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COMPARATIVE AND ONTOGENIC BIOCHEMISTRY

Kinetic Analysis of the "Substrate Protective Effect" in Cholinesterases of Different Origin

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Abstract—The authors' own and literature data are summarized on interaction of 17 irreversible organophosphorus inhibitors with different types of cholinesterases (ChE): erythrocyte acetylcholinesterase (AChE), serum butyrylcholinesterase (BuChE), and cholinesterase of the Pacific squid *Todarodes pacificus,* in the presence of 9 substrates. The kinetic analysis of the "substrate protective effect" based on A.P. Brestkin's equation is performed, and the current interpretation of individual components of this process is done. An essential effect of the inhibitor structure on individual phases of the reaction is revealed. Among choline substrates, only formylcholine did not show a protective effect. The inability of an uncationic substrate, phenylacetate, to regulate ChE reactivity is confirmed. Among the studied ChE, the highest substrate protective effect was revealed in the Pacific squid ChE.

INTRODUCTION

The main comparative-enzymologic characteristics of related enzymes is their substrate specificity, which is clearly demonstrated in studies on the cholinesterase (ChE) family [1, 2]. The substrate specificity is the function of the "working" enzyme, hence, the kinetic analysis is necessary here to provide the quantitative parameters so important for comparison of hydrolysis rates of structurally different substrates [2]. Meanwhile, the role of substrates in enzymatic catalysis is polyfunctional: apart from being an object of the ChE hydrolytic action, they also perform regulatory functions (activation of ChE at acid pH and inhibition by excess of substrate [2]), participate in interaction with competitive reversible inhibitors [3], and, at last, the rate of ChE inhibition by irreversible organophosphorus inhibitors (OPI) decreases in the presence of substrates; this is the so-called "substrate protective effect" [4–6]. The protective function of substrate is extremely important, as it models the situation *in vivo*: when entering the organism, xenobiotic interacts with enzyme, as a rule, in the presence of substrate.

In 1964, Brestkin has carried out a detailed kinetic analysis of the substrate protective effect [6]. He disproved the common concept [4, 5] that the decrease of rate of the ChE activity inhibition with OPI in the presence of substrate occurs simply because the OPI interacts only with free ChE, rather than with that bound to acetylcholine. The equation developed by Brestkin [6] has made it possible to evaluate quantitatively individual constants of rates of certain reactions. It has allowed revealing an essentially new aspect of the substrate protective effect, a decrease of the butyrylcholinesterase (BuChE) reactivity to OPI under effect of acetylcholine. Subsequent studies have shown the correctness and usefulness of his conclusions [7–12].

The analyzed system is three-component: participating in it are enzyme, substrate, and inhibitor. The nature and structure of each component should affect character of the protective effect. In the present paper, using our own as well as literature data, we are analyzing for the first time the A.P. Brestkin's equation for structurally

different substrates and OPI, and for ChE of different nature, which opens a new approach for comparative-enzymologic investigations.

MATERIALS AND METHODS

As sources of enzymes we used partially purified preparations of acetylcholinesterase (AChE) from human erythrocytes (EC 3.1.1.7) and BuChE (EC 3.1.1.8) from the horse blood serum with specific activities of 1.2 and 9.6 U/mg , respectively (Perm' Research Institute of Vaccines and Sera), and also supernatant (800 g, 15 min) of water homogenate (3 mg/ml) of the optical ganglia tissue from the Pacific squid *Todarodes pacificus* [3].

Catalytic activity of the enzymes was determined by a method of continuous potentiometric titration at pH 7.5 and 30°C [1]. When studying kinetics of ChE interaction with substrate and OPI, the reaction mixture containing ChE and substrate was titrated during 1–2 min with NaOH solution, then an inhibitor was added and the titration was continued for 8–10 min. The experimental curve allowed calculating the initial and the current reaction rates from the slope angle of tangent to the curve. To evaluate the interaction rate constant of ChE with OPI in the absence of substrate, the ChE solution was incubated with an inhibitor solution under the same conditions, and samples for determination of the residual ChE activity were taken from the incubation mixture at regular time intervals for 15–20 min. The ChE reaction with inhibitor was stopped in the sample by its 7–8-fold dilution with water and addition of the substrate solution up to a final concentration of 1.6×10^{-2} M.

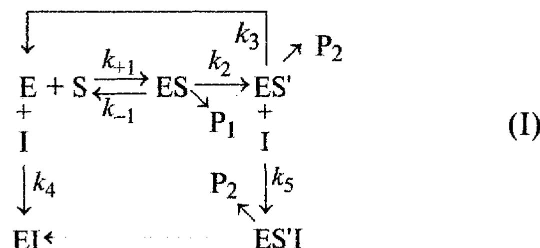
Calculation and analysis of kinetic parameters of the process of the irreversible OPI interaction with various ChE in the presence of different substrates was carried out according to methods described in papers [6–8].

As shown in several works [4, 13], the substrate protective effect consisted in a decrease of the value of the bimolecular rate constant of irreversible OPI interaction with ChE in the presence of acetylcholine (S):

$$k_{II,S} = \frac{1}{t\,[I]} \ln(v_0/v_{i,S}), \qquad (1)$$

where $[I]$—OPI concentration; v_0—enzymatic reaction rate in the absence of OPI; $v_{i,S}$—enzymatic reaction rate after t min of ChE incubation with OPI in the presence of S. A gradual decrease of the $k_{II,S}$ value with increase of the acetylcholine concentration was shown.

Brestkin was the first to carry out a complex kinetic analysis of the substrate protective effect [6]. Initially he analyzed mathematically the current concept [4, 5] the protective effect is based on the ability of OPI to interact only with free ChE (scheme I),



(I)

whose concentration $[E]_f$ in the presence of substrate is described by the formula

$$[E]_f = K_M\,[E]/(K_M + [S]), \qquad (2)$$

where K_M—Michaelis constant, [S]—substrate concentration, [E]—active concentration of enzyme equal to $[E]_0$—[EI]. The rate of interaction of OPI in the presence of S:

$$-d\,[E]/dt = k_4\,K_M\,[E]\,[I]/(K_M + [S]), \qquad (3)$$

where k_4—the rate constant of interaction of OPI with free ChE (see scheme I). After integration (3) and several transformations considering (1), it is obtained

$$k_{II,S} = \frac{1}{t\,[I]} \ln(v_0/v_{i,S}) = k_4\,K_M/(K_M + [S]).$$

When ChE interacts with OPI in the absence of substrate ($[S] = 0$),

$$k_{II,0} = \frac{1}{t\,[I]} \ln(v_0/v_i) = k_4,$$

where v_i—rate of enzymatic reaction after t min of ChE incubation with OPI in absence of S. If the concept is correct,

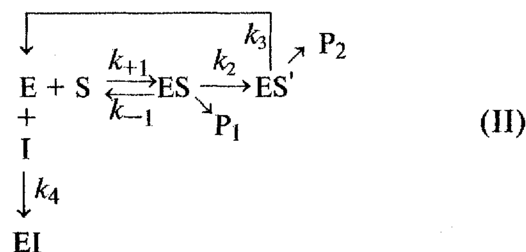
$$k_{II,0}\,/\,k_{II,S} = (K_M + [S])/K_M, \qquad (4)$$

Table 1. Protective effect of acetylcholine at interaction of OPI of various structure with butyrylcholinesterase (the primary protective effect $(K_M + [S])/K_M = 9$)

	OPI	k_{II}, $M^{-1}\ min^{-1}$	Substrate protective effect		
			total, [ACh] = 10^{-2} M $k_{II,0}/k_{II,S}$	secondary, $k_{II,0}$ / k_4	tertiary, α (%)
I	$[(CH_3)_2CHO]_2P(O)F$	6.7×10^6	10	4	2.5
II	$C_2H_5O(C_2H_5)P(O)OC_6H_4(4{-}NO_2)$	2.6×10^5	15	4	5
III	$C_2H_5O(CH_3)P(O)S(CH_2)_2\overset{+}{S}(CH_3)C_2H_5$	2.8×10^6	20	3	16
IV	$C_2H_5O(CH_3)P(O)S(CH_2)_2SC_2H_5$	6.3×10^3	10	6.5	7.5
V	$C_3H_7O(CH_3)P(O)S(CH_2)_2\overset{+}{S}(CH_3)C_2H_5$	6.5×10^6	15	2	18
VI	$C_3H_7O(CH_3)P(O)S(CH_2)_2SC_2H_5$	8.1×10^4	10	1.5	17
	$C_2H_5O(CH_3)P(O)SC_nH_{2n+1}$				
VII	$n = 5$	2.6×10^3	15	2.5	7.5
VIII	$n = 6$	3.8×10^4	40	4.5	0
IX	$n = 7$	3.3×10^4	15	2	0
X	$n = 8$	4.0×10^4	15	2	0
XI	$n = 10$	3.8×10^4	15	2	0
	$C_2H_5O(CH_3)P(O)S(CH2)_mC(CH_3)_3$				
XII	$m = 1$	2.1×10^4	30	8	16
XIII	$m = 2$	4.7×10^3	20	4.5	11
XIV	$m = 3$	1.8×10^4	20	3.5	7
XV	$m = 4$	9.0×10^4	70	9	0
XVI	$m = 5$	8.9×10^4	45	5	0
XVII	$m = 6$	9.9×10^4	50	5	0

i.e., this reflects a decrease of the free enzyme concentration (2). For each enzyme–substrate pair, the ratio $k_{II,0}/\ k_{II,S}$ (4) should be the same regardless of the OPI structure, however, this does not agree with the experimental data (Table 1). Besides, the ratio (4) for different substrates should have certain calculable values, which was not observed in the experiment, either (Table 2). Hence, the concept of the decrease of the OPI inhibitory effect in the presence of substrate only due to the decrease of concentration of the free enzyme able to interact with OPI, does not reflects mechanism of the substrate protective effect.

Brestkin was the first to put forward a suggestion that OPI can interact in the presence of substrate not only with free (E), but also with the acylated (ES′) enzyme according to the scheme (II):



(II)

Table 2. Effect of substrate structure on different components of the protective effect at inhibition of butyrylcholinesterase by $C_2H_5O(CH_3)P(O)S(CH_2)_2SC_2H_5$ (IV)

Substrates		Hydrolysis parameters		Substrate protective effect				
		$a_c \times 10^4$, min^{-1}	$K_M \times 10^{-3}$, M	total, $k_{II,0}/k_{II,S}$		primary, $(K_M + [S])/K_M$, $[S] = 10^{-2}$ M	secondary, $k_{II,0}/k_4$	tertiary, α (%)
				$[S] = 10^{-2}$ M	$[S]/K_M = 10$			
FCh	$HC(O)OC_2H_4\overset{+}{N}(CH_3)_3$	12	17	–	1.5	–	–	–
ACh	$CH_3C(O)OC_2H_4\overset{+}{N}(CH_3)_3$	6.5	1.2	10	10	9	1.5	17
PCh	$C_2H_5C(O)OC_2H_4\overset{+}{N}(CH_3)_3$	10	1.0	12	12	11	2	8.5
BCh	$C_3H_7C(O)OC_2H_4\overset{+}{N}(CH_3)_3$	14	1.0	15	15	11	3	0
VCh	$C_4H_9C(O)OC_2H_4\overset{+}{N}(CH_3)_3$	1.9	0.8	19	11	13	3	0
AThCh	$CH_3C(O)SC_2H_4\overset{+}{N}(CH_3)_3$	3.8	1.8	9	8	7	2.5	10
PThCh	$CH_3C(O)SC_2H_4\overset{+}{N}(CH_3)_3$	6.0	1.3	12	12	9	3.5	7
BThCh	$C_3H_7C(O)SC_2H_4\overset{+}{N}(CH_3)_3$	8.5	1.7	14	14	7	4	2.5
PhA	$CH_3C(O)OC_6H_5$	6.4	4.8	3	–	3	1	0

Note: FCh—formylcholine, ACh—acetylcholine, PCh—propionylcholine, BCh—butyrylcholine, VCh—valerylcholine, AThCh—acetylthiocholine, PThCh—propionylthiocholine, BThCh—butyrylthiocholine, PhA—phenylacetate.

Reaction of OPI with the Michaelis complex (ES) is hardly probable, as in this complex, in which the substrate molecule is sorbed as a whole, all functional groups of the ChE active center, which participate in catalytic process, are occupied. In the acylated enzyme (ES′), whose acyl group of substrate is bound to serine hydroxyl, the site of binding of the alcoholic part of the substrate molecule can be free, if the desorption of alcohol occurs earlier than deacylation (see below); in this connection, the interaction of OPI with ES′ cannot be ruled out, which results in formation of the ES′I complex able hypothetically be deacylated, with transformation into the enzyme–inhibitor complex EI.

According to the scheme (II), the initial ChE concentration, $[E]_0$, decreases due to interaction of OPI both with the free $[E]_f$ and with the acylated [ES′] ChE: $-d[E] / dt = k_4 [E]_f [I] + k_5 \times [ES'] [I]$, where $[E]_f = [E]_0 - [EI] - [ES'I] - [ES] - [ES']$.

After several transformations, the linear form of A.P. Brestkin's equation was obtained

$$k_{II,S} (K_M + [S])/K_M = k_4 + a\, k_5 [S]/K_M, \qquad (5)$$

where k_4—the rate constant of reaction with free ChE; $a \times k_5$—the value proportional to the rate constant of the reaction of I with acylated ChE; $a = k_2 / (k_2 + k_3)$.

The equation (5) is the chief achievement of the performed kinetic analysis of the scheme II, as it allows evaluating values of individual constants for certain stages.

The value of the a factor seems to depend both on the substrate structure and on the enzyme nature. According to the data of the paper [14], $a = 0.86$ at acetylcholine hydrolysis by AChE. Volkova believes [9] that processes of hydrolysis of acetylcholine and phenylacetate by BuChE are characterized by different limiting stages: deacetylation in the case of acetylcholine ($k_3 << k_2$) and acetylation in the case of phenylacetate ($k_2 << k_3$) (see below). According to Yakovlev [15], on the contrary, the limiting stage at hydrolysis of acetylcholine by BuChE is acetylation ($k_2 << k_3$).

Table 3. Effect of the enzyme nature on different components of the substrate protective effect at inhibition by $C_2H_5O(CH_3)P(O)S(CH_2)_2SC_2H_5$ (IV) in the presence of acetylcholine (ACh), propionylcholine (PCh), butyrylcholine (Bch)

Enzymes	Substrates	Hydrolysis parameters		Substrate protective effect					
		$a_c \times 10^4$, min^{-1}	$K_M \times 10^{-3}$, M	total, $k_{II,0}/k_{II,S}$		primary, $(K_M + [S])/K_M$		secondary, $k_{II,0}/k_4$	tertiary, α (%)
				$[S]/K_M = 1$	$[S]/K_M = 10$	$[S]/K_M = 1$	$[S]/K_M = 10$		
Horse serum BuChE	ACh	6.5	1.2	5	10	2	11	3.5	22
	PCh	10	1.0	6	15	2	11	3.5	14
	BCh	14	1.0	7	25	2	11	4.5	5.5
Human erythrocyte AChE	ACh	30	0.1	14	30	2	11	8	14
	PCh	22	0.1	12	25	2	11	7.5	20
Pacific squid ChE	ACh	11	0.06	55	80	2	11	25	20
	PCh	9	0.11	25	55	2	11	8	6
	BCh	7	0.08	20	70	2	11	5.5	0

Thus, the value of the a factor can vary from 1 down to 0.1–0.02.

From the equation (5), it follows that

$$k_{II,S} = k_4\, K_M/(K_M + [S]) + a\, k_5\, [S]/(K_M + [S]). \quad (6)$$

The first summand characterizes interaction of OPI with the free ChE, while the second, with the acylated enzyme. To estimate contribution of reaction with acylated ChE to the $k_{II,S}$ value, we are to consider a special case, when $[S] = K_M$. The equation (6) takes a form $k_{II,S} = k_4/2 + a \times k_5/2$. Then the portion of reaction with acylated ChE (α, %) is $\alpha = 100 \times a \times k_5/(k_4 + a \times k_5)$.

It might have been expected that the rate constant of OPI reaction with free ChE (k_4) should correspond to the value of the bimolecular rate constant of OPI interaction with ChE, when this value is measured in the absence of substrate ($k_{II,0}$) [1]. However, in the experiment, as a rule, $k_{II,0} >> k_4$ (Table 1), i.e., the reaction rate of OPI with ChE that was not in contact with substrate is much higher than the reaction rate of OPI with free ChE participating in the substrate hydrolysis. With decrease of the substrate concentration in the interval $[S] < K_M$, this difference disappears. The substrate seems to somehow change conformation of the ChE active center, preventing reaction with OPI, this effect reaching maximum at saturation of enzyme with substrate.

The fact that some OPI can also react with the acylated enzyme should reduce the summarized substrate protective effect. However, as seen from our experimental data (Tables 1–3), $k_5 < k_4$, which seems to be due to steric difficulties at sorption of OPI on the acylated enzyme. The ability of OPI to interact slower with acylated ChE, than with free ChE, also results in a decrease of the enzyme inactivation rate.

We can try formulating what the "substrate protective effect" is. In our opinion, it is a phenomenon associated with that the irreversible inhibitor inhibits the enzyme activity in the presence of substrate less effectively, than in its absence. As a whole, it is expressed quantitatively by the ratio $k_{II,0}/k_{II,S}$ and can be called the "summarized substrate protective effect." What are possible features of the phenomenon of the substrate protective effect for ChE?

First, when OPI is added to the reaction mixture containing ChE and substrate, the inhibitor has to interact not with all ChE ($[E]_0$, but only with free ($[E]_f$, whose concentration is lower $(K_M + [S])/K_M$ times. Thus, this is the "primary substrate protective effect" that depends on the enzyme nature and substrate structure (the K_M value), but should not depend on the OPI structure.

Second, as established experimentally, the sensitivity of free ChE to OPI action is different (as a rule, lower), than of ChE without contact with substrate. This may be considered the "secondary substrate protective effect" that is associated both with the enzyme nature and with the structure of substrate and OPI.

Third, the previous works [6–11] have shown that in some cases the interaction of OPI is possible not only with free, but also with acylated ChE. In fact, this should have reduced the "primary substrate protective effect" due to elevation of the concentration of ChE, with which the OPI can interact, which increases the degree of the ChE inactivation in the presence of substrate. However, as it was to expect, the OPI reaction with acylated ChE runs with a lower rate, than with free enzyme due to steric hindrances at sorption. This effect may be called the "tertiary substrate protective effect," which, like the secondary one, depends both on the enzyme nature and on the structures of substrate and OPI.

RESULTS AND DISCUSSION

The analysis of Table 1 data allows the following conclusions to be made.

1. The summarized protective effect of acetylcholine ($k_{II,0} / k_{II,S}$) practically does not correlate with anticholinesterase action of OPI ($k_{II,0}$).

2. Since presented here are the data on interaction of OPI varying in structure with BuChE in the presence of acetylcholine ($[S] = 10^{-2}$ M, $K_M = 1.25 \times 10^{-3}$ M [6]), the primary protective effect reflecting decrease of the free ChE concentration $(K_M + [S])/K_M$ times, should be equal to 9 regardless of the OPI structure.

3. As seen from Table 1, the total protective effect is higher than the primary one, these excesses being 5–8-fold for OPI XV–XVII.

4. The secondary protective effect of acetylcholine turned out to be more expressed for hydrophobic OPI with *tert*-butyl group (XII–XVII). Probably, changes in the BuChE active center under action of acetylcholine affect configuration of hydrophobic areas.

5. The tertiary protective effect of acetylcholine was absent in cases of hydrophobic OPI with alkyl chain length of 6 and more carbon atoms (VIII–XI, XV–XVII) and was low in the case of DFP, which seems to be due to spatial hindrances at sorption on acetylated enzyme.

The analysis of Table 2 data also allows several conclusions.

1. No correlation has been revealed between parameters of the enzymatic hydrolysis and protective effect of the studied substrates.

2. The total protective effect at the identical concentration of substrate ($[S] = 10^{-2}$ M) rose in a row ACh < PCh < BCh < VCh, whereas at the identical degree of enzyme saturation by substrate, ($[S]/K_M = 10$), this dependence was somewhat different: ACh < PCh < BCh > VCh.

3. The dependence of the primary protective effect on the substrate structure repeated qualitatively the total effect at the identical substrate concentrations. In the case of the identical degree of the enzyme saturation by substrate (($[S]/K_M = 10$), the values of the primary protective effect should have been 11, which does not agree with the obtained experimental data.

4. The secondary protective effect had a tendency for rise with increase of the size of the acyl radical of both choline and thiocholine substrates: ACh < PCh < BCh = VCh and AThCh < PThCh < BThCh.

5. The tertiary protective effect of ACh was higher than of PCh and was absent for BCh and VCh. In the case of thiocholine substrates, the decrease was observed throughout the row AThCh > PThCh > BThCh.

6. We failed to reveal the summarized protective effect for formylcholine, which made it impossible to carry out further kinetic analysis.

7. The phenylacetate (PhA) showed a low total protective effect that seems to be completely realized as the primary effect due to a decrease of the free ChE concentration. In the presence of

phenylacetate there was no decrease observed of the BuChE reactivity to one of the studied OPI, compound VI, i.e., the secondary protective effect was absent. It is also shown that compound VI did not interact with acetylated ChE ($\alpha = 0$). This is in accordance with Volkova's data [9].

As shown by Volkova [7, 9], in the former case, at inhibition of BuChE by the group of OPI (I–IV) in the presence of ACh and PhA, the pronounced interaction of inhibitors with acetylated enzyme was observed, whereas in the latter case, this effect was absent. In this connection, it was suggested [9, 16] that the limiting stages are different at the process of hydrolysis of these substrates: deacetylation (ACh) or acetylation (PhA), and, hence, concentration of acetylated enzyme is very low in the latter case, so the inhibitor merely has nothing to interact with. However, physical-chemical and kinetic controversies occur here. First, stability of the ester bond in PhA should be much lower than in ACh: the pK value for phenol is 9.98, and for choline, the aliphatic alcohol, it is 15.5 [17]; hence, the acetylation reaction should run at a higher rate in the case of PhA. Second, it is not clear kinetically at which stage, acetylation or deacetylation, the alcohol desorption occurs; therefore, it is impossible to state that the acetylated enzyme in both cases is identical [16]. The problem of desorption of alcohol (even of choline) is also actual in cases of different acyl groups (compare the ACh, PCh, and BCh hydrolysis) (Table 2) and at catalysis by various enzymes (Table 3).

Based on the analysis of the Table 3 data, the following conclusions may be made:

1. The total protective effect of the studied substrates both at high and at low concentrations depended essentially on the enzyme nature: it was the highest in squid ChE, lower in AChE, and the lowest in BuChE.

2. The total substrate protective effects was different in different enzymes depending on the substrate structure: it was ACh > PCh > BCh in BuChE, and ACh > PCh in AChE both at high and low substrate concentrations, while for the squid ChE the effect depended on the substrate concentration: ACh > PCh > BCh at low, and ACh > BCh > PCh at high concentrations.

3. The primary protective effect in the present experiment design, when we compared substrates at the identical degree of enzyme saturation ($[S]/K_M$), also is the constant value: it is 2 at the low substrate concentration, and 11, at the high one. In other words, the degree of decrease of the free ChE concentration did not depend either on the enzyme nature, or on the substrate structure. Nevertheless, the primary protective effect was lower, than the total effect in all cases (and essentially lower in the case of squid ChE).

4. The secondary protective effect in squid ChE decreased in the row ACh > PCh > BCh (ACh exceeded BCh 3 times); in BuChE and AChE it practically did not depend on the substrate structure. It should be noted that ACh reduced sensitivity of squid ChE to compound IV 25 times (!).

5. The tertiary protective effect in different enzymes depended differently on the substrate structure: in BuChE and squid ChE this factor decreased in the row ACh > PCh > BCh, and compound IV did not interact with acylated squid ChE in the presence of BCh; the tertiary protective effect of ACh was higher than of PCh in the case of AChE.

CONCLUSION

In the present work, an attempt has been made to summarize the authors' own data as well as the data from the literature on the problem of interaction of irreversible OPI with ChE in the presence of substrate (the substrate protective effect). The kinetic analysis of this triple system, enzyme–substrate–inhibitor, is carried out by studies on effect of the structure of both effectors and the enzyme nature. This has allowed us to define and to evaluate more precisely the major components of the studied phenomenon. Such approach provides a new important information both in the field of enzymatic kinetics and for further development of comparative-enzymologic investigations.

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