KINETICS OF INHIBITION OF RAT BRAIN ACETYLCHOLINESTERASE BY 3-DIETHYLAMINOPHENYL-N-METHYL- AND -N-PHENYLCARBAMATE METHOIODIDE *in vitro*

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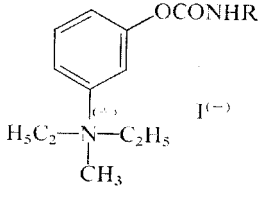
The kinetics was examined of the *in vitro* inhibition of rat brain acetylcholinesterase by 3-diethylaminophenyl-N-methylcarbamate methoiodide and by 3-diethylaminophenyl-N-phenylcarbamate methoiodide. The constants characterizing the affinity of both carbamates for acetylcholinesterase and the rate constants of carbamylation and decarbamylation were measured.

Some carbamic acid esters are known for their acetylcholinesterase-inhibiting properties[1]. Their inhibitory effect on the cholinesterases consists in the carbamylation of the active site of these enzymes and can be described by the following scheme[2]

$$E + I \overset{K_a}{\rightleftharpoons} E{\cdot}I \xrightarrow{k_c} EC \xrightarrow{k_d} E + \text{products},$$

where E is the free enzyme, I the carbamate inhibitor, E.I the reversible enzyme–inhibitor complex, and EC the carbamylated enzyme. The reaction is analogous to the reaction of the enzyme with its substrate and the carbamate actually plays the role of a substrate characterized by an extremely low turnover number[2]. The first stage of the reaction, which involves the formation of a temporary complex E·I, is characterized by dissociation constant K_a. The rate of conversion of this complex into the carbamylated enzyme is determined by the size of the carbamylation rate constant k_a and the rate of decarbamylation by the size of the decarbamylation rate constant k_d.

The kinetics of inhibition of rat brain acetylcholinesterase by 3-diethylaminophenyl-N-methylcarbamate methoiodide (*I*) and 3-diethylaminophenyl-N-phenylcarbamate methoiodide (*II*) is investigated in the present study.



I, R = CH_3; *II*, R = C_6H_5

EXPERIMENTAL

Chemicals. 3-Diethylaminophenyl-N-methylcarbamate methoiodide (*I*) and 3-diethylaminophenyl-N-phenylcarbamate methoiodide (*II*) were prepared by Dr J. Socha. Triton X-100 was from Koch-Light. Acetylcholine iodide and the remaining chemicals of analytical purity were from Lachema, Brno.

Acetylcholinesterase. A Triton extract of brains of rats of both sexes served as a source of the enzyme. Rats (180—200 g) were sacrificed by scission of the carotid arteries and bleeding. The brains were rapidly excised, washed with physiological saline and homogenized with physiological saline containing 1% of Triton X-100 to a 10% (w/v) homogenate in an Ultra-Turrax blender. The homogenate was stirred 30 min at room temperature and then centrifuged (105 000 *g*, 60 min). The clear supernatant served as a source of acetylcholinesterase. The activity of acetylcholinesterase was measured titrimetrically as the initial rate (v) of acetylcholine hydrolysis in a Radiometer pH-stat. The titration was effected with 0·05M-NaOH. The measurement was made at 25°C and pH 8·0 in a total volume of 20 ml. The final concentration of acetylcholine iodide as substrate was 5 mM.

Determination of kinetic constants. The dissociation constant K_a, the carbamylation rate constant k_c, and the bimolecular rate constant k^{II} were determined by the method of Main[3] as modified by O'Brien and coworkers[4]. The enzyme solution (0·5 ml) and the carbamate inhibitor solution (0·5 ml) were incubated 30 s at 25°C and pH 8·0. Afterwards the mixture was rapidly diluted with substrate solution in physiological saline to a total volume of 20 ml and the residual enzyme activity was measured. The difference in the activity of noninhibited (v_0) and inhibited (v_i) acetylcholinesterase was used for the calculation of $\Delta t/2{\cdot}303\ \Delta \log v$, where $\Delta \log v = \log v_0 - \log v_i$. After the values of $\Delta t/2{\cdot}303\ \Delta \log v$ had been plotted *versus* $1/[I]$ according to equation[3] $\Delta t/2{\cdot}303\ \Delta \log v = K_a/k_c \,.\, 1/[I] + 1/k_c$ a straight line not passing through the origin was obtained and the bimolecular rate constant k^{II} was calculated from its slope ($\mathrm{tg}\ \alpha = K_a/k_c = 1/k^{II}$). The dissociation constant K_a was similarly calculated from the slope ($\mathrm{tg}\ \alpha = K_a$) of the plot $2{\cdot}303\ \Delta \log v/\Delta t$ *versus* $2{\cdot}303\ \Delta \log v/[I]\ \Delta t$ according to equation $2{\cdot}303\ \Delta \log v/\Delta t = k_c - K_a\ (2{\cdot}303\ \Delta \log v/[I]\ \Delta t)$, and the carbamylation rate constant k_c from the slope ($\mathrm{tg}\ \alpha = 1/k_c$) of the plot $[I]\ \Delta t/2{\cdot}303\ \Delta \log v$ *versus* $[I]$ according to $[I]\ \Delta t/2{\cdot}303\ \Delta \log v = [I]/k_c + K_a/k_c$. These operations involve three linear transformations of the same relation derived by Main[3]. Each of the constants was calculated by a transformation in which the magnitude calculated exists in the expression for the slope since the calculation of the slope is more exact than the calculation of the intersections of the straight line with the axes[4,5]. The decarbamylation rate constant k_d was determined by the method of Wilson and coworkers[2]. These measurements were likewise carried out at 25°C and pH 8·0. The interpolation of the lines through the experimental points and the calculations of the kinetic constants were performed in a MINSK-22 computer. The values of the constants are means ± confidence interval for $P = 0{\cdot}95$.

RESULTS

The dependence of the inhibition of acetylcholinesterase on the concentration of both carbamates is shown in Fig. 1. The concentration of the carbamate which brings about a 50% inhibition after 10 min incubation with the enzyme (I_{50}) was calculated from the linear probit-logarithmic transformation. The negative decadic logarithm of this constant (pI_{50}) has for compound *I* a value of 8·79 ± 0·05 and for compound *II* a value of 4·19 ± 0·04. The dissociation constants K_a and the carbamylation constants

k_c were calculated from transformations of the Main equation[3] and their numerical values together with the values of the bimolecular rate constant $k^{II} = k_c/K_a$ are given in Table I.

TABLE I

Kinetic Constants of Inhibition of Rat Brain Acetylcholinesterase by 3-Diethylaminophenyl-N-methylcarbamate Methoiodide (*I*) and by 3-Diethylaminophenyl-N-phenylcarbamate Methoiodide (*II*)

Compound	K_a M	k_c min^{-1}	k_d min^{-1}	k^{II} M^{-1} min^{-1}
I	$(3{\cdot}06 \pm 0{\cdot}86) \cdot 10^{-6}$	$26{\cdot}0 \pm 5{\cdot}2$	$(1{\cdot}84 \pm 0{\cdot}96) \cdot 10^{-2}$	$(8{\cdot}70 \pm 1{\cdot}54) \cdot 10^{6}$
II	$(6{\cdot}68 \pm 1{\cdot}42) \cdot 10^{-4}$	$0{\cdot}57 \pm 0{\cdot}08$	—[a]	$(8{\cdot}50 \pm 1{\cdot}39) \cdot 10^{2}$

[a] No decarbamylation observed.

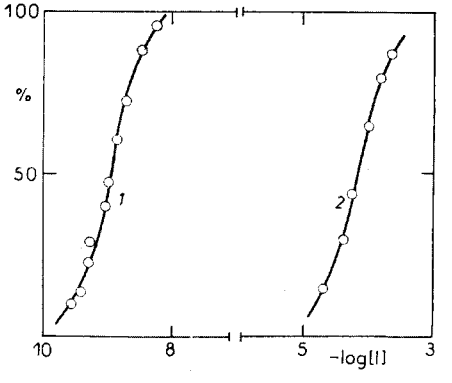


FIG. 1

Dependence of Inhibition of Rat Brain Acetylcholinesterase on Molar Concentration of Carbamate

1 3-Diethylaminophenyl-N-methylcarbamate methoiodide; *2* 3-diethylaminophenyl-N-phenylcarbamate methoiodide. Each point represents a mean of two parallel measurements. The enzyme was incubated with the inhibitor 10 min at 25°C and pH 8·0. %, inhibition; I, concentration of inhibitor.

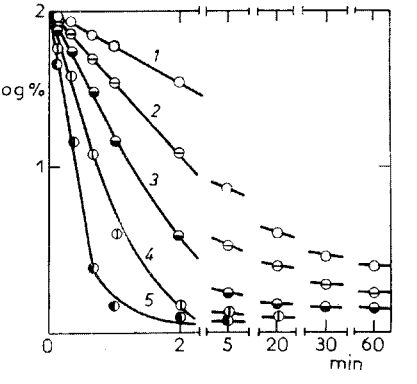


FIG. 2

Dependence of Activity of Rat Brain Acetylcholinesterase on Time of Incubation with 3-Diethylaminophenyl-N-methylcarbamate Methoiodide

Concentration of 3-diethylaminophenyl-N-methylcarbamate methoiodide: *1*, $6 \cdot 10^{-8}$, *2* $1 \cdot 10^{-7}$, *3* $2 \cdot 10^{-7}$, *4* $4 \cdot 10^{-7}$, and *5*, $1 \cdot 10^{-6}$ M. The inhibition proceeded at 25°C and pH 8·0. The activity is given in log %.

The incubation of acetylcholinesterase with the carbamate inhibitor leads to progressive inhibition which follows first the kinetics of first order and arrives at an equilibrium later, as shown for compound *I* in Fig. 2. The initial linear portion of the log % *i versus t* plot, where *i* stands for fractional inhibition and *t* for time of incubation of the inhibitor with the enzyme, was used for the calculation of pseudomonomolecular rate constants k_{obs} from the formula $k_{obs} = -2{\cdot}303 \operatorname{tg} \alpha$. The bimolecular rate constants were obtained by dividing the latter by the corresponding concentration of the carbamate ion, *i.e.* $k_i^{II} = k_{obs}/[I]$. The values of k_{obs} and k_i^{II} for five concentrations of compound *I* are given in Table II. The average value of $k_i^{II} = (8{\cdot}68 \pm \pm 1{\cdot}92) \cdot 10^6 \text{M}^{-1} \text{min}^{-1}$.

The decarbamylation rate constants k_d were measured by the dilution method[2] and calculated from the slope of the linear log % *i versus t* plot, where *t* is the time after the dilution of the mixture of the enzyme with the inhibitor according to the formula $k_d = -2{\cdot}303 \operatorname{tg} \alpha$. A value of $k_d = (1{\cdot}84 \pm 0{\cdot}96) \cdot 10^{-2} \text{min}^{-1}$ was obtained for compound *I* whereas for compound *II* $k_d = 0$ since no measurable decarbamylation was observed within 6 h after the dilution. The decarbamylation of compound *I* follows the kinetics of first order.

DISCUSSION

The two carbamates studied, differing in the character of the N-substituent only, inhibit rat brain acetylcholinesterase. As follows from a comparison of the size of the pI_{50}-constants, the N-methyl derivative is a far stronger inhibitor than the N-phenyl

TABLE II

Rate Constants of Inhibition of Rat Brain Acetylcholinesterase by 3-Diethylaminophenyl--N-methylcarbamate Methoiodide

The monomolecular rate constant of the inhibition was calculated from formula $k_{obs} = -2{\cdot}303 \operatorname{tg} \alpha$ where $\operatorname{tg} \alpha$ is the slope of the linear portion of the plot of the log % activity *versus* time of incubation of the enzyme with the inhibitor (Fig. 2). The bimolecular rate constant of the inhibition was calculated from formula $k_i^{II} = k_{obs}/[I]$.

[I] M	k_{obs} min^{-1}	k_i^{II} $\text{M}^{-1} \text{min}^{-1}$
$6 \cdot 10^{-8}$	0·505	$8{\cdot}40 \cdot 10^6$
$1 \cdot 10^{-7}$	1·07	$10{\cdot}70 \cdot 10^6$
$2 \cdot 10^{-7}$	1·88	$9{\cdot}40 \cdot 10^6$
$4 \cdot 10^{-7}$	3·15	$7{\cdot}89 \cdot 10^6$
$1 \cdot 10^{-6}$	6·93	$6{\cdot}93 \cdot 10^6$

derivative ($4{\cdot}2 \cdot 10^4$ times). This result is in agreement with the data of Metcalf[6] who found that $I_{50} = 4 \cdot 10^{-7}$M for 3-tert-butylphenyl-N-methylcarbamate and a value $> 10^{-3}$M for the N-phenyl derivative in experiments with fly head acetylcholinesterase. Likewise the inhibition rate expressed by the size of the bimolecular rate constant k^{II} ($8{\cdot}7 \cdot 10^6 M^{-1} min^{-1}$ for compound *I* and $8{\cdot}5 \cdot 10^2 M^{-1} min^{-1}$ for compound *II*) is higher by four orders for the N-methyl derivative. The bimolecular rate constant k^{II} for compound *II* calculated from the Main plot ($k^{II} = (8{\cdot}70 \pm \pm 1{\cdot}54) \cdot 10^6 M^{-1} min^{-1}$) is identical with the constant calculated from the time profile of the inhibition ($k_i^{II} = (8{\cdot}68 \pm 1{\cdot}92) \cdot 10^6 M^{-1} min^{-1}$).

The reason for this difference in the magnitude of the inhibitory effect of the two carbamates is both their different affinity for acetylcholinesterase (K_a $3{\cdot}06 \cdot 10^{-6}$M for compound *I* and $6{\cdot}68 \cdot 10^{-4}$M for compound *II*) and also the difference in the decarbamylation rate of the temporary complex (k_c $26{\cdot}0\ min^{-1}$ for compound *I* and $0{\cdot}57\ min^{-1}$ for compound *II*). The methyl derivative shows a higher affinity for acetylcholinesterase than the phenyl derivative and is carbamylated at a higher rate. The half-life of the complex E·I calculated from formula $t_{0{\cdot}5} = 0{\cdot}6933/k_c$ is 0·027 min for compound *I* whereas a 45 times higher value, *i.e.* 1·22 min was obtained for compound *II*.

The values measured of the carbamylation rate constant k_c for compound *I* fall in the range of values measured with a series of substituted phenyl-N-methylcarbamates and bovine erythrocyte acetylcholinesterase at 25°C by Hastings and coworkers[7]; these values vary between $0{\cdot}45\ min^{-1}$ and $80{\cdot}7\ min^{-1}$. The greatest differences were found to exist in the decarbamylation rate of methylcarbamylated and phenylcarbamylated acetylcholinesterase. The k_d-value ($(1{\cdot}84 \pm 0{\cdot}96) \cdot 10^{-2}\ min^{-1}$) measured for methylcarbamylated acetylcholinesterase by us is in excellent agreement with the data characterizing the enzyme from electric eel[8] and from bovine erythrocytes[9,10]. The decarbamylation of the phenylcarbamylated enzyme was not observed; this finding is in accordance with the observation[11] that the decarbamylation rate decreases with higher N-alkylcarbamate substituents. No data, however, have been reported on the decarbamylation of N-phenylcarbamylated acetylcholinesterase.

The ratio of values of the k_c- and k_d-constants permits us to assess the concentration of the temporary complex E.I under equilibrium conditions. The rate of formation of the carbamylated enzyme EC is given by $k_c[E{\cdot}I]$ and the rate of its decomposition by $k_d[EC]$; hence under equilibrium conditions $[E{\cdot}I]/[EC] = k_d/k_c$. After substituting for k_c and k_d we obtain for compound *I* (Table I) $[E{\cdot}I]/[EC] = 0{\cdot}00071$; this shows that under equilibrium conditions of inhibition of acetylcholinesterase by compound *I* 0·071% of the enzyme only is in the form of temporary complex E·I.

Assuming that K_a is the true dissociation constant of complex E·I we can calculate the change in free energy of binding of the carbamate to the enzyme. The value

of ΔF is 7·55 kcal mol^{-1} for compound *I* and 4·35 kcal mol^{-1} for compound *II* at 25°C. Most likely it is electrostatic forces which predominantly participate on the interaction of both carbamates with the enzyme; the bulkier-N-substituent of compound *II* prevents the carbamate from closer contact with the active surface of acetylcholinesterase, a phenomenon which manifests itself by an increase of the equilibrium distance d_e and a decrease[12] of the binding energy ΔF.

Disperse interactions obviously do not participate on the binding of the carbamate to acetylcholinesterase since the polarizability of the benzene ring is higher that of a methyl, a fact which is reflected in differences in interaction energies. Complex E·I should be more stable with compound *II*; no such observation, however, has been made. These disperse interactions however can play a role on carbamylated enzyme EC and this could explain its high stability in the case of compound *II*. It is because of this stability that there is no decarbamylation and compound *II* is an irreversible inhibitor.

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