Kinetics of 13 New Cholinesterase Inhibitors

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Kinetics of hydrolysis of acetylcholine and acetylthiocholine by two types of acetylcholinesterase and butyrylcholinesterase inhibited by 13 new inhibitors (5 carbamates and 8 carbazates – hydrazinium derivatives) was measured *in vitro* in a batch reactor at 25 °C, pH 8, ionic strength 0.11 M and enzyme activity 3.5 U by four nondependent analytical methods. Sevin®, rivastigmin (Exelon®) and galantamin (Reminyl®) served as comparative inhibiting standards. Kinetics of hydrolyses inhibited by all studied carbamates, sevin, carbazates (with exceptions) and rivastigmin (with exceptions) can be simulated by the competitive inhibition model with irreversible reaction between enzyme and inhibitor. Galantamin does not fulfil this model. In positive simulations, the value of inhibition (carbamoylation) rate constant k_3 was calculated, describing the reaction velocity between the given enzyme and inhibitor. Physiologically important hydrolyses of acetylcholine catalyzed by acetylcholinesterase from electric eel or bovine erythrocytes and butyrylcholinesterase from horse plasma can be most quickly inhibited by carbamoylation of the mentioned enzymes by the 3-*N,N*-diethylaminophenyl-*N′*-(1-alkyl) carbamates **4** and **5**. Probably this is due to a long enough hydrocarbon aliphatic substituent (hexyl and octyl) on the amidic nitrogen atom. The tested carbazates failed as inhibitors of cholinesterases. The regeneration ability of the inhibited enzymes was not measured.

Key words: Cholinesterases, Inhibition, Kinetics

Introduction

Our research group is studying already for a longer time the kinetics and mechanism of *in vitro* enzymatic hydrolysis of acetylcholine inhibited by new substances which could be potential drugs against Alzheimer disease. In our last three papers two new methods for kinetic research of acetylcholine (ACH) and acetylthiocholine (ATCH) hydrolyses (Štěpánková *et al.*, 2005), kinetics of total hydrolyses of ACH and ATCH by acetylcholinesterase (ACHE) and butyrylcholinesterase (BCHE) using four independent analytical methods (Zdražilová *et al.*, 2006), and values of the half-inhibition index pI_{50} of ATCH hydrolysis inhibited by 13 new potential cholinesterase inhibitors (5 carbamates and 8 hydrazinium derivatives – carbazates) (Zdražilová *et al.*, 2004) were described. This work deals with the kinetics and mechanism of *in vitro* hydrolyses of ACH and ATCH by two types of ACHE and two types of BCHE inhibited by the 13 inhibitors mentioned above.

Materials and Methods

Chemicals

ACHE chloride (99%, p.a.), ATCH iodide (99%, p.a.), choline (CH) chloride (99%, p.a.), ACHE1 (from electric eel, type VI-S), ACHE2 (from bovine erythrocytes, type XII-S), BCHE2 (from horse plasma), 5,5′-dithiobis-2-nitrobenzoic acid (Ellman′s reagent, DTNB) were all from Sigma-Aldrich, Praha, CZ; BCHE1 (from horse plasma) was a gift of the Department of Toxicology, Purkyně Military Medical Academy, Hradec Králové, CZ; galantamin (Reminyl®), (4a*S*,6-*R*,8a*S*)-4a,-5,9,10,11,12-hexahydro-3-methoxy-11-6*H*-benzofuro-[3a,3,2-*ef*][2]benzazepin-6-ol were from Janssen, Belgium; rivastigmin (Exelon®), (+)*S*-*N*-ethyl-3-[(1-dimethylamino)ethyl]-*N*-methylphenyl carbamate hydrogentartrate were from Novartis, Switzerland; Sevin®, 1-naphthyl-*N*-methyl carbamate were from Merck-Schuchard, Munich, Germany. The studied 5 carbamates and 8 carbazates were synthesized in the Department

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of Organic Chemistry, University of Pardubice, CZ. Their purity was verified by thin layer chromatography (carbamates) and by comparison of melting points with published data (carbazates). Their structures are described in Table I. Dioxane (p.a.), $Fe(NO_3)_3 \cdot 9H_2O$ (p.a.), hydrochloric acid (35%, p.a.), KCl (p.a.), KOH (p.a.), KH_2PO_4 (p.a.), $Na_2HPO_4 \cdot 12H_2O$ all from Lachema, Brno, CZ; hydroxylamine (p.a.) was from Reactivul Bucuresti, Romania; argon was from Linde, CZ. Demineralized water was used as the solvent for all substances; just carbamates were first solved to 0.05 M solutions in dioxane and then diluted to smaller concentrations with water. The freshly prepared aqueous solutions of ACHE and BCHE were immediately divided into portions kept at −6 °C and then separately melted for daily use.

Methods and apparatus

The measurements proceeded in a batch ideally mixed glass reactor at 25 °C, pH 8, ionic strenght 0.11 M (adjusted by KCl) and catalytic activity 3.5 U (ACHE2: only 0.5 U, according to its small solubility). U is the international unit of catalytic (specific) activity; the activity of 1 U has that amount of the enzyme production which transforms 1 μmol of substrate in 1 min under given conditions (temperature, pH, ionic strength, etc.).

The kinetic experimental data were determined at least by two of four independent analytical methods: Ellman's (ELM), pH-stat (PHS), hydroxylamine (HXA) and chromatographic (HPLC). Instruments, principles and measuring techniques of these methods are described in detail in Štěpánková *et al.* (2005) and Zdražilová *et al.* (2006).

The kinetic informations about the measured inhibitions were calculated from the mentioned dependences by three independent mathematical procedures described in Zdražilová *et al.* (2006), using the following considerations.

Theory

According to results in Zdražilová *et al.* (2006) all uninhibited *in vitro* hydrolyses of ACH or ATCH by ACHE or BCHE (aqueous solution, 25 °C, pH 8, ionic stregth 0.11 M) can be explained by a Michaelis-Menten (Briggs-Haldane) reaction scheme with the second step being irreversible:

$$\mathrm{E} + \mathrm{S} \underset{k_{-1}}{\overset{k_1}{\leftrightarrows}} \mathrm{ES}, \tag{1}$$

$$\mathrm{ES} + \mathrm{H_2O} \overset{k_2}{\rightarrow} \mathrm{E} + \mathrm{P} + \mathrm{HA}, \tag{2}$$

where E is the enzyme, S the substrate, ES the complex enzyme-substrate, P the product and HA acetic acid. The determined rate constants k_i must be, under identical conditions, valid also for the same but inhibited enzymatic hydrolysis. Carbamates and carbazates, used as inhibitors (I), are irreversible (covalent) inhibitors, because they are structurally similar to ACH or ATCH and create in the first step a covalent bond at the esteratic site of ACHE (BCHE) between the hydroxy group of Ser200 and the nitrogen atom of the carbamate (carbazate):

$$\mathrm{E} + \mathrm{I} \overset{k_3}{\rightarrow} \mathrm{EI}. \tag{3}$$

The carbamoylated enzyme is relatively stable, so that the configuration EI can exist for a much longer time than the complex ES. Therefore, in this reaction time interval step (3) can be taken as irreversible. So, the reaction scheme (1)–(3) represents the competitive inhibition with irreversible formation of EI.

Measuring procedures

The measurements proceeded as described in the same chapter of Zdražilová *et al.* (2006), but simultaneously with the dose of enzyme preparation solution (starting the hydrolysis) the dose of given inhibitor solution was (separately) added to the mixture of water, substrate, buffer, DTNB, KCl (according to the analytical method).

Calculations

From the original measurements the experimental dependences actual concentrations ([S] and/or [P] and/or [HA]) *vs.* reaction time t were calculated. These dependences were tested for validity of the Michaelis-Menten equation

$$-\mathrm{d[S]}/\mathrm{d}t = \mathrm{d[P]}/\mathrm{d}t = \mathrm{d[HA]}/\mathrm{d}t = V_\mathrm{m}' \, [\mathrm{S}]/(K_\mathrm{M}' + [\mathrm{S}]), \tag{4}$$

and competitive irreversible inhibition reaction scheme (1)–(3) by means of the PC program GEPASI (Mendes, 1993, 1997; Mendes and Kell, 1998; http://gepasi.dbs.aber.ac.uk/softw/...).

The fulfilment of the Michaelis-Menten equation (4) in Briggs-Haldane modification [$V_m = k_2' [E]_0$, $K_M' = (k_{-1}' + k_2')/k_1'$] gave the optimal values of the modified rate constants k_i' and therefore also V_m' and K_M'.

The same dependences were compared with the solution of the system of differential kinetic equations derived from scheme (1)–(3) of competitive irreversible inhibition using as constants the average values of the rate constants k_1, k_{-1} and k_2, obtained for the uninhibited hydrolysis of the given combination of (S + E), and given analytical method under the same reaction conditions. From positive comparison (fitting) of experimental and theoretical dependences the optimal values of the inhibition rate constant k_3 were obtained.

Results

Hydrolyses of all (S + E) combinations (S: ACH, ATCH; E: ACHE1, ACHE2, BCHE1, BCHE2) inhibited by (some or all of) 5 carbamates and 8 carbazates from Table I and 3 comparative inhibitors (sevin, galantamin, rivastigmin)

Table I. Names of the studied inhibitors [**1**–**5**, carbamates, **6**–**13**, carbazates (hydrazinium derivatives)].

Inhibitor	Name
1	3-*N,N*-Diethylaminophenyl-*N'*-(1-ethyl) carbamate
2	3-*N,N*-Diethylaminophenyl-*N'*-(1-propyl) carbamate
3	3-*N,N*-Diethylaminophenyl-*N'*-(1-butyl) carbamate
4	3-*N,N*-Diethylaminophenyl-*N'*-(1-hexyl) carbamate
5	3-*N,N*-Diethylaminophenyl-*N'*-(1-octyl) carbamate
6	Phenylester of 2-methyl-hydrazinocarboxylic acid
7	2-Chloro-phenylester of 2-methyl-hydrazinocarboxylic acid
8	3-Chloro-phenylester of 2-methyl-hydrazinocarboxylic acid
9	4-Chloro-phenylester of 2-methyl-hydrazinocarboxylic acid
10	Phenylester of 2,3-dimethyl-hydrazinocarboxylic acid
11	2-Chloro-phenylester of 2,3-dimethyl-hydrazinocarboxylic acid
12	3-Chloro-phenylester of 2,3-dimethyl-hydrazinocarboxylic acid
13	4-Chloro-phenylester of 2,3-dimethyl-hydrazinocarboxylic acid

Table II. Average values of rate constants of competitive irreversible inhibition (k_3) of ACH hydrolysis by ACHE1, ACHE2 and BCHE2 with the tested inhibitors and their concentration separation coefficients 1-octanol/water (K_S).

I[a]	K_S	ACH + ACHE1 k_3 [l/(mol s)]			ACH + ACHE2 k_3 [l/(mol s)]			ACH + BCHE2 k_3 [l/(mol s)]		
		PHS[b]	HXA[b]	HPLC[b]	PHS[b]	HXA[b]	HPLC[b]	PHS[b]	HXA[b]	HPLC[b]
S		96.36	63.51		12.09	6.92		122.1	87.51	
R	0.087	M N	N	M N	24.9	13.77	17.87	187.6	653.4	593.5
G	0.108	M N	M 7.90		M N	M N		M N	5.562	
1	2.49	0.8623	2.7		29.15	76.79	54.12	386.3	122.8	
2	11.24	38.31	3.891		39.36	67.84		567	210.5	
3	12	76.14	58.43		43.63	78.6		673.1	3258	
4	18.57	99.07	100.7	6.904	144.8	69.46	140.4	2183	2488	2597
5	19.75	M 11.6	51.92	16.65	73.55	90	128.4	2162	7837	9137
6	0.084	63.84	4.155		1.487	1.89		28	21.81	
7	0.077	M N	M 0.689		1.06	M N		230.6	225.1	
8	0.091	M N	M 0.541		0.4504	1.533		77.43	6.14	
9	0.088	M N	1.065		0.0845	0.2463		73.81	5.504	
10	0.082	M N	M		M 0.0103	0.0568		35.97	31.53	
11	0.106	M N	M 0.353		1.108	1.318		135.4	91.72	
12	0.114	M N	M N		M N	0.1127		63.21	38.25	
13	0.68	M N	M N		0.0243	0.0507		2.841	5.497	

[a] I, inhibitor; S, sevin; R, rivastigmin; G, galantamin.
[b] PHS, pH-stat method; HXA, hydroxylamine method; HPLC, chromatographic method.
N, experimental data do not comply with the model; M, data fulfil the Michaelis-Menten equation; free window, unmeasured combination.

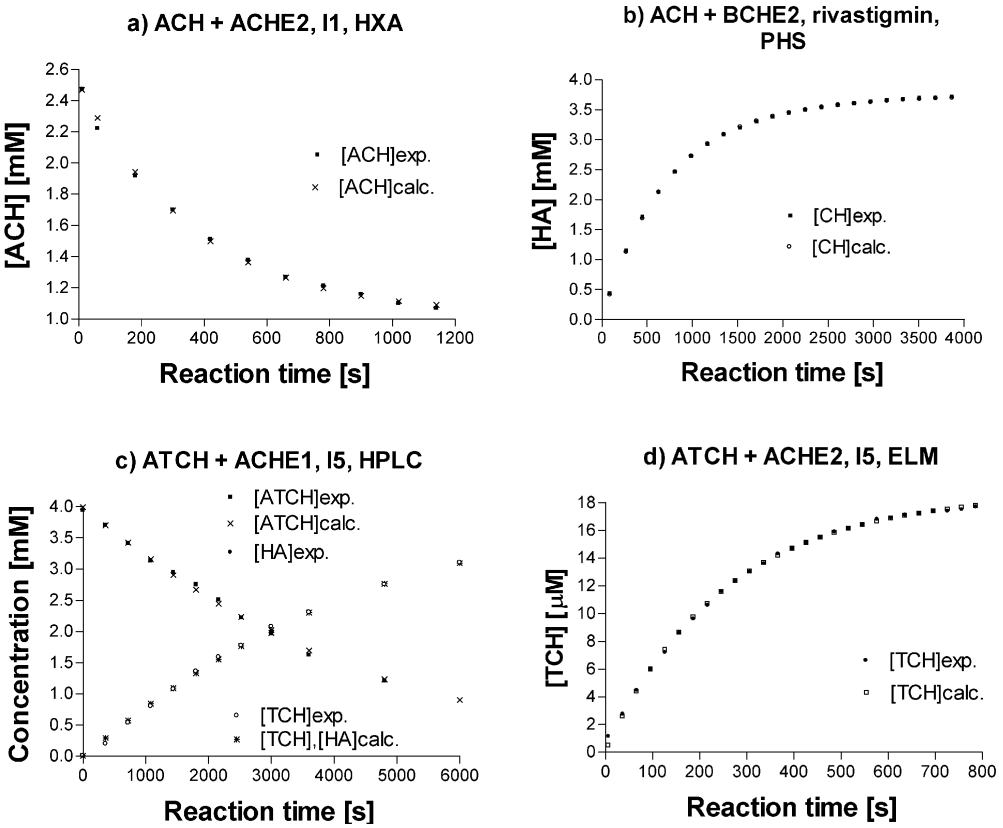

Fig. 1. Inhibited hydrolysis of: a) ACH by ACHE2: $[ACH]_0$ = 2.5 mM; $[I1]_0$ = 8 μM; optimal k_3 = 72.09 (M s)$^{-1}$; optimal $[E]_0$ = 0.478 nM; standard deviation sd = 27.2 μM; HXA method; b) ACH by BCHE2: $[ACH]_0$ = 4 mM; $[R]_0$ = 4 μM.; optimal k_3 = 189.5 (M s)$^{-1}$; optimal $[E]_0$ = 0.2342 μM; sd = 12 μM; PHS method; c) ATCH by ACHE1: $[ATCH]_0$ = 4 mM; $[I5]_0$ = 0.16 mM; optimal k_3 = 1.269 (M s)$^{-1}$; optimal $[E]_0$ = 21.24nM; sd = 75.2mM; HPLC method; d) ATCH by ACHE2: $[ATCH]_0$ = 40 μM; $[I5]_0$ = 16 μM; optimal k_3 = 287.5 (M s)$^{-1}$; optimal $[E]_0$ = 25.88 nM; sd = 0.164 mM; ELM method.

were studied by at least two from four above mentioned independent analytical methods (ELM, PHS, HXA, HPLC). Based on orientation experiments, such an initial concentration of inhibitors $[I]_0$ was chosen to evoke a visible inhibition effect. The values of $[I]_0$ varied in the interval 0.32 μM [combination (ACH + BCHE2), inhibitor **5**] to 2 mM [(ATCH + ACHE2), inhibitor **10**)]. Every inhibited hydrolysis was 2–5 times reproduced with constant or different $[I]_0$ value.

The obtained dependences concentration *vs.* time were tested as described above. Examples of validity of the assumed mechanism are presented in Fig. 1.

The summary of average values of rate constants k_3 for all or some inhibitors, all combinations (S + E) and all used analytical methods is given in Table II (ACH) and Table III (ATCH). For the combination (ACH + BCHE1) only the inhibition power of **4** was determined with the result k_3 = 138.1 (M s)$^{-1}$. For more serious appreciation of the effect of the studied inhibitors, in Table II the values of concentration separation coefficients 1-octanol/water at ca. 25 °C for all used inhibitors (except for sevin), as the measure of ability of single inhibitors to cross through the hematoencephalic (brain) biomembrane (Štěpánková, 2006, unpublished results), and in Table III the values of half-inhibition indices pI_{50} (Zdražilová *et al.*, 2004) are given.

Table III. Average values of rate constants of competitive irreversible inhibition (k_3) of ATCH hydrolysis by ACHE1, ACHE2 and BCHE1, BCHE2 with the tested inhibitors and their pI_{50} values.

I[a]	ATCH + ACHE1				ATCH + ACHE2				ATCH + BCHE1		ATCH + BCHE2		
	k_3 [l/(mol s)]				k_3 [l/(mol s)]				k_3 [l/(mol s)]		k_3 [l/(mol s)]		
	ELM[b]	HXA[b]	HPLC[b]	pI_{50}	ELM[b]	HXA[b]	HPLC[b]	pI_{50}	ELM[b]	pI_{50}	ELM[b]	HXA[b]	HPLC[b]
S	959.5	131.2			671.1	M 30.02			290.6		1802	23.77	
R	M 431	M N	M N	3.3	63.17	M 42.76	M 16.16	4.1	720.1	4.7	688.1	452.9	174.8
G	M N	M N		6.4	N	M 256.8		6.4	M 816.8	5.1	M 539	M 186.4	
1	397.4	4.593		6.2	500.7	30.24		4.8	2296	6	4452	717.8	
2	335	2.38		6	430.3	M N		6.3	1452	6.2	1482	434.8	
3	577.7	7.256		5.7	632.3	86.33		6	1515	5.5	2884	3876	
4	306.9	4.519	3.394	5.3	485.8	856.6	M 444.1	5.6	2935	6.5	1734	2896	902
5	188.4	2.417	M 1.38	4.8	321.6	49.72	M 47.76	4.9	4624	6.9	1994	4612	1648
6	M 269.6	N		3.4	2.252	M 2.011		3.3	335.2	4.4	73.07	9.22	
7	444.3	N		3.7	148.9	M 8.596		5	6328	5.5	1252	M 30.62	
8	M 226.4	N		3.8	45.06	M 2.744		4.3	3679	5.3	237	22.9	
9	263.6	N		3.1	3.987	M 0.304		3.3	1909	3.45	25.19	M 3.795	
10	M 45.41	N		2.7	1.545	M 0.130		2.9	1373	4.1	50.64	46.98	
11	M 141.2	N		3.7	10.56	M 3.427		4.2	1366	4.9	130.7	M 66.88	
12	M 616.3	N		3.1	2.471	M 0.317		3.1	1096	4.2	175.8	N	
13	M 96.31	N		2.5	1.156	M 0.666		2.3	341.1	2.9	6.201	N	

[a] I, inhibitor; S, sevin; R, rivastigmin; G, galantamin.
[b] ELM, Ellman's method; HXA, hydroxylamine method; HPLC, chromatographic method.
N, experimental data do not comply with the model; M, data fulfil the Michaelis-Menten equation; free window, unmeasured combination.

Discussion

1.) Not all 13 inhibitors were tested in all 8 combinations of (S + E) by all suitable analytical methods mentioned above. The reasons were a) limited amounts of specially synthesized inhibitors and of enzyme BCHE1; b) time demand factor: over 700 experiments were done; some of them lasted 10 min but another also several hours; c) the most complicated HPLC analytical method was used only for the most promising inhibitors.

2.) All inhibitions with studied carbamates can be described by the model of competitive inhibition with irreversible synthesis of EI according to equations (1) – (3). The same model is valid also for other studied carbazates, but with several exceptions (see Tables II and III). This mechanism fulfil also sevin and rivastigmin (with exceptions). Galantamin, as could be expected, does not fulfil this model, because it is not a covalent (irreversible) inhibitor.

3.) A number of experimental dependences which do not fulfil the model of competitive irreversible inhibition (but also some which fulfil it) comply with the Michaelis-Menten model (see Tables II and III). The determined values of k_i' or K_M' and V_m' (4) are generally complicated functions of true k_i, K_M, V_m, initial concentration of inhibitors and other parameters (*e.g.* equilibrium concentration constants of the reactions between S, E and I) (Kotyk and Horák, 1977). No relationship was found between our relevant K_M', V_m' and K_M, V_m values.

4.) The most important informations about the inhibited hydrolyses in Tables II and III are the average rate constants k_3 for single combinations of (S + E + I). The k_3 value is the quantitative measure of the velocity of inhibition of the given enzyme (carbamoylation for carbamates and carbazates) by the given inhibitor (at given conditions). The exactly defined quantity k_3, *i.e.* the number of inhib-

ited moles of the enzyme in 1 l of the reaction mixture in 1 s at unit molarities of enzyme and inhibitor depends (if it includes no other parameters, such as pH value, ionic strength etc.) only on temperature. It describes therefore, in our view, better the inhibition power of individual inhibitors than the currently used parameters as pI_{50} or enzyme activity, which depend always on the measurement method (at given conditions) and so their values are mostly incomparable due to variable assay techniques.

5.) On the other hand, the value of k_3 predicates nothing about the rate of the possible (total or partial) regeneration (decarbamoylation) of the given enzyme. To discover this effect it is necessary, *e.g.*, to measure the time dependence of the increasing activity of the inhibited enzyme to hydrolyze the substrate.

6.) Every reaction of (S + E + I) was reproduced at least three times, mostly with various initial concentrations $[I]_0$ at else identical conditions. At smaller $[I]_0$ the k_3 values were either greater or practically identical without any dependence on the combination of (S + E + I). These differences disappeared using the same $[I]_0$ values.

7.) All combinations of (S + E), see Tables II, III, show that the greatest inhibition power, expressed by the k_3 value, are found with inhibitors **4** and **5**. The inhibition by these two inhibitors was therefore studied for all combinations (S + E) by all suitable analytical methods except for BCHE1, which was not available in sufficient quantity.

8.) Most of the experimental inhibited dependences of concentration *vs.* time are nonlinear and produce monotonic curves limited to a certain value. This is in agreement with their theoretical course according to the reaction scheme (1)–(3) or the Michaelis-Menten equation (4). But some obtained dependences are evidently linear. This experimental reality can be explained in compliance with the mentioned reaction model: Scheme (1)–(3) is described, according to the laws of formal reaction kinetics for isochoric reactions, by a relevant system of differential kinetic equations concentration *vs.* time for all reaction components. If the condition [ES] ≈ const. is matched (most probable for inhibited hydrolyses, where the initial concentration $[E]_0$ and therefore also [ES] decrease substantially owing to the formation of EI), then [ES] is very small in comparison with [S], [P] and [HA], and therefore practically constant during the reaction time. From the combination of this steady state condition for ES ([ES] ≈ const., *i.e.* $d[ES]/dt \approx 0$) with the system of differential kinetic equations mentioned above it follows:

$$-d[S]/dt = d[P]/dt = d[HA]/dt = k_2\,[ES] = k. \quad (5)$$

After integration this relation leads to the linear dependences between [S], [P] and [HA] and reaction time t

$$[S]_0 - [S] = [P] = [HA] = k\,t \quad (6)$$

at zero initial concentrations of P and HA. The reaction scheme (1)–(3) complies at such conditions the reaction of zero order on the mentioned components. Some experiments show even a combined course: The dependence concentration *vs.* time begins with a short nonlinear part which passes (sharply or continuously) to a longer linear part.

9.) In Tables II and III the summary of average values of the rate constant k_3 is given for all studied inhibitors, (S + E) combinations and used analytical methods. For complex checking of effectivity of studied inhibitors, Table II (ACH) includes also their separation concentration coefficients in the milieu 1-octanol (1)/water (2), K_S = [I](1)/[I](2), which are the measure of the ability of single inhibitors to cross over the hematoencephalic membrane. In Table III (ATCH) the values of indices pI_{50} measured by the ELM method with incubation are presented (except of combination with BCHE2) .

10.) At a majority of combination of (S + E + I) in Tables II and III the average values of k_3 determined by various methods are of the same order. Exceptions can be explained by different reaction conditions necessary for realization of the individual analytical methods: ELM, HXA and HPLC methods are buffered, the PHS method must work without any buffer, HXA and HPLC methods require collection of samples, for the ELM method the surplus of a strange aggresive substance (DTNB) is in the reaction mixture, which can in greater surplus evidently affect the course of hydrolysis (Komersová, 2006, unpublished results). For searching more effective inhibitors of ACHE it is important, that the k_3 values for every physiologically relevant combination (ACHE + E + I) and used analytical method (even if the absolute values are different) determine the same order of studied inhibitors according to the velocity of blocking the given enzyme. Unphysiogical combinations (ATCH + E + I) show a series of excep-

tions. From Tables II and III follows also, that the inhibition of BCHE by the tested inhibitors proceeds about ten times faster than the inhibition of ACHE.

11.) The determined values of k_3, pI_{50} and K_S of the 13 studied inhibitors lead to the conclusion, that the most suitable for the inhibition (blocking, carbamoylation) of ACH and ATCH *in vitro* hydrolysis by ACHE and BCHE are the carbamates **4** and **5**. They differ from the other tested carbamates **1**–**3** by a longer hydrocarbon chain (hexyl and octyl to ethyl, propyl and butyl) at the amidic nitrogene atom. With physiologically most important combinations of (ACH + ACHE) the difference in the k_3 values of tested and standard (sevin, rivastigmin) carbamates is not too significant (average ratio 5.4). For the combination (ACH + BCHE2) the differences between the k_3 values of **4**, **5** and sevin, rivastigmin are greater (average ratio 21.2).

On the other hand the substituents used in the tested carbazates do not amplify enough the inhibition effect of the carbazate structure, except for the combination (ATCH + BCHE1), which is indeed not physiogically important.

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