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Characterization of the Active Site of Acetylcholinesterases by Application of Sterically Modified Acetylcholine Homologues

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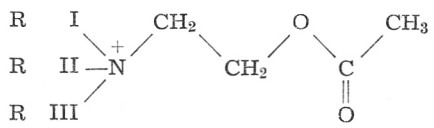
Our aim was to find steric limitations for the model of the active site of acetylcholinesterase and cholinesterase. For this purpose we used homologues of acetylcholine with hydrocarbon chains of increasing size at the cationic head. Catalysis of hydrolysis ceased for both enzymes, when the methyl groups of the cationic head of acetylcholine were substituted by three *n*-propyl groups. With data already documented in the literature and our additional experiments, under the same conditions, we were able to present models of the active sites for both enzymes.

For our understanding of cholinergic mechanisms we use a model representing the active site of biologically active macromolecules. It serves for nearly all molecules dealing with acetylcholine mediated transmission in the nervous system, or its modification. This model composed of an anionic centre and an esteratic centre was proposed by Wilson and colleagues[1–3] by ingenious considerations and methods. It is mainly used to represent the active site of acetylcholinesterase (Acetylcholine Acetyl-Hydrolase, EC 3.1.1.7), cholinesterase (Acylcholine Acyl-Hydrolase, EC 3.1.1.8), and with limitations even for cholinergic receptor proteins.

Our aim was to find steric limitations of the active site of acetylcholinesterase and cholinesterase. For this purpose we synthesized some homologues of acetylcholine with the structure shown in Table I.

TABLE I.

Acetylcholine homologues



S1	RI = — CH_3	S4	RI = RII = RIII = — C_3H_7
	RII = RIII = — C_2H_5	S5	RI = RII = — CH_3
S2	RI = RII = RIII = — C_2H_5		RIII = — C_4H_9
S3	RI = — CH_3	S6	RI = RII = — CH_3
	RII = RIII = — C_3H_7		RIII = — C_6H_{13}

Use of these substances in addition to tetraethylammonium- and tetra-n-propylammonium-chloride served to characterize the space around the anionic centre.

To characterize the space around the esteratic centre we conducted experiments which can be easily repeated in the laboratory, the results of which are already well documented by Augustinsson[4-6] in the literature.

MATERIALS AND METHODS

For our experiments with acetylcholinesterase we used enzyme from *Torpedo marmorata*[a] electric organ, prepared according to Hopff *et al.*[7,8] For experiments with cholinesterase we used »pseudocholinesterase« from horse serum[b]. All experiments were carried out with the pH-stat method[9] (Autotitrierstand »Methrohm«[c]) 0.15 M NaCl and 0.02 M $MgCl_2$ solution without buffer, and 0.01 M NaOH (Fixanal »Merck«) were used for titration.

Both enzymes were standardized to 1 µM unit with acetylcholine bromide[d], 4×10^{-3} M.

Dreiding models and Ångstroemscala[e] served as a basis for our considerations and for measuring atomic distances. Substances S1—S6 were synthesized according to the methods of Holton and Ing[10] and Mehrotra and Dauterman[11].

1. 2-n-Dipropylaminoethanol

To 45 ml (0.34 M) *n*-dipropylamine in a 250 ml roundbottomed flask, 17 ml (0.25 M) 2-chloroethanol in small quantities were added. The reaction mixture was refluxed for 8 hours and after cooling 150 ml of 10% NaOH were added. Extraction was carried out 4 times with benzene and dried with $MgSO_4$. Evaporation at the rotatory evaporator and twofold redistillation at 71—73 °C (9—10 mm Hg) yielded 18.45 g (50,9%) of 2-*n*-dipropylaminoethanol.

2. Acetic acid-β-dialkylaminoethylester

To an ice-cooled solution of 0.125 M dialkylaminoethanole in 200 ml of absolute chloroform, magnetically stirred, were added 10 ml (0.14 M) acetylchloride in 100 ml of absolute chloroform. After 2 hours enough water was added to dissolve the precipitation and then the reaction mixture was stirred with 15 g potassiumcarbonate for another 2 hours. In a separator the aqueous solution was separated from the chloroform layer and again extracted for 6 more times with chloroform. The combined extracts were dried with $MgSO_4$ and the chloroform was evaporated at the rotatory evaporator (25 °C, 11 mm Hg). Data and yields are given in Table II.

TABLE II.

Data from acetic acid-β-dialkylaminoethylesters

R	Bp.	Yield
$-CH_3$	40—43 °C/10 mm Hg	70%
$-C_2H_5$	76 °C /18 mm Hg	74%
$-C_3H_7$	85—90 °C/10 mm Hg	68%

[a] *Torpedo marmorata* originated from the French Atlantic coast and was purchased from Institut Universitaire de Biologie Marine, F-33120 Arcachon, France.

[b] Pseudo cholinesterase Type IV »Sigma« St. Louis, Mo. USA.

[c] pH-Meter E 512, Impulsomat E 473 fitted with a device to vary the impulse--time, and Dosimat-Recorder. Metrohm, CH 9100 Herisau, Switzerland.
Electrode type: Lot-401-M8-NS, from Ingold, Urdorf-ZH, Switzerland.

[d] Acetylcholinbromid, reinst, Siegfried, Zofingen, Switzerland.

[e] Dreiding models purchased from: Büchi, Glasapp.-Fabrik, Flawil, Switzerland.

3. Acetoxyethyl-N,N,N-trialkylammoniumhalogenide

3 g acetic acid-dialkylaminoethylester was dissolved in 20 ml absolute benzene and the alkylhalogenide was added in small quantities. The precipitations were filtered off, washed with absolute benzene and recrystallized. Specific data are given in Table III.

TABLE III.

Chemical data for acetylcholine homologues

Substance	Melting pt./°C and recrystallization medium	Yield in %	SF	Analysis			
				Calcd. C %	Calcd. H %	Found C %	Found H %
S1	66—68 Methylethylketone	78.7	$C_9H_{20}NO_2I$	35.89	6.70	36.18	6.74
S2	120,5—121 Acetone	81.1	$C_{10}H_{22}NO_2I$	38.10	7.05	37.99	7.02
S3	123,5—125 Acetone	77.6	$C_{11}H_{24}NO_2I$	40.13	7.35	40.19	7.26
S4	137—139 Methylethylketone	61.3	$C_{13}H_{28}NO_2I$	43.69	7.89	43.53	7.88
S5	105—106 Acetone	54.7	$C_{10}H_{22}NO_2Br$	44.78	8.27	44.67	7.99
S6	73—74,5 Acetone/Benzene	64.3	$C_{12}H_{26}NO_2I$	41.99	7.64	42.24	7.69

Enzyme assay

Substances S1—S6 were tested the following way: From standard dilutions the concentrations documented in Figs. 1—6 were assayed 6 times each, and mean and standard deviation were calculated with a hp 45 computer. Enzyme activity in μM/min. was plotted against negative logarithm (pS) of substrate concentration. The lines connecting the experimental points do not represent curves, but demonstrate the different behavior of the two enzymes with increasing substrate concentration.

To compare data collected in fragments from the literature with our own data, we had to repeat those data already published. All results were obtained under the same assay conditions.

RESULTS

The three methyl groups around the acetylcholine quaternary nitrogen successively replaced by ethyl groups lead to the compound with 2 ethyl groups S1, which compound reduced the catalysis of hydrolysis slightly for both enzymes. As already found by Holton and Ing[10] even the hydrolysis of the acetylcholine homologue S2, with three ethyl groups around the quaternary nitrogen is catalyzed by both enzymes. Further extension of the cationic head leads to the compound S3 with 2 *n*-propyle groups around the quaternary nitrogen. This compound was lately assayed by Krupka and Hellenbrand[12] for erythrocyte acetylcholinesterase. They found the compound to be still active. This is in good agreement with our findings for acetylcholinesterase from *Torpedo* electric organ. Cholinesterase from horse serum catalyzes its hydrolysis almost negligibly.

When all three methyl groups are replaced by *n*-propyle groups (S4), neither with cholinesterase nor with acetylcholinesterase any catalysis of hydrolysis could be observed. This almost fits the results of Mehrotra and Dauterman[11] who had used this compound earlier but still found little activity. Their only »enzyme« however, was rat brain powder and the activity was assayed with the Warburg technique[11]. Curiously enough we found, when compound S4 is added to a solution of 4×10^{-3} M of acetylcholine bromide in decreasing concentrations, (Fig. 4), it shows inhibiting activity for both enzymes. These findings in addition to our other results contribute to our active-site-model. Detailed data are given in Figs. 1—4. Acetylcholine homologues with one hydrocarbon chain of increasing length C_4H_9- (S5), and C_6H_{13}- (S6) at the cationic head show relative hydrolysis rates, catalized by acetylcholinesterase of 52.00 ± 2.79 (S5) and 36.78 ± 2.79 (S6). This may be attributed to the slower rotation velocity of the $\overset{+}{N}—CH_2$ bond in the S6 rotamere, compared with S5. Further we paid special attention to the homologues of the tetramethylammonium ion. In this series experimental data are well established by Bergmann and Wurzel[13], Long[14], and Krupka and Hellenbrand[12]. When these competitive inhibitors are added to acetylcholine its hydrolysis rate catalized by both enzymes decreases from tetramethylammonium ion to tetraethylammonium ion to tetra-*n*-propylammonium ion, which is the most powerful reversible inhibitor in this series.

TABLE IV.

Relative rates of hydrolysis catalyzed by acetylcholinesterase

		V_{rel}
$(CH_3)_3\overset{+}{N}—$	Acetylcholine	100.00
$(C_2H_5)_2\overset{+}{N}(CH_3)—$	S1	77.87 ± 7.09
$(C_2H_5)_3\overset{+}{N}—$	S2	61.85 ± 5.99
$(C_3H_7)_2\overset{+}{N}(CH_3)—$	S3	28.14 ± 2.09
$(C_3H_7)_3\overset{+}{N}—$	S4	0
$C_4H_9—\overset{+}{N}(CH_3)_2—$	S5	52.00 ± 2.79
$C_6H_{13}—\overset{+}{N}(CH_3)_2—$	S6	36.78 ± 2.79

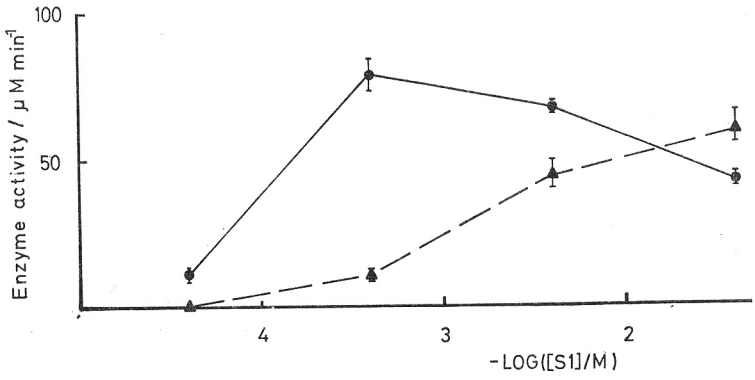


Fig. 1. Hydrolysis of $(C_2H_5)_2N^+(CH_3)(CH_2)_2OC(O)CH_3$ (S1) by acetylcholinesterase (●) and cholinesterase (▲).

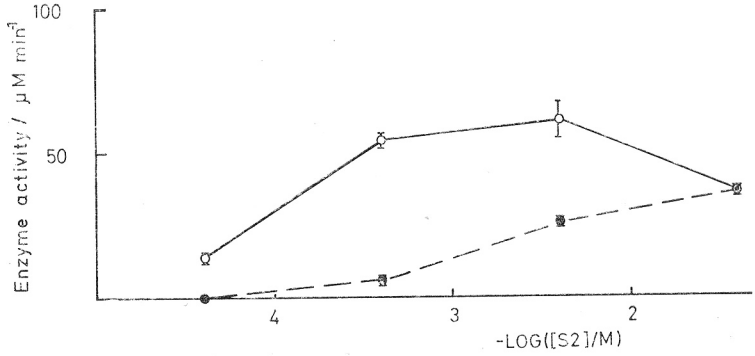


Fig. 2. Hydrolysis of $(C_2H_5)_3N^+(CH_2)_2OC(O)CH_3$ (S2) by acetylcholinesterase (○) and cholinesterase (●).

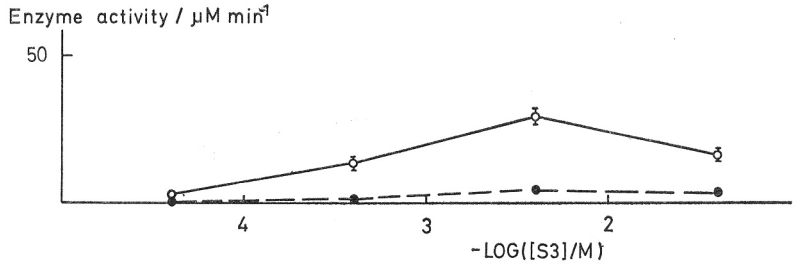


Fig. 3. Hydrolysis of $(C_3H_7)_2N^+(CH_3)(CH_2)_2OC(O)CH_3$ (S3) by acetylcholinesterase (○) and cholinesterase (●).

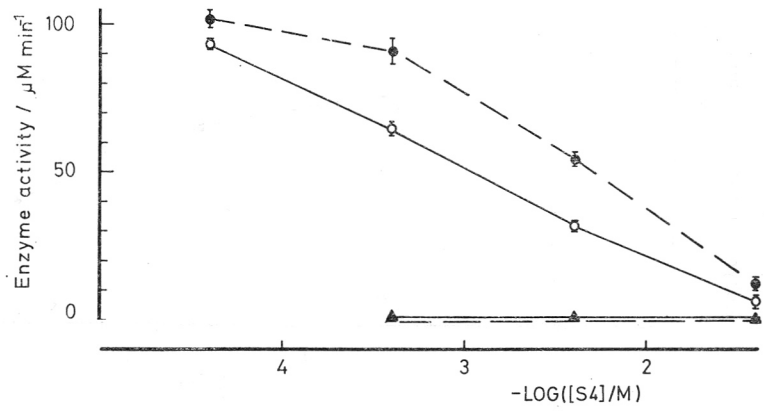


Fig. 4. Hydrolysis of acetylcholine (4.0 mM) by acetylcholinesterase (○) and cholinesterase (●) in presence of $(C_3H_7)_3N^+(CH_2)_2OC(O)CH_3$ (S4). The concentration of S4 is plotted on the abscissa and its hydrolysis by the two enzymes in the absence of acetylcholine is also plotted (▲).

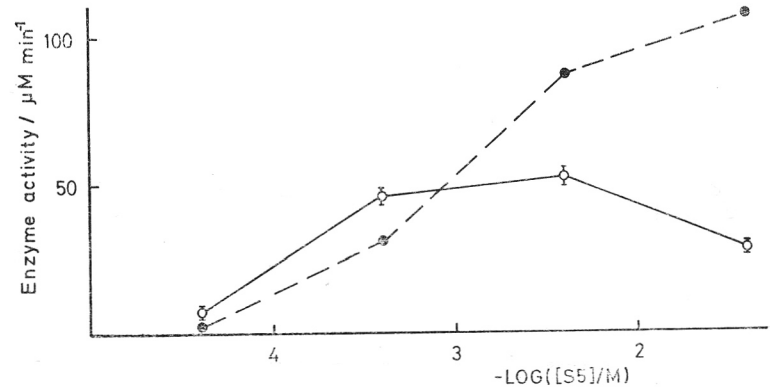


Fig. 5. Hydrolysis of $(C_4H_9)N^+(CH_3)_2(CH_2)_2OC(O)CH_3$ (S5) by acetylcholinesterase (○) and cholinesterase (●).

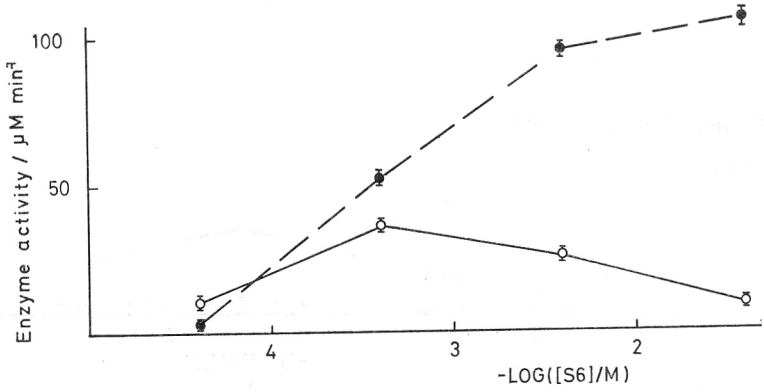


Fig. 6. Hydrolysis of $(C_6H_{13})N^+(CH_3)_2(CH_2)_2OC(O)CH_3$ (S6) by acetylcholinesterase (○) and cholinesterase (●).

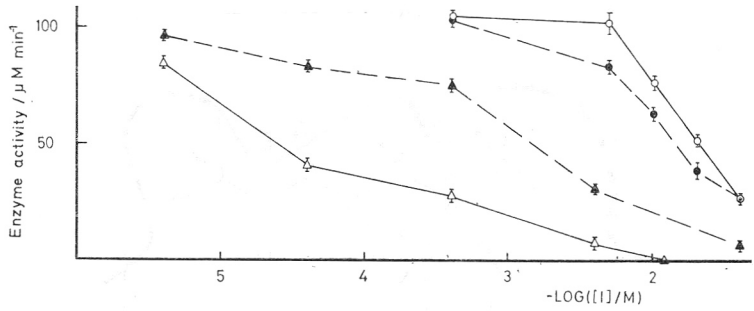


Fig. 7. Hydrolysis of acetylcholine (4.0 mM) by acetylcholinesterase (○, △) and cholinesterase (●, ▲) in presence of the inhibitors $(C_2H_5)_4N^+$(○, ●) and $(C_3H_7)_4N^+$ (△, ▲). Concentrations of the inhibitors (I) are plotted on the abscissa.

Tetra-*n*-butylammonium ion and even tetra-*n*-pentylammonium ion are still inhibitors, but with increasing chain length, their inhibiting power decreases. This phenomenon can be easily understood when we use Dreiding models for its explanation. The molecules can be arranged with the quaternary nitrogen at the top and all four substituents hanging down grapelike. The greatest distance around the nitrogen atom is then smaller than 4.5 Å as it can be also measured in tetraethylammonium ion.

CONCLUSIONS AND DISCUSSION

According to the forementioned references[1–14] with the help of our own findings, we suggest steric limitations for the active sites of acetylcholinesterase and cholinesterase. Our suggestions are based on hydrolysis rates from acetylcholine homologues and acetylcholine hydrolysis in presence of tetramethylammonium ion homologues, catalyzed by acetylcholinesterase and cholinesterase.

Based on his studies on acetylcholine conformation, Peter Pauling claims: »the conformation relevant to esterase is a fairly open structure«[15]. This is in good agreement with our model, because the open structure only fits and can be used to explain our findings. When the acetylcholine molecule is attracted by Coulomb forces acting between the anionic centre of the enzyme and the cationic head of acetylcholine, it is further fixed by the δ^-/δ^+ binding of the esteratic oxygen to the enzyme. The resulting conformation, expressed in Figs. 8 and 9 claims a close contact between ester oxygen and enzyme and could be attributed to a hydrogen-bridge-bond.

The resulting conformation at the cationic head representing the lowest energy conformation, is thus a little different from the ideal conformation with closest contact between anionic centre and cationic head, because one methyl group points directly toward the enzyme. Considering the conformation with two methyl groups pointing toward the enzyme only, the closest contact between cationic head and anionic centre is established. This really can be demonstrated when tetra-*n*-propylammonium ion fits into the crevice at the anionic centre. Also it is a possible explanation for the fact that tetra-n-propylammonium ion is the most powerful inhibitor in the homologue series of alkylammonium ions. On the other hand when compound S4, the acetylcholine homologue with three *n*-propyl groups at the cationic head, is in the conformation of acetyl-

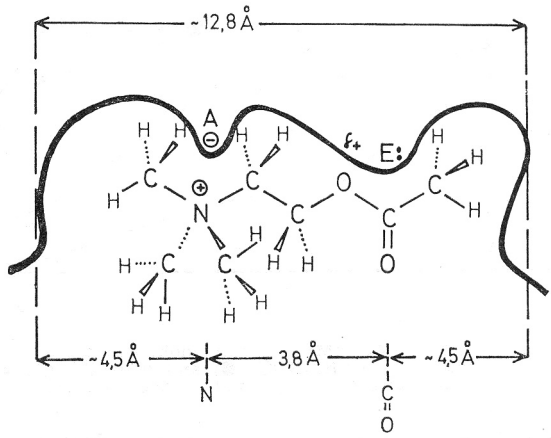


Fig. 8. Active site of acetylcholinesterase (EC 3.1.1.7) A = anionic centre, E = esteratic centre.

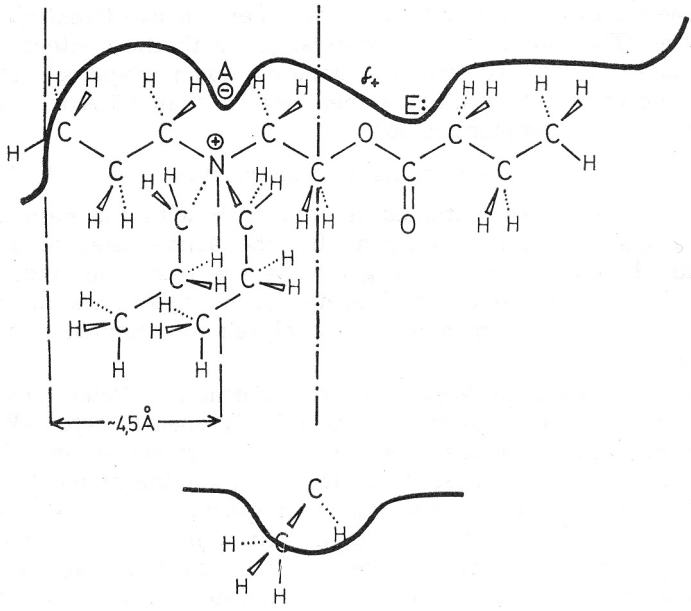


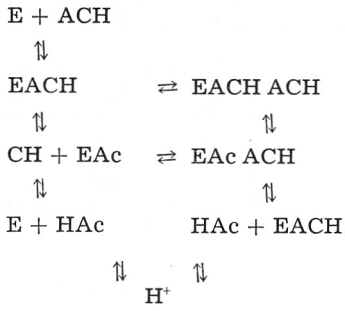
Fig. 9. Active site of cholinesterase (EC 3.1.1.8) A = anionic centre, E = esteratic centre.

choline when it is »ready for hydrolysis« as it is demonstrated in Figs. 8 and 9, the γ-C does not fit in the anionic crevice of the active sites of both enzymes. When the $\overset{+}{N}$—CH_2 bond is turned, so that the $\overset{+}{N}$ comes closest to the anionic centre, two out of the three similar substituents point toward the enzyme. Now both the α-C and β-C atoms fit into the anionic crevice, whilst both the γ-C atoms are turned out of the crevice. The molecule is now shifted to the right side, twisted out of the position with the possibility for δ^-/δ^+ binding of the esteratic oxygen and thus the esteratic centre cannot act on the carbonyle C atom. The catalysis of hydrolysis ceases.

The model of the active site of cholinesterase can be further limited considering the fact, that the hydrolysis of acetyl-β-methylcholine is not catalyzed by this enzyme. In a twodimensional model, as pointed out in Fig. 9 we suggest, that the crevice at the anionic centre decreases teaspoon-like towards the —CH—CH_3 group. This is not the case with acetylcholinesterase (Fig. 8) where the model has an open structure. The difference in catalysis, when acetylcholine is compared with acetyl-β-methylcholine may be fully attributed to the slower velocity of rotation in the β-methyl-homologue. Fig. 8 also explains the fact of substrate-inhibition. When two quaternary nitrogens of the competing acetylcholine molecules are attracted by the cationic head of acetylcholinesterase, one molecule can be arranged for hydrolysis, whilst the second molecule only gets its head into the anionic crevice and thus hampers choline from leaving the active site. Another substrate-inhibiting effect could be, when the hydrolysis of the acetylenzyme is hampered by an acetylcholine molecule. Chemical data for possible reaction-mechanisms at the active site of both enzymes are presented in a simplified form in Table V.

TABLE V.

Simplified mechanism of catalysis of acetylcholinesterase



E = enzyme, ACH = acetylcholine, EACH = enzyme-acetylcholine-complex, EACHACH = enzyme-acetylcholine-acetylcholine-complex, CH = choline, EAc = acetylenzyme, EAcACH = acetylenzyme-acetylcholine-complex, HAc = acetic acid, H^+ = Proton.

For steric limitations of the esteratic centre we could use data already well established in the literature and first published by Bergmann and Nachmansohn[16]. The hydrolysis of propionylcholine is still catalyzed by acetylcholinesterase, also the velocity of hydrolysis slows down remarkably. The hydrolysis of butyrylcholine however is not catalyzed by acetylcholinesterase. This might be explained by an esteratic crevice with almost the same diameter of the cationic centre.

The structure of cholinesterase at the esteratic centre, which catalyzes the hydrolysis of butyrylcholine even more than the hydrolysis of acetylcholine, is evidently an open structure, as suggested in Fig. 9.

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DISCUSSION

P. W. Taylor:

I wonder if you have extended your considerations to calculations of the hydrophobic and electrostatic contributions of tetraalkylammonium binding to acetylcholinesterase. A Lennard-Jones type analysis of these factors would be of interest particularly if combined with quantum mechanical considerations of the orientation of the hydrophobic chains.

W. H. Hopff:

We did not calculate hydrophobic binding forces of tetraalkylammonium groups *versus* the electrostatic forces. We consider the electrostatic forces to be in favour when we compare the possible van der Waals binding forces of one or even two propyl groups with the strong coulombic forces of the positively charged nitrogen interacting with the negatively charged anionic centre.

R. D. O'Brien:

If coulombic forces are dominant in binding of tetraalkylammonium ions why is ammonium such an exceedingly poor inhibitor? Even with 0.1 M NH_4^+ there is only partial inhibition.

W. H. Hopff:

Considering the series TMA, TEA and TPA we suggest that the smallest cation is most easily displaced by acetylcholine. The bigger cations fit more tightly into the crevice at the anionic centre, so beside hydrophobic forces there will be sterical reasons that account for their displacement.

E. A. Barnard:

I think there may be an alternative explanation of the existing data on cation interactions on acetylcholinesterase, that is entirely compatible with your model. In the original analyses of Wilson and others the loss of binding strength as alkyl groups are successively removed was interpreted in terms of a mainly hydrophobic contribution to binding of the cholinium head, rather than an electrostatic one. However, one would get the same series for the effect on solvation of the cation. The ammonium ion is very strongly solvated and if removal of water plays a role in the interaction of the enzyme surface (as the results of Belleau suggest) then the alkylated derivatives of ammonium would give the order of effectiveness that is actually observed. Does this not fit in with your model?

W. H. Hopff:

This fits indeed very well. Your contribution reminds me of the well known fact, that when we are dealing with size and behavior of Na^+ and K^+, we have to take the solvation into consideration.

W. N. Aldridge:

You have shown inhibition of acetylcholine hydrolysis by its tripropyl analogue. Have you contemplated doing such experiments with the carbon analogue of the tripropyl compound?

W. H. Hopff:

We have taken carbon analogs into account, but with *n*-propyl groups around the »cationic head« we certainly will have problems to get this compound or homologues into aqueous solution.

R. M. Krupka:

We have found that any substrate inhibition with acetylcholine analogues having a relatively low V_{max} occurred at concentrations considerably higher than that for acetylcholine. Have you measured the substrate inhibition constants for your substrates and do they fit this pattern?

W. H. Hopff:

We did not measure substrate inhibition constants of the acetylcholine homologues. In our opinion we will have problems in expressing such numbers. You might have had similar problems.

SAŽETAK

Karakterizacija aktivnih mjesta acetilkolinesteraza primjenom prostorno modificiranih homologa acetilkolina

W. H. Hopff, G. Riggio, A. Hofmann i P. G. Waser

Svrha je ovog rada naći prostorna ograničenja aktivnog mjesta acetilkolinesteraze i kolinesteraze, primjenom homologa acetilkolina s ugljikovodičnim lancima koji se razlikuju po veličini kationske strane. Zamjenom metilnih skupina na dušiku trima *n*-propil-skupinama, dobije se spoj koji nije supstrat ni acetilkolinesteraze niti kolinesteraze. Već postojeći literarni podaci i rezultati pokusa provedenih pod istim uvjetima omogućili su predložiti modele za aktivna mjesta na oba enzima.

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CH 8006 ZÜRICH, ŠVICARSKA