (of Table I).

The synthesis and properties of thioesters of diethylphosphoric acid were described in earlier papers [8,9]. A special preparative chromatographic procedure was applied to free organophosphorus thioesters from contaminants of high anticholinesterase activity, supposedly pyrophosphates [10]. The purity of the inhibitor preparations used in the kinetic measurements was checked as described by Aldridge and Davison [11] and Maglothin and Wilson [12]. Solutions of organophosphorus compounds were made in 0.15 M KCl. The concentration of organophosphorus thioesters in the solution was checked by our method of spectrophotometric titration [13]. Butyrylcholine iodide and other reagents from Reakhim were of analytical grade.

Kinetic measurements

Butyrylcholinesterase inhibition was carried out in 0.15 M KCl at pH 7.5 and 25°C. At zero time, the enzyme and inhibitor solutions were mixed in a temperature-controlled vessel with a pH-stat (pH-121/BAT-15/B-705; USSR). At appropriate time intervals, 0.01—0.2 ml reaction mixture were diluted into 5 ml $4 \cdot 10^{-3}$ M butyrylcholine/0.15 M KCl in a second temperature-controlled vessel also with a pH-stat (Radiometer Titrigraph TTT1c/SBR2c/ABU1; Denmark) and the substrate hydrolysis residual velocities v_t were measured titrimetrically at pH 7.5 and 25.0°C.

When the conditions [E] << [Q] were used, the pseudo-first-order rate constants k_I were calculated from ln v_t vs. t plots:

$$\ln v_t = \ln v_0 - k_I t, \tag{5}$$

where v_0 is the enzyme activity at zero time. The inhibition reactions were followed for at least two half-life periods and at least five velocities were measured. The k_I values were analyzed by means of Eqns. 3 and 4 to obtain k_i, k_2 and K_Q. Both latter constants were calculated from $1/k_I$ vs. $1/[Q]$ plots, according to the appropriate linear transformation of Eqn. 3 and inhibitor concentrations in the same range as K_Q were used. An example of the $1/k_I$ vs. $1/[Q]$ plot for compound $(C_2H_5O)_2P(O)S(CH_2)_3SC_2H_5$ is presented in Fig. 1. The straight line does not pass through the origin and the separate determination of the constants k_2 and K_Q is quite reliable.

Under the second-order conditions, where $[E] \approx [Q]$, the constants k_i were calculated by means of the following equation:

$$k_i = \frac{1}{([Q]_0 - [E]_0)t} \ln \frac{[E]_0[Q]_t}{[Q]_0[E]_t}, \tag{6}$$

where $[E]_0$ and $[Q]_0$ are the initial enzyme and inhibitor concentrations and $[E]_t$ and $[Q]_t$ denote the appropriate concentrations at time t. The enzyme concentrations were calculated from the initial velocities of butyrylcholine hydrolysis, $[E] = v/a_m$, making use of the enzyme molecular activity a_m obtained from active site titration experiments.

All calculations and statistical treatment of the experimental data were performed on a Nairi 2 computer.

Substituent constants for correlation analysis

The Hansch π constants and Taft σ^* constants were used to account the hydrophobicity and electronic effects. The π values were calculated additively taking $\pi = 0.5$ for CH_2 and CH_3 groups [14] and $\pi = 1.05$ for SC_2H_5 [15]. For the sulfonium group, $S^+(CH_3)C_2H_5$, $\pi = -4.35$ was used [16]. The inductive constants for the electronegative groups $(CH_2)_nSC_2H_5$ and $(CH_2)_nS^+(CH_3)C_2H_5$

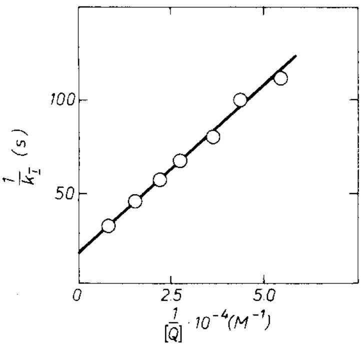


Fig. 1. Plot of $1/k_I$ vs. $1/[Q]$ for the inhibition of butyrylcholinesterase by compound $(C_2H_5O)_2P(O)S(CH_2)_3SC_2H_5$. The conditions of measurement are given in the text.

were calculated from the following expression:

$$\sigma^*_{(CH_2)_nX} = Z^{(n-1)}\,\sigma^*_{CH_2X} \qquad (7)$$

where the attenuation factor, $Z = 0.5$, by analogy with the reaction of the alkaline hydrolysis of the same series of diethylthiophosphates [4]. For $CH_2SC_2H_5$ and $CH_2S^+(CH_3)C_2H_5$, $\sigma^* = 0.56$ and 2.2, respectively, were used [17]. For the alkyl groups C_nH_{2n+1} with $n \geqslant 3$ and for C_2H_5, $\sigma^* = 0$ and 0.07, respectively, were applied [18]. These, somewhat different from the primary σ^* values, permit the treatment of both alkyl and electronegative substituents within the framework of a common reaction series, since the σ^* constants for all of these groups approach the same limit value zero as the inductive effects is attenuated [18].

Results

Kinetic constants

The values of k_i, k_2 and K_Q are listed in Table I. The data indicate that the second-order rate constants k_i, determined under the pseudo-first-order conditions, are more precise than k_2 and K_Q. For most hydrophobic compounds of the reaction series, the separate determination of k_2 and K_Q was complicated by their limited solubility. In the case of very active compounds, the measurement of these constants by the conventional method was prevented by the high velocity of the enzyme phosphorylation reaction.

In the case of $[(C_2H_5O)_2P(O)S(CH_2)_2S^+(CH_3)C_2H_5]\,CH_3SO_4^-$ the rate constant k_i was obtained under the bimolecular conditions where $[E] \approx [Q]$.

TABLE I

KINETIC CONSTANTS FOR BUTYRYLCHOLINESTERASE REACTION WITH ORGANOPHOSPHORUS THIOESTERS $(C_2H_5O)_2P(O)SX$ IN 0.15 M KCl AT pH 7.5 AND 25°C

Compound	X	$10^{-3} \cdot k_i$ ($M^{-1} \cdot s^{-1}$)	$10^2 \cdot k_2$ (s^{-1})	$10^4 \cdot K_Q$ (M)
1	C_2H_5	0.00313 ± 0.00024	0.78 ± 0.18	22.1 ± 1.3
2	C_3H_7	0.00550 ± 0.00016	0.62 ± 0.19	11.9 ± 1.8
3	C_4H_9	0.0222 ± 0.0008	0.55 ± 0.17	2.51 ± 0.29
4	C_5H_{11}	0.0994 ± 0.0046	0.67 ± 0.17	0.778 ± 0.065
5	C_6H_{13}	0.500 ± 0.043	1.42 ± 0.39	0.285 ± 0.048
6	C_7H_{15}	0.910 ± 0.049	1.10 ± 0.56	0.123 ± 0.039
7	C_8H_{17}	0.780 ± 0.050	—	—
8	$CH_2SC_2H_5$	2.66 ± 0.18	—	—
9	$(CH_2)_2SC_2H_5$	0.272 ± 0.015	16.0 ± 2.5	4.79 ± 0.89
10	$(CH_2)_3SC_2H_5$	0.479 ± 0.019	5.36 ± 0.95	0.939 ± 0.132
11	$(CH_2)_4SC_2H_5$	0.660 ± 0.023	1.69 ± 0.23	0.225 ± 0.028
12	$(CH_2)_5SC_2H_5$	0.250 ± 0.016	—	—
13	$(CH_2)_6SC_2H_5$	0.244 ± 0.013	—	—
14	$(CH_2)_2S^+(CH_3)C_2H_5$	199.5 ± 9.9	—	—
15	$(CH_2)_3S^+(CH_3)C_2H_5$	9.58 ± 0.29	—	—
16	$(CH_2)_4S^+(CH_3)C_2H_5$	3.46 ± 0.12	11.1 ± 4.7	0.282 ± 0.087
17	$(CH_2)_5S^+(CH_3)C_2H_5$	0.543 ± 0.015	5.8 ± 1.1	0.111 ± 0.035
18	$(CH_2)_6S^+(CH_3)C_2H_5$	0.546 ± 0.023	2.41 ± 0.64	0.429 ± 0.097

Hydrophobicity effects

Hydrophobicity is the only variable structural parameter within the subseries of the inhibitors with alkyl groups X that makes these compounds useful for investigating the hydrophobic region of the enzyme active center. The plot of log k_i vs. π for compounds with n-alkyl groups X is presented in Fig. 2. The linear relationship supports the idea that the hydrophobic forces play an essential role in the interaction of the leaving part of organophosphorus inhibitors with the butyrylcholinesterase active center. A break is revealed in this linearity at the *n*-heptyl group and further increase in both the length and hydrophobicity of the substituent seems to have no effect on the antibutyrylcholinesterase activity of the compounds. Similar results have been obtained earlier for butyrylcholinesterase reaction with another series of organophosphorus compounds $(C_2H_5O)(CH_3)P(O)SC_nH_{2n+1}$, where the constancy of log k_i was demonstrated up to the *n*-decyl radical [19]. These results may be taken as an indication of the limited size of the hydrophobic region in which the inhibitor's leaving group binds. In further calculations, it will be considered that the enzyme active center provides the hydrophobic sorption of the substituents X no longer than the *n*-heptyl radical.

The data in Table I indicate that the hydrophobicity of the inhibitors manifests only in the non-covalent binding Step 1 and plays no role in the reaction Stage 2 as the k_2 for the inhibitors with alkyl groups X remains unchanged within the limits of error, although the hydrophobicity of X increases. The plot of pK_Q vs. π in Fig. 3 gives a common linearity for both alkyl and non-ionic electronegative groups X. Consequently, the butyrylcholinesterase specificity in the binding step may be generalized in terms of structure-activity relationships by the equation:

$$pK_Q^X = pK_Q^0 + \phi_b \pi_X . \tag{8}$$

The value of the intensity factor ϕ_b in Table II approaches the theoretical limit

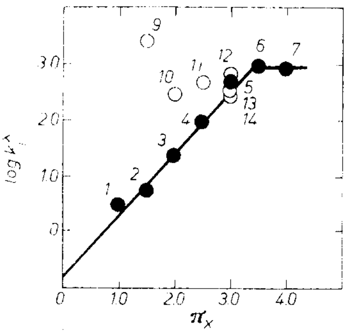


Fig. 2. Plot of log k_i^X vs π_X for butyrylcholinesterase reaction with organophosphorus inhibitors $(C_2H_5O)_2P(O)SX$. ●, Inhibitors with *n*-alkyl groups X; ○, inhibitors with non-ionic electronegative groups. The numbers in the figure refer to the compounds listed in Table I. For $(CH_2)_nSC_2H_5$ with $n > 4$ the apparent π values were used.

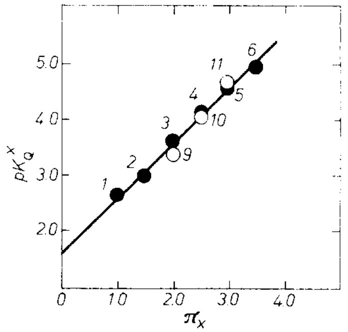


Fig. 3. Plot of pK_Q^X vs. π_X for butyrylcholinesterase reaction with organophosphorus inhibitors $(C_2H_5O)_2P(O)SX$. ●, Inhibitors with *n*-alkyl groups; ○, inhibitors with non-ionic electronegative groups. The numbers in the figure refer to the compounds listed in Table I.

TABLE II

STRUCTURE-ACTIVITY CORRELATIONS FOR BUTYRYLCHOLINESTERASE INHIBITION BY ORGANOPHOSPHORUS THIOESTERS $(C_2H_5O)_2P(O)SX$

The numbers of the compounds included in the analyses refer to the inhibitors in Table I. The values of correlation coefficient (r) and standard deviation (s) are given.

Equation		Constants	r	s	Compounds used in correlation
$pK_Q^X = pK_Q^0 + \phi_b\pi_X$	(8)	$pK_Q^0 = 1.55 \pm 0.15$	0.99	0.13	1—6, 9—11
		$\phi_b = 0.98 \pm 0.06$			
$\log k_2^X = \log k_2^0 + \rho^*\sigma_X^*$	(10)	$\log k_2^0 = -2.05 \pm 0.16$	0.95	0.16	1—6, 9—11, 16—18
		$\rho^* = 4.30 \pm 0.45$			
$\log k_i^X = \log k_i^0 + \rho^*\sigma_X^* + \phi\pi_X$	(9)	$\log k_i^0 = -0.89 \pm 0.17$	0.989	0.145	1—13
		$\rho^* = 4.51 \pm 0.29$			
		$\phi = 1.06 \pm 0.09$			

unity deriving from the extraction model of the hydrophobic interaction [14]. A similar slope, $\phi = 1.07 \pm 0.08$, was also found for the linearity between log k_i and π in Fig. 2. In the calculation of ϕ, the k_i values were used for $(C_2H_5O)_2$-$P(O)SC_nH_{2n+1}$, where $n = 2$—7.

It should be noted that no break is seen in the pK_Q versus π plot, as the relationship does not involve the most hydrophobic inhibitors of the reaction series in which the length of the group X exceeds that of the n-heptyl radical.

Inductive effect

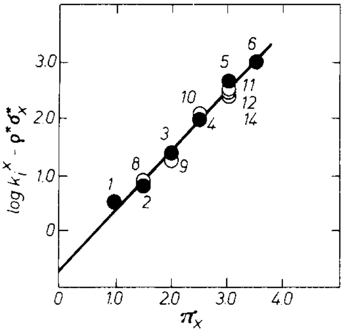
Phosphorylation of the butyrylcholinesterase active center is considered to be a nucleophilic displacement reaction that is intrinsically accelarated by introduction of the electronegative substituent into the leaving part of organophosphorus inhibitors. The data in Fig. 2 show that k_i values for the inhibitors with electronegative substituents in their leaving part are significantly higher than is required by their hydrophobicity. This deviation of the points from the straight line with the slope unity was accepted as the contribution of the inductive effect, and the general structure-activity relationship for all the non-ionic compounds is expressed by the following equation:

$$\log k_i^X = \log k_i^0 + \rho^*\sigma_X^* + \phi\pi_X, \qquad (9)$$

where $\rho^*\sigma_X^*$ and $\phi\pi_X$ stand for the inductive effect and hydrophobicity respectively. In data processing by means of this equation the limited range of the hydrophobic binding site was taken into consideration and for the substituents $(CH_2)_nSC_2H_5$ with $n > 4$ the apparent π values were calculated assuming that a part of the radical had no influence on hydrophobic binding. The results of the calculations are given in Table II. The validity of Eqn. 9 is illustrated in Fig. 4, where the relationship between $\log k_i^X - \rho^*\sigma_X^*$ and π constants is given.

The increase in k_2 for organophosphorus inhibitors with electronegative leaving groups reflects the inductive effect only and can be described by the Taft equation:

$$\log k_2^X = \log k_2^0 + \rho^*\sigma_X^*. \qquad (10)$$



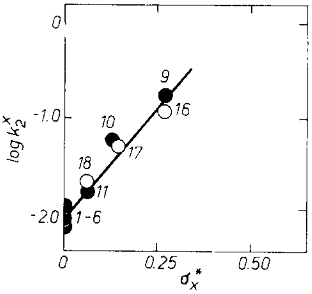


Fig. 4. Plot of log k_i^X — $\rho^*\sigma^*_X$ vs. π_X for butyrylcholinesterase reaction with organophosphorus inhibitors $(C_2H_5O)_2P(O)SX$. ●, Inhibitors with n-alkyl groups X; ○, inhibitors with non-ionic electronegative groups X. The numbers in the figure refer to the compounds listed in Table I. For $(CH_2)_nSC_2H_5$ with $n > 4$ the apparent π values were used.

Fig. 5. Evaluation of ρ^* for butyrylcholinesterase phosphorylation reaction by inhibitors $(C_2H_5O)_2$-P(O)SX. ○, Inhibitors with ionic leaving groups X; ●, inhibitors with non-ionic leaving groups X. The numbers in the figure refer to the compounds listed in Table I.

In Fig. 5 the plot of log k_2^X vs. π_X provides a common line for inhibitors with both non-ionic and ionic substituents X. Consequently, the sufficient increase in the reactivity of the sulfonium compounds in the bond-breaking Step 2 is caused solely by the electronic effects and involve no specific influence of the ionic charge.

It should be pointed out that the ρ^* values obtained for both k_i and k_2 are well within the limits of error. This is only to be expected since Eqn. 9 is a combination of Eqns. 8 and 10 just as constant k_i consists of both k_2 and K_Q. Analogically, equality was observed between ϕ_b and ϕ in Eqns. 8 and 9 (see Table II).

Charge effect

Introduction of a cationic group into a substituent strongly hampers its transition from water into hydrophobic medium. This results in the negative π values for ionic groups [15,16]. However, the data in Table I indicate that the affinity constants K_Q for both ionic and nonionic compounds lie in the same range of magnitude. Consequently, besides the hydrophobic forces an additional factor reveals in the binding of cationic compounds in the active center of butyrylcholinesterase. This additional influence may be attributed to the anionic site in butyrylcholinesterase specificity. To take into account this influence an extra term θ was inserted into Eqns. 8 and 9:

$$pK_Q^X = pK_Q^0 + \phi_b\pi_X + \theta_b, \tag{11}$$

$$\log k_i^X = \log k_i^0 + \rho^*\sigma_X^* + \phi\pi_X + \theta. \tag{12}$$

Making use of the parameters pK_Q^0, ϕ_b, log k_i^0, ϕ and ρ^* given in Table II, and appropriate σ^* and π constants to take into account the inductive effect and hydrophobicity of the sulfonium substituents $(CH_2)_nS^+(CH_3)C_2H_5$, the θ values listed in Table III were calculated for the ionic inhibitors. It is evident from

TABLE III

CHARGE EFFECT IN BUTYRYLCHOLINESTERASE REACTION WITH CATIONIC ORGANOPHOSPHORUS INHIBITORS $[(C_2H_5O)_2P(O)S(CH_2)_nS^+(CH_3)C_2H_5]CH_3SO_4^-$

$\theta_b = pK_Q^{X^+} - pK_Q^0 - \phi_b\pi_{X^+}$

$\theta = \log k_i^{X^+} - \log k_i^0 - \rho^*\sigma^*_{X^+} - \phi\pi_{X^+}$

n	θ_b	θ
2	—	4.8
3	—	5.5
4	5.5	5.7
5	5.8	5.6
6	5.6	6.0

Table III that both k_i and K_Q give coincidental results in agreement with the fact that no charge effect appeared in the enzyme phosphorylation step.

In the calculation of θ it was presumed that both the ionic and non-ionic leaving groups would interact with the enzyme active center in the same manner. Support for this assertion may be found in the dependence of log k_i of the ionic organophosphorus compounds $(C_2H_5O)(CH_3)P(O)SC_2H_4S^+(CH_3)C_nH_{2n+1}$ [19] and $(C_6H_5)_2P(O)SC_2H_4S^+(CH_3)C_nH_{2n+1}$ [20] upon the hydrophobicity of the variable alkyl radical directly bound to the onium atom and in the close values of the appropriate intensity factors ϕ for both ionic and non-ionic compounds within the respective reaction series. A similarity in the mode of binding of both ionic and non-ionic substituents of organophosphorus inhibitors in the active center had been recognized earlier in the case of acetylcholinesterase [2,5].

The data in Table III indicate that θ remains practically constant for ionic compounds with $n > 3$ and only for $n = 2$ the charge effect has somewhat lower value. Thus, the nature of the dependence of θ upon the length of polymethylene chain intervening between the cationic group and the reaction center is strikingly different from the dependence of inductive effect and hydrophobicity on the reagent structure.

Discussion

In butyrylcholinesterase reactions with organophosphorus inhibitors both the leaving group hydrophobicity and inductive effect are manifested and the structure-activity relationships in the non-covalent binding and phosphorylation steps can be expressed by Eqns. 8 and 10. The same equations may also be used in the case of the malaoxon derivatives $(CH_3O)_2P(O)SCH(COOR)$-CH_2COOR, where R is a variable alkyl radical. On the basis of the experimental data published by Main and Hastings [21] we found $\phi_b = 0.74 \pm 0.06$ for this reaction series, while k_2 may be taken as being practically unchanged. This agrees well with Eqn. 10 as the $\rho^*\sigma^*$ term for the malaoxon derivatives is constant.

It is evident from those results that no interrelationship between binding and catalysis specificities is revealed in butyrylcholinesterase inhibition by organophosphorus compounds with variable leaving parts. However, such interrelationship can be found in butyrylcholinesterase-catalyzed hydrolysis of substrates C_3H_7COOR with n-alkyl radicals R in their alcohol portion. It is evident from the data given by Main and Hastings [20] that in this reaction the leaving group hydrophobicity stabilizes both the Michaelis complex and the transition state of the acylation reaction. For the later process the following equation is valid:

$$\log k_2^X = \log k_2^0 + \phi_a \pi_X, \qquad (13)$$

as the $\rho^*\sigma^*$ term for the n-alkyl butyrates $C_3H_7COOC_nH_{2n+1}$ may be taken as zero. The plots of pK_m and $\log k_{cat}$ vs. π are given in Fig. 6. Provided that $K_m = K_s$ and $k_{cat} = k_2$ the values $\phi_b = 0.99 \pm 0.16$ and $\phi_a = 1.05 \pm 0.10$ were found for the binding step and the enzyme acylation reaction respectively. The deviation of the point for methyl butyrate may be a result of the inductive and steric influences that differ in the case of CH_3 in comparison with other n-alkyl radicals.

It is remarkable that substrate leaving group hydrophobicity makes an equal contribution in both steps of the enzyme reaction, $\phi_a = \phi_b$. The equality of ϕ_a with ϕ_b has been established earlier for acetylcholinesterase-catalyzed hydrolysis of the substrates CH_3COOR [2] and for some reactions of α-chymotrypsin [22,23]. The phenomenon has been connected with a conformational change of the enzyme that consists of "shutting of a jaws-like hydrophobic slit", in which the hydrophobic substituent is located in the enzyme active center [23]. The present comparison of the data on butyrylcholinesterase substrates and inhibitors indicates that the stabilization of the transition state in the bond-breaking step by hydrophobicity is not a general feature of the enzyme action and may be connected with some definite properties of the active center and reagent structure. A more detailed discussion of the binding-reaction interrelationship in butyrylcholinesterase catalysis will be possible after a more complete structure-activity analysis of the enzymic hydrolysis of substrates.

Butyrylcholinesterase reaction with organophosphorus inhibitors shows a remarkably high sensitivity to the inductive effect of the leaving group. The

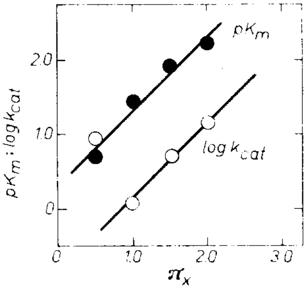


Fig. 6. Dependence of pK_m and $\log k_{cat}$ upon substrate leaving group hydrophobicity in the butyrylcholinesterase-catalyzed hydrolysis of n-alkyl butyrates $C_3H_7COOC_nH_{2n+1}$. The data were taken from the article by Main and Hastings [21].

intensity factor ρ^* in Table II exceeds twice the appropriate value 2.2 for alkaline hydrolysis of the same organophosphorus compounds [4], but is similar to that of acetylcholinesterase phosphorylation [4]. Moreover, the inductive effect has been demonstrated to be an important structural factor in acetylcholinesterase-catalyzed hydrolysis of acetic esters and ρ^* for the enzyme acetylation reaction was also found to be almost twice as high as that for alkaline hydrolysis [2]. Thus, high sensitivity to electronic effects seem to be a common feature of reactions of both types of cholinesterases. It should be noted that some earlier data on the anticholinesterase activity of organophosphorus compounds seem to fit in with the same regularity [11,24,25].

In the reaction series X-Y, where X denotes the variable substituent and Y is the reaction center, the inductive effect intensity factor ρ^* is determined by the difference between the σ^* constants for the reaction center in the initial and transition states, σ^*_{Y} and $\sigma^*_{\mathrm{Y}\neq}$, respectively [26]:

$$\rho^* = \frac{\alpha^*(\sigma^*_{\mathrm{Y}} - \sigma^*_{\mathrm{Y}\neq})}{2.303\,RT} \qquad (14)$$

where the universal constant of inductive interaction $\alpha^* = 2.6$ [27]. Making use of $\sigma^*_{\mathrm{Y}} = 2.20$ for the reaction center $(C_2H_5O)_2P(O)S$- [4], $\sigma^*_{\mathrm{Y}\neq} = -0.15$ was found for the butyrylcholinesterase phosphorylation reaction. The latter value is close to $\sigma^* = -0.4$ for the S^- group [27], pointing to extensive cleavage of the phosphorus-sulfur bond in the transition state of butyrylcholinesterase phosphorylation reaction. The same conclusion was drawn about acetylcholinesterase inhibition reaction by the same series of organophosphorus inhibitors in a previous report from our laboratory [4]. However, another possible explanation of the phenomenon would be the assumption of different solvation effects in the transition states of the enzymic and non-enzymic reactions. In fact, the quantity ρ^* for heterolytic reactions increases when passing from hydroxylic media to aprotic ones, probably due to the weakened electrophilic solvation of the reaction center in the transition state (or, in the final state in the case of equilibrium processes) [28], while the solvent effects on the constants σ^* of neutral groups in the initial state may be considered to be neglible. According to this explanation the enzyme active center seems to be like aprotic media which are unable to solvate the reaction center. Thus, the heightened sensitivity to the inductive effect in enzyme catalysis in comparison with non-enzymic reactions may well be a general feature of enzyme action.

The role of the anionic site in butyrylcholinesterase catalysis has been widely discussed, but it is only now that we can make a quantitative statement of its extent in the enzyme reaction with organophosphorus inhibitors. It is evident from the results in Table III that the charge effect strongly stabilizes the non-covalent enzyme-inhibitor complex, making a large contribution to butyrylcholinesterase specificity. This contribution was found to be much greater than had been expected in any earlier attempt to establish the role of the "anionic point" in specificity of cholinesterases by simple comparison of kinetic or equilibrium data for pairs of cationic reagent and its non-ionic analogue [29–33]. In contrast, in the present study the other strucutral effects were also considered in the calculation of θ values.

The hydrophobicity constant π for a charged substituent X^+ may be expressed as follows [5,16]:

$$\pi_{X^+} = \pi_X + \pi_+, \qquad (15)$$

where π_+ is the "antihydrophobicity" parameter for the ionic charge. The validity of this expression has been demonstrated for both ammonium and sulphonium groups, where $\pi_+ = -5.7$ [16] independently of the nature of the onium atom. One can conclude that the decrease of π values for onium groups characterizes the thermodynamically unfavourable transfer of a charged substituent from water into hydrophobic phase, octanol in the case of π constants. It is natural that the same "antihydrophobicity" effect should occur in transfer of the ionic substituent into the hydrophobic region of the enzyme active center and that its quantity should be proportional to the intensity factor ϕ. However, in butyrylcholinesterase reaction with cationic inhibitors the corresponding negative increment $\phi\pi_+$ in both pK_Q and $\log k_i$ is compensated for by θ. As a result the effectiveness of butyrylcholinesterase interaction with cationic compounds becomes comparable in magnitude with that of non-ionic compounds. Only the inhibitor with $n = 2$ deviates from this general regularity.

It is noteworthy, that earlier exactly the same situation had been observed in the reactions of acetylcholinesterase with the same series of organophosphorus inhibitors [5] and acetic esters with onium groups in their leaving parts [3]. Thus, the compensation of the cationic group "antihydrophobicity" seems to be a general function of the "anionic point" in cholinesterases. It should also be emphasized that no difference between butyrylcholinesterase and acetylcholinesterase specificities appears in this connection, although many earlier papers have discussed the problem, including the existence of different numbers of anionic sites in these enzymes [1,29–31,34].

The extrathermodynamic approach used by us in the calculation of θ did not reveal any information on the nature of the charge effect observed. But some suggestions can be made on the basis of the regularities observed for both cholinesterases.

According to the view mentioned above the "antihydrophobicity" effect has been related to the presence of an ionic charge in the substituent. Therefore, one may assume that the neutralization of this charge by an ion of opposite sign unloads the "antihydrophobicity" as the substituent becomes formally non-ionic. In cholinesterases the ion of opposite sign probably belongs to the "anionic point" in the enzyme active center.

The charged group of the anionic site may be buried in the hydrophobic environment. This arrangement is thermodynamically unfavourable, but, it may be stabilized by successive interactions between the anionic group and other parts of the protein molecule if the surroundings of the active center are rigid enough to keep the "anionic point" out of water. On the other hand, the anionic group may be located in the external medium near the boundary of the hydrophobic phase and "dive" into it together with the cationic substituent. This model assumes remarkable flexibility of the peptide chain that contains the anionic group. It is noteworthy that the latter idea is in good agreement with the permanence of θ values independently of the chain length between the onium group and reaction center.

Thus, the major part of the charge effect may be attributed to this "ionic charge neutralization effect". However, it seems reasonable that θ may also include some other contributions, for example a part of the electrostatic interaction between the cationic group and the "anionic point". Therefore, as has been pointed out in our previous report [5] a more detailed study of θ content calls for further experimental work, including the investigation of salt effects in the enzyme reactions in order to get information about the ion-ion interaction.

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