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. Galantamin does not fulfill this model. 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-diethylamino-phenyl-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 \( \text{pI}_{50} \) 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®), (4aS,6R,8aS)-4a,5,9,10,11,12-hexahydro-3-methoxy-11-6H-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...
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(NO₃)₃⋅9H₂O (p.a.), hydrochloric acid (35%, p.a.), KCl (p.a.), KOH (p.a.), KH₂PO₄ (p.a.), Na₂HPO₄⋅12H₂O all from Lachema, Brno, CZ; hydroxylamine (p.a.) was from Reactivul Bucuresti, Romania; argon was from Linde, CZ. De-mineralized 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 Štepánková 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 strenght 0.11 m) can be explained by a Michaelis-Menten (Briggs-Haldane) reaction scheme with the second step being irreversible:

\[ \begin{align*}
E + S & \rightleftharpoons ES, \\
ES + H₂O & \rightarrow E + P + HA,
\end{align*} \]

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):

\[ \begin{align*}
E + I & \rightarrow EI.
\end{align*} \]

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

\[ -d[S]/dt = d[P]/dt = d[HA]/dt = V_m' [S]/(K_M' + [S]), \]

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 \( V_m = \frac{k_2}{[E]_0} \), \( K_{M'} = \frac{(k_1' + k_2')/k_1'}{k_2} \) gave the optimal values of the modified rate constants \( k_1' \) 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 \( k_1, k_\text{-1}, \) 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 (\( S: \text{ACH, ATCH}; E: \text{ACHE1, ACHE2, BCHE1, BCHE2} \)) inhibited by (some or all of) 5 carboxylates and 8 carbazates from Table I and 3 comparative inhibitors (sevin, galantamin, rivastigmin)

<table>
<thead>
<tr>
<th>Inhibitor Name</th>
<th>( k_3 ) [l/(mol s)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 3-N,N-Diethylaminophenyl-N’-(1-ethyl) carbamate</td>
<td>( k_3 ) [l/(mol s)]</td>
</tr>
<tr>
<td>2 3-N,N-Diethylaminophenyl-N’-(1-propyl) carbamate</td>
<td>( k_3 ) [l/(mol s)]</td>
</tr>
<tr>
<td>3 3-N,N-Diethylaminophenyl-N’-(1-butyl) carbamate</td>
<td>( k_3 ) [l/(mol s)]</td>
</tr>
<tr>
<td>4 3-N,N-Diethylaminophenyl-N’-(1-hexyl) carbamate</td>
<td>( k_3 ) [l/(mol s)]</td>
</tr>
<tr>
<td>5 3-N,N-Diethylaminophenyl-N’-(1-octyl) carbamate</td>
<td>( k_3 ) [l/(mol s)]</td>
</tr>
<tr>
<td>6 Phenylester of 2-methyl-hydrazinocarboxylic acid</td>
<td>( k_3 ) [l/(mol s)]</td>
</tr>
<tr>
<td>7 2-Chloro-phenylester of 2-methyl-hydrazinocarboxylic acid</td>
<td>( k_3 ) [l/(mol s)]</td>
</tr>
<tr>
<td>8 3-Chloro-phenylester of 2-methyl-hydrazinocarboxylic acid</td>
<td>( k_3 ) [l/(mol s)]</td>
</tr>
<tr>
<td>9 4-Chloro-phenylester of 2-methyl-hydrazinocarboxylic acid</td>
<td>( k_3 ) [l/(mol s)]</td>
</tr>
<tr>
<td>10 Phenylester of 2,3-dimethyl-hydrazinocarboxylic acid</td>
<td>( k_3 ) [l/(mol s)]</td>
</tr>
<tr>
<td>11 2-Chloro-phenylester of 2,3-dimethyl-hydrazinocarboxylic acid</td>
<td>( k_3 ) [l/(mol s)]</td>
</tr>
<tr>
<td>12 3-Chloro-phenylester of 2,3-dimethyl-hydrazinocarboxylic acid</td>
<td>( k_3 ) [l/(mol s)]</td>
</tr>
<tr>
<td>13 4-Chloro-phenylester of 2,3-dimethyl-hydrazinocarboxylic acid</td>
<td>( k_3 ) [l/(mol s)]</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Inc.</th>
<th>( K_S )</th>
<th>( \text{ACH + ACHE1} )</th>
<th>( \text{ACH + ACHE2} )</th>
<th>( \text{ACH + BCHE2} )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>( k_3 ) [l/(mol s)]</td>
<td>( k_3 ) [l/(mol s)]</td>
<td>( k_3 ) [l/(mol s)]</td>
</tr>
<tr>
<td>S</td>
<td>0.087</td>
<td>63.51</td>
<td>12.09</td>
<td>122.1</td>
</tr>
<tr>
<td>R</td>
<td>0.108</td>
<td>7.90</td>
<td>13.77</td>
<td>187.6</td>
</tr>
<tr>
<td>G</td>
<td>2.49</td>
<td>0.8623</td>
<td>2.7</td>
<td>24.9</td>
</tr>
<tr>
<td>1</td>
<td>11.24</td>
<td>2.7</td>
<td>29.15</td>
<td>54.12</td>
</tr>
<tr>
<td>3</td>
<td>12</td>
<td>58.43</td>
<td>43.63</td>
<td>140.4</td>
</tr>
<tr>
<td>4</td>
<td>18.57</td>
<td>100.7</td>
<td>144.8</td>
<td>140.4</td>
</tr>
<tr>
<td>5</td>
<td>19.75</td>
<td>51.92</td>
<td>73.55</td>
<td>128.4</td>
</tr>
<tr>
<td>6</td>
<td>0.084</td>
<td>4.155</td>
<td>1.487</td>
<td>28</td>
</tr>
<tr>
<td>7</td>
<td>0.077</td>
<td>0.689</td>
<td>1.06</td>
<td>230.6</td>
</tr>
<tr>
<td>8</td>
<td>0.091</td>
<td>0.541</td>
<td>0.4504</td>
<td>77.43</td>
</tr>
<tr>
<td>9</td>
<td>0.088</td>
<td>0.0655</td>
<td>0.0845</td>
<td>73.81</td>
</tr>
<tr>
<td>10</td>
<td>0.082</td>
<td>M</td>
<td>M</td>
<td>5.045</td>
</tr>
<tr>
<td>11</td>
<td>0.106</td>
<td>M</td>
<td>0.0103</td>
<td>35.97</td>
</tr>
<tr>
<td>12</td>
<td>0.114</td>
<td>M</td>
<td>0.1127</td>
<td>135.4</td>
</tr>
<tr>
<td>13</td>
<td>0.68</td>
<td>M</td>
<td>0.0243</td>
<td>63.21</td>
</tr>
</tbody>
</table>

\( a \) Inc. inhibitor; S, sevin; R, rivastigmin; G, galantamin.

<table>
<thead>
<tr>
<th>( b )</th>
<th>Method</th>
<th>( k_3 ) [l/(mol s)]</th>
<th>( k_3 ) [l/(mol s)]</th>
<th>( k_3 ) [l/(mol s)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHS</td>
<td>pH-stat method</td>
<td>M</td>
<td>M</td>
<td>M</td>
</tr>
<tr>
<td>HXA</td>
<td>hydroxylamine method</td>
<td>M</td>
<td>M</td>
<td>M</td>
</tr>
<tr>
<td>HPLC</td>
<td>chromatographic method</td>
<td>M</td>
<td>M</td>
<td>M</td>
</tr>
</tbody>
</table>

N, experimental data do not comply with the model; M, data fulfil the Michaelis-Menten equation; free window, unmeasured combination.
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 \(k_3 = 138.1 \text{ (m s)}^{-1}\). 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 (Štepánková, 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.

<table>
<thead>
<tr>
<th>I</th>
<th>ATCH + ACHE1</th>
<th>ATCH + ACHE2</th>
<th>ATCH + BCHE1</th>
<th>ATCH + BCHE2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k_3$ [l/(mol s)]</td>
<td>$k_3$ [l/(mol s)]</td>
<td>$k_3$ [l/(mol s)]</td>
<td>$k_3$ [l/(mol s)]</td>
</tr>
<tr>
<td></td>
<td>ELM$^b$</td>
<td>HXA$^b$</td>
<td>HPLC$^b$</td>
<td>pI$^{50}$</td>
</tr>
<tr>
<td>S</td>
<td>959.5</td>
<td>131.2</td>
<td>671.1</td>
<td>M 30.02</td>
</tr>
<tr>
<td>R</td>
<td>M .431</td>
<td>M N</td>
<td>M N</td>
<td>6.4</td>
</tr>
<tr>
<td>G</td>
<td>M N</td>
<td>M N</td>
<td>6.4</td>
<td>430.3</td>
</tr>
<tr>
<td>1</td>
<td>397.4</td>
<td>4.593</td>
<td>632.3</td>
<td>86.33</td>
</tr>
<tr>
<td>2</td>
<td>335</td>
<td>2.38</td>
<td>321.6</td>
<td>49.72</td>
</tr>
<tr>
<td>3</td>
<td>306.9</td>
<td>4.519</td>
<td>3.394</td>
<td>5.3</td>
</tr>
<tr>
<td>4</td>
<td>188.4</td>
<td>2.417</td>
<td>M 1.38</td>
<td>4.8</td>
</tr>
<tr>
<td>5</td>
<td>M 269.6</td>
<td>N</td>
<td>2.252</td>
<td>M 2.011</td>
</tr>
<tr>
<td>6</td>
<td>444.3</td>
<td>N</td>
<td>3.7</td>
<td>148.9</td>
</tr>
<tr>
<td>7</td>
<td>M 226.4</td>
<td>N</td>
<td>3.8</td>
<td>45.06</td>
</tr>
<tr>
<td>8</td>
<td>263.6</td>
<td>N</td>
<td>3.1</td>
<td>3.987</td>
</tr>
<tr>
<td>9</td>
<td>M 45.41</td>
<td>N</td>
<td>2.7</td>
<td>1.545</td>
</tr>
<tr>
<td>10</td>
<td>M 141.2</td>
<td>N</td>
<td>3.7</td>
<td>15.56</td>
</tr>
<tr>
<td>11</td>
<td>M 616.3</td>
<td>N</td>
<td>3.1</td>
<td>2.471</td>
</tr>
<tr>
<td>12</td>
<td>M 96.31</td>
<td>N</td>
<td>2.5</td>
<td>1.156</td>
</tr>
<tr>
<td>13</td>
<td>M</td>
<td>269.6</td>
<td>N</td>
<td>3.7</td>
</tr>
</tbody>
</table>

$^a$ 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 carbanates 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 carbanates, but with several exceptions (see Tables II and III). This mechanism (full also sevin and rivastigmin) 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 carbanates, but with several exceptions (see Tables II and III).
concentration $[E]_0$ and therefore also $[ES]$ de-
probable for inhibited hydrolyses, where the initial
(1) reaction with the mentioned reaction model: Scheme
experimental reality can be explained in compli-
obtained dependences are evidently linear. This
or the Michaelis-Menten equation (4). But some
ences of concentration $[I]_0$ 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.

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 neces-
sary, e.g., to measure the time dependence of the
increasing activity of the inhibited enzyme to hy-
drolyze the substrate.

6.) Every reaction of $(S + E + I)$ was reproduced
at least three times, mostly with various initial con-
centrations $[I]_0$ 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, ex-
pressed 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 depend-
ences 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 compli-
ance with the mentioned reaction model: Scheme
(1)–(3) is described, according to the laws of for-
amal reaction kinetics for isochoric reactions, by a
relevant system of differential kinetic equations
concentration vs. time for all reaction components.
If the condition $[ES] = \text{const}.$ is matched (most
probable for inhibited hydrolyses, where the initial
concentration $[E]_0$ and therefore also $[ES]$ de-
crease substantially owing to the formation of $E_I$),
then $[ES]$ is very small in comparison with $[S]$, $[P]$,
and $[HA]$, and therefore practically constant dur-
ing the reaction time. From the combination of
this steady state condition for $ES$ ($[ES] = \text{const}$.,
$i.e.$ $d[ES]/dt = 0$) with the system of differential
kinetic equations mentioned above it follows:
\[
-d[S]/dt = d[P]/dt = d[HA]/dt = k_2\;[ES] = k. \tag{5}
\]

After integration this relation leads to the linear
dependences between $[S]$, $[P]$ and $[HA]$ and reac-
tion time $t$
\[
[S]_0 - [S] = [P] = [HA] = k\;t \tag{6}
\]
at zero initial concentrations of $P$ and $HA$. The
reaction scheme (1)–(3) complies at such condi-
tions the reaction of zero order on the mentioned
components. Some experiments show even a com-
bined course: The dependence concentration vs.
time begins with a short nonlinear part which
passes (sharply or continuously) to a longer lin-
ear part.

9.) In Tables II and III the summary of average
values of the rate constant $k_3$ is given for all stud-
ied inhibitors, $(S + E)$ combinations and used ana-
lytical methods. For complex checking of effective-
ity of studied inhibitors, Table II (ACH) includes
also their separation concentration coefficients in
the milieu 1-octanol (1)/water (2), $K_S = [I]/[I]_0(2)$,
which are the measure of the ability of sin-
gle inhibitors to cross over the hematoencephalic
membrane. In Table III (ATCH) the values of in-
dices $\pi_{50}$ measured by the ELM method with in-
cubation 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 $k_3$ deter-
mined by various methods are of the same order.
Exceptions can be explained by different reaction
conditions necessary for realization of the individ-
ual 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 hy-
drolysis (Komersová, 2006, unpublished results).
For searching more effective inhibitors of ACHE
it is important, that the $k_3$ values for every physio-
logically 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. Unphysiological com-
binations (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 nitrogen 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 + BCHE) 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 + BCHE), which is indeed not physiologically important.

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