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Conference Paper

Regulatory Properties of Membrane Bound Acetylcholinesterase from Red Cell Ghosts

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Activating and inhibiting properties of quaternary pyridinium compounds on acetylcholinesterase (AChE), bound to bovine red cell membranes, were investigated. The action of these compounds is mainly due to their quaternary structure, while the effect of the substituents on the pyridinium moiety is less important. The effect of the compounds on the rate of hydrolysis of acetyl- β -methylcholine (MeCh) is different from the effect on acetylcholine (ACh) hydrolysis. The effect of toxogonin on the structure-bound enzyme is different from the effect on the water-soluble enzyme, the latter not being activated by toxogonin.

V_m values were determined by the method of Hofstee. Hill plots yielded several straight lines. It is postulated that AChE exists in several states of cooperativity (negative, non- and positive cooperativity). The different states are assumed to correspond to different allosteric conformations of the protein. The reactions of structure-bound and water-soluble enzyme are different. In contrast to the bound form, the soluble form could not be activated.

INTRODUCTION

It was first suggested by Changeux¹ that acetylcholinesterase (AChE) might be a regulatory enzyme with allosteric properties, and this assumption was taken up by several workers²⁻¹³. Most studies were carried out with water-soluble enzymes from electroplax of *Electrophorus electricus* or torpedo species. Only some investigators considered that reactions and properties of a water-soluble and membrane-bound enzyme might not be the same. The aim of our present work was to study the properties of a membrane-bound enzyme by investigating the effect of some pyridinium compounds on the enzymic hydrolysis of two substrates.

METHODS

Freshly prepared bovine red cell membranes were used as the enzyme source. The cells were brought stepwise into a more and more hypotonic solution, followed each time by ultracentrifugation. By this procedure, the content of the cells was washed out. Finally, the cells were suspended in distilled water and brought back into an isotonic Tyrode solution. If kept at 4 °C their activity remained unaltered up to six weeks.

In some experiments, the water-soluble AChE from *Electrophorus electricus* (Boehringer, Mannheim) was used as the enzyme source.

Acetylcholine chloride (ACh) and acetyl- β -methylcholine chloride (MeCh) were used as substrates. The rate of substrate hydrolysis was measured by the pH-stat method, with a modified Kombititratator (Metrohm). In this modification the original

single syringe is replaced by a double-syringe system. The acetic acid formed from substrate hydrolysis is neutralized by addition of NaOH from one syringe, while the same amount of substrate is added into the reaction vessel from the other syringe. In that way it is possible to measure the enzyme activities at substrate concentrations as low as 3 μM .

The pyridinium compounds investigated are listed in Table I. The unsubstituted parent compounds MePyJ, PM₂ OP and PM₃P, as well as GK 3, were prepared in our laboratory. HS 6 and HS 8 were a gift from Dr. K. Schoene, Institut für Aerobiologie. The other compounds were obtained commercially. All experiments were carried out at 37 °C and pH = 7.4, in a solution of 0.14 M NaCl, 5.4 mM KCl and 1.6 mM CaCl₂ (ionic strength 0.15).

RESULTS AND DISCUSSION

Plots of the activity of AChE *vs.* log acetylcholine concentration result in a family of bell-shaped curves for each antagonist and every concentration investigated (*e.g.* Fig. 1). For nearly all antagonists and most of their concentrations the dose-response curves are sigmoidal. A presentation of the results in Lineveawer-Burk plots is impossible, because the Michaelis-Menten equation is not valid for these enzyme—substrate—antagonist systems. The effects with different pyridinium compounds are dependent on their own concentration and on that of the substrate ACh. Most of the ligands are able to produce either an inhibitory or activating effect on the enzyme. The example of toxogonin (Fig. 1) shows that antagonist concentrations below 0.3 mM enhance the enzyme activity, whereas concentrations up to 3 mM decrease the activity. Furthermore the maximum of the substrate decomposition rate is shifted and this shift is different for each compound depending also on its concentration. In case of toxogonin, the maximum rate is shifted to lower substrate concentrations by low antagonist concentrations (30 to 0.3 μM), and to higher substrate concentrations by higher concentrations (0.3 to 3 mM). At these concentrations the curves become less sigmoidal.

Mono- and bis-pyridinium derivatives have a similar action on AChE. Primarily their properties are due to the structure of the parent compounds.

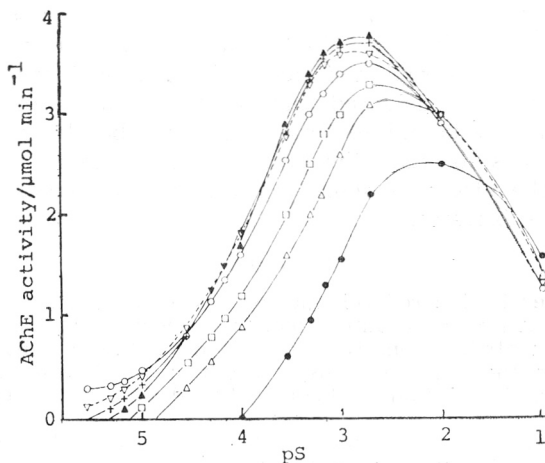
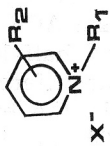
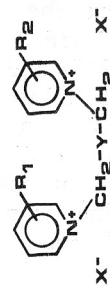


Fig. 1. Effect of toxogonin on the activity of acetylcholinesterase (AChE). Substrate: acetylcholine. Concentrations of toxogonin: (○) zero, (▲) 0.3 μM , (+) 3 μM , (∇) 30 μM (□) 0.3 mM, (△) 1 mM and (●) 3 mM. Each point is the mean of six experiments. The SEM does not exceed size of the symbols.

TABLE I.

Formula	X ⁻	R ₁	R ₂	-Y-	Name
	J	-CH ₃	-H		Me-Py-J
	J	-CH ₃	2-CH=NOH		2-PAM
	J	-CH ₃	4-CH=NOH		4-PAM
	Cl	-CH ₂ -O-CH ₂ -CH ₂ Cl	4-CH=NOH		GK3
	J	-H	-H	O	PM ₂ OP
	Cl	4-CH=NOH	4-CH=NOH	O	Toxogonin
	Cl	2-CH=NOH	3-C(O)-NH ₂	O	HS 6
	Cl	3-C(O)-NH ₂	3-C(O)-NH ₂	O	HS 8
	J	-H	-H	CH ₂	PM ₃ P
	Br	4-CH=NOH	4-CH=NOH	CH ₂	TMB 4

This action can be increased or decreased by introduction of an appropriate substituent on the pyridine, and by the position of the substituting groups in the ring. The effects of mono-pyridinium antagonists can be influenced by variation of the side chain on the quaternary nitrogen. Also the chemical structure of the bridge, by which the bis-pyridinium compounds are linked, plays a role in their mode of action. PM_3P , for example, is only an inhibitor of the enzyme. PM_2OP , however, has activating or inhibitory properties, depending on its own and the substrate concentration. On the other hand, if two oxime groups are introduced into position 4- in the pyridine ring, the resulting compound, TMB 4, acts nearly in the same way as PM_2OP does. 4-PAM acts *in vitro* similar to 2-PAM. Their action can be compared with that of toxogonin. A replacement of the side chain $-CH_3$ by $-CH_2-O-CH_2-$ $-CH_2Cl$ changes the properties of the 4-oxime derivative. The resulting compound GK 3 is a pure competitive inhibitor of AChE. This is due to the longer side chain with a chlorine at the end. In comparison with the bis-quaternary pyridinium compounds, the mono-pyridinium derivatives show significantly weaker effects, either activation or inhibition of the enzyme activity.

The mechanism of action of these compounds with ACh as substrate may be interpreted by their attachment at the catalytic centre and at a secondary binding site. Binding of antagonists at secondary binding sites was described by several authors (cf. Introduction). At this secondary, noncompetitive binding site, competition between ligands and substrate may occur. This reaction takes place beside the competitive mechanism at the active centre of the enzyme. Thus, a competitive—noncompetitive mechanism appears. Furthermore, an interdependence between the two binding sites exists. Occupation of one or both by substrate and/or antagonist may result not only in conformational changes, but also in allosteric reactions. By this way, two reactions are taking place at the same time and are overlapping. The resulting dose-response curve represents phenomena of which the maximum reaction velocity V_m cannot be calculated. The curves do not even fit the Hill equation. Appropriate methods for the calculation of kinetic data of these curves will be discussed at the end of this paper.

Other phenomena elucidating the mechanism of action of the pyridinium compounds with AChE are the different effects which they produce with different substrates. Figs. 2 and 3 show the different mode of action on AChE when the substrates are ACh and MeCh. Under our experimental conditions MeCh showed less substrate inhibition than ACh does. The maximal substrate decomposition rate of MeCh is more than half lower than that of ACh and shifted towards higher concentrations. This difference is not due to the enzyme concentration. The amount of enzyme in the experiments with MeCh was the same as in the experiments with ACh and both studies were done with ghosts from the same batch. Bis-pyridinium compounds are not able to activate AChE significantly in presence of MeCh as substrate, as it happens in presence of ACh. Assuming that the action of the active centre of AChE is influenced by ligand binding at the secondary binding site, binding of ACh at the latter is possibly one of the reasons for the enzyme inhibition at high substrate concentrations. An inhibitory effect at the active centre is induced by this reaction. This effect would be additional to the competition of the substrate

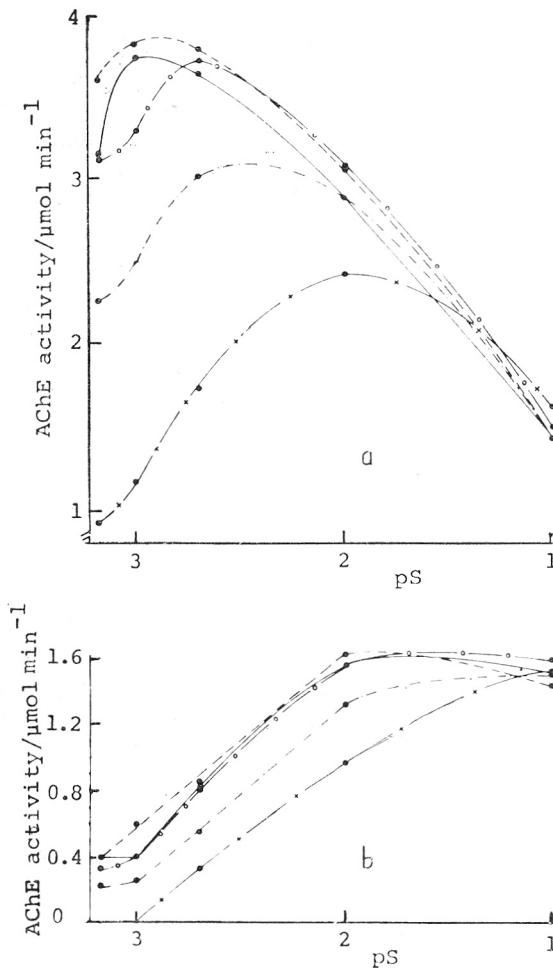


Fig 2. Effect of TMB-4 on the activity of acetylcholinesterase (AChE). Substrates: (a) acetylcholine and (b) acetyl- β -methylcholine. Concentrations of TMB-4: —●— zero, - - -○- 3 μ M, -○- 30 μ M, -·-·- 0.3 mM and -×- 3 mM. Data from Kuhn¹¹

molecules involving a two-point attachment at the catalytic site. Probably the affinity of TMB 4 and other quaternary compounds for the secondary binding site is higher than that of ACh. If these ligands are bound, potentiation or prevented inhibition of the enzyme results (provided that no competition at the active site takes place at the same time).

The substrate MeCh may also be bound at the secondary binding site. However, nearly no substrate inhibition results. The inhibitory effect on the active site is highly diminished. The affinity of MeCh for the secondary site may be even higher than that of TMB 4. When this binding site is occupied by MeCh an increase of the enzyme activity by pyridinium compounds cannot occur. Therefore the activity of AChE remains unchanged in presence of TMB 4, if MeCh is chosen as substrate.

The reaction of AChE with two substrates added simultaneously is given in Fig. 3. At a concentration of 0.1 M MeCh the activity of AChE is not influenced by ACh ($3 \mu\text{M} \leq [\text{ACh}] \leq 0.1 \text{ M}$). The dose-response curve is nearly a straight line, parallel to the abscissa. The enzyme activity with ACh as substrate and in presence of millimolar concentration of MeCh results in bell-shaped curves. At higher concentrations MeCh does not produce an additional effect; only at 1 mM MeCh, and 3 and 10 μM ACh, the rate

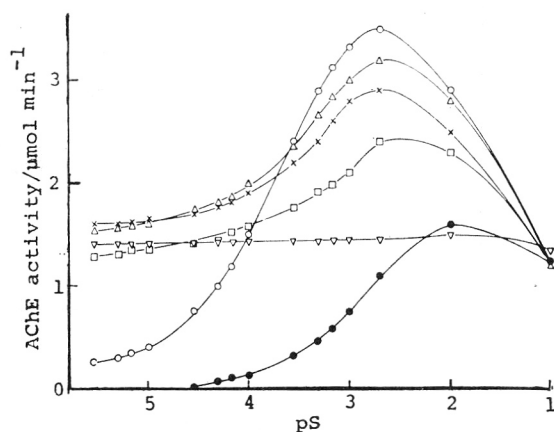


Fig. 3. The hydrolysis of acetylcholine(ACh) and acetyl- β -methylcholine (MeCh) by acetylcholinesterase (AChE). The symbols correspond to the following reactions: Abscissa-ACh; concentrations of MeCh: (O) zero, (Δ) 1 mM, (\times) 2 mM, (\square) 10 mM and (∇) 0.1 M. Abscissa- MeCh; concentration of ACh: (\bullet) zero.

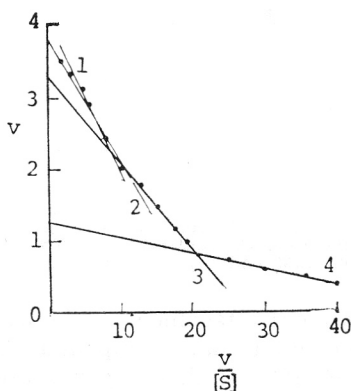


Fig. 4. Hofstee plot for the rate of acetylcholine hydrolysis by acetylcholinesterase. Results from Fig. 1 (with no toxogonin). The enzyme activity (v) is expressed in $\mu\text{mol min}^{-1}$ and the substrate concentration in mM. From the slopes and intercepts of the four lines the following V_m and K_m were calculated:

Line	$V_m/\mu\text{mol min}^{-1}$	K_m/mM
1	4.1	1.88
2	3.8	1.48
3	3.3	1.2
4	1.25	0.21

of substrate decomposition is faster, than one would expect when the single decomposition rates of both substrates are summarised. The effects of both substrates are not simply additive, but cooperative. As already mentioned the results cannot be presented by Lineweaver-Burk plots, because the enzyme does not obey the Michaelis-Menten kinetics. On the other hand the results have to fit the Hill equation in a certain way, if the enzyme undergoes allosteric reactions, that is to say, it obeys sigmoidal kinetics. Here too, the question arises, how to calculate the maximum reaction velocity V_m . This is possible when the Michaelis-Menten equation is used in the form of the Hofstee transformation instead of the Lineweaver-Burk plot

$$v = -K_m \frac{v}{[S]} + V_m$$

In the Hofstee presentation v is plotted *vs.* $v/[S]$. The intercept with the ordinate yields V_m , and the slope of the resulting straight line is the negative value of the Michaelis-Menten constant K_m . Plots of our experimental data according to Hofstee showed not a single straight line, but several lines with various slopes and different intercepts on the ordinate (Fig. 4). If these points, of which the straight lines consist, are compared with the original sigmoidal dose-response curve, it is evident that they correspond to quasi-straight sections of the bell-shaped curve. Within the range of each section the Michaelis-Menten kinetics is valid, but this is not the case for the over-all reaction.

The V_m values obtained by this graphical determination can be inserted into the Hill equation

$$\log \frac{v}{V_m - v} = n \cdot \log [S] - \log K'$$

Plotting $\log v/(V_m - v)$ *vs.* $\log [S]$ yields a straight line. Its slope equals the Hill coefficient n , and the intercept with the ordinate $-\log K'$. Inserting the experimental values of the enzyme activity with ACh as substrate according to this method (with an assumed V_m of 3.8) we obtained Hill plots as shown in Fig. 5. The conformation of the protein changes in two steps from two

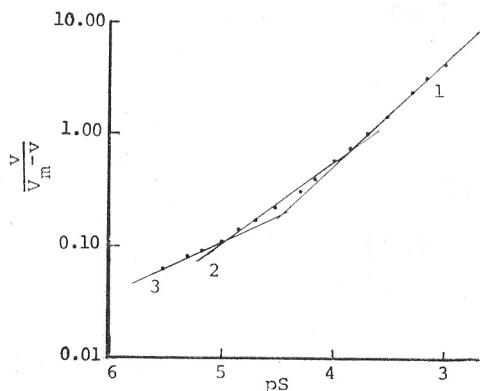


Fig. 5. Hill plot for the hydrolysis of acetylcholine by the membrane-bound acetylcholinesterase. Results from Fig. 1 (with no toxogonin). The value of V_m was taken to be $3.8 \mu\text{mol min}^{-1}$ (cf. Fig. 4). The calculated Hill coefficients (n) from the slopes of the lines 1, 2 and 3 are 0.93, 0.75, and 0.45, respectively.

different states of negative cooperativity to non-cooperativity. The Hill coefficient approaches to one, what means that the Michaelis-Menten kinetics is valid. The intercepts of the straight lines represent those substrate concentrations, where the enzyme undergoes conformational changes. Therefore we call them transition-points. Fig. 6 shows the Hill plots obtained in presence of toxo-

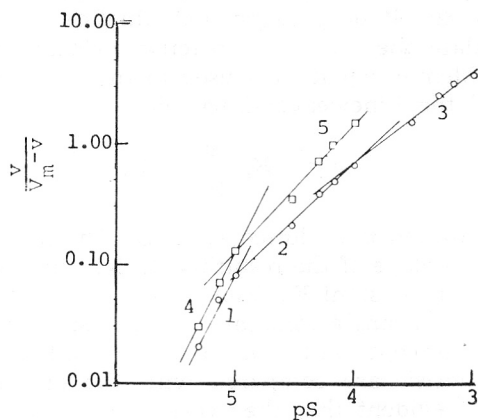


Fig. 6. Hill plot for the effect of toxogonin (3 μM) on the activity of acetylcholinesterase (AChE). Substrate: acetylcholine. V_m and v are expressed in $\mu\text{mol min}^{-1}$. The points are calculated for V_m (O) 4.60 and (\square) 3.06. Hill coefficients (n) calculated from the slopes of the lines 1, 2, 3, 4 and 5 are 1.95, 0.95, 0.78, 2.05 and 1.07.

gonin as antagonist, with ACh as substrate. Toxogonin influences the conformation of the enzyme until a state of positive cooperativity is reached, which is expressed by a Hill coefficient greater than one (in our case nearly two). Fig. 7 represents the reaction of the enzyme with MeCh as substrate. It can be seen that with MeCh, AChE approaches more to the Michaelis-Menten kinetics than it does with ACh. Our interpretation of this phenomenon is the following: ACh has at lower concentrations a greater affinity for an activator binding site, which results in a positive cooperativity of the enzyme. Concentrations

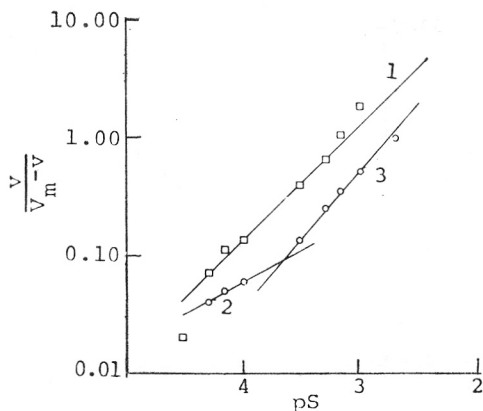


Fig. 7. Hill plot for the hydrolysis of acetyl- β -methylcholine by acetylcholinesterase. V_m and v are expressed in $\mu\text{mol min}^{-1}$. The points are calculated for V_m (O) 2.22 and (\square) 1.15. Hill coefficients (n) calculated from the slopes of the lines 1, 2 and 3 are 1, 0.54 and 1.2 resp.

of 0.01 to 1 mM indicate a greater affinity for the catalytic site, and concentrations more than 10 mM produce an allosteric inhibition. MeCh has a greater affinity for the inhibitory binding site than ACh has, but it is less effective, when the ligand is bound there. This results in a prevented allosteric inhibition, as shown by the two-substrate reaction.

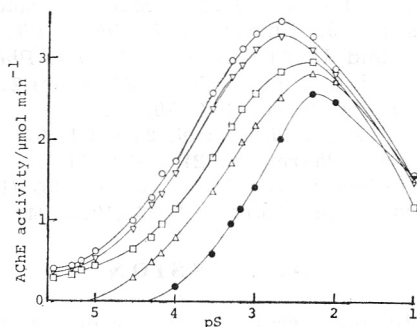


Fig. 8. Effect of toxogonin on the activity of water-soluble acetylcholinesterase (AChE) from electric eel. Substrate: acetylcholine. Concentrations of toxogonin: (O) zero, (∇) 30 μ M, (\square) 0.3 mM, (\triangle) 1 mM and (\bullet) 3 mM.

Toxogonin inhibits the enzyme at low concentrations (0.3 to 30 μ M) when at the same time the substrate concentration is also low ($3 \leq [S]/\mu\text{M} \leq 10$). An allosteric activation occurs at higher ACh concentrations (0.1 to 2 mM). Here ligand binding at a secondary binding site is postulated. This site is different from the catalytic centre (but close to it) and its occupation produces an effect on the active site. At high toxogonin concentration (0.3 to 3 mM) the reaction of the enzyme approaches the Michaelis-Menten kinetics. At toxogonin concentrations between 0.03 to 0.3 mM, AChE shows already transition states. The ligands are bound at both, the catalytic and the secondary binding sites. However, the apparent properties of the enzyme, demonstrated by the over-all reaction, are dependent on the partition of the ligand between the different binding sites.

At last a short remark concerning the different properties of structure bound and water soluble AChE. Fig. 1 shows the reaction of toxogonin with the enzyme bound to red cell membranes. In contrast to these findings the water soluble AChE from *Electrophorus electricus* could not be activated by the same concentration of toxogonin (Fig. 8), and this form of AChE showed more inhibition than the membrane bound enzyme does. Certainly, these two types of enzyme cannot be compared in a strict sense, because of their different origin. Nevertheless, there must be a reason for their different properties. We are not able to decide if the divergent reactions are due to different influences of ligand binding, or to unspecific binding, at membrane proteins, interacting with the enzyme.

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DISCUSSION

G. Kato:

Did the enzyme activation occur only at lower concentrations of toxogonin?

H. Kuhnen:

Yes.

M. E. Eldefrawi:

Why did you stop your measurements at 3×10^{-6} M substrate? It would be interesting to decrease the concentration to 10^{-8} M or even lower.

H. Kuhnen:

We measured the enzyme activity by the pH-stat, and 3×10^{-6} M substrate was the limit for the sensitivity of our electrode.

M. E. Eldefrawi:

How did you reach this substrate concentration using the pH-stat method?

H. Kuhnen:

By a double syringe system.

R. M. Krupka:

Could it not be that the activation of the membrane bound enzyme is produced by binding of toxogonin to the membrane and through interaction of unspecific proteins?

H. Kuhnen:

I would not exclude that possibility, but why does activation occur only at low antagonist concentrations?

R. M. Krupka:

How do you explain that only the membrane bound enzyme is activated?

H. Kuhnen:

We assume that interactions of the subunits occur easier when the enzyme is bound than soluble.

T. L. Rosenberry:

What kinetic explanation do you have for the properties of the enzyme?

H. Kuhnen:

I can only explain it by different degrees of cooperativity and change in cooperativity.

SAŽETAK

Regulatorna svojstva acetilkolinesteraze govedih eritrocita vezane na membranu

H. Kuhnen

Aktivatorska i inhibitorska svojstva kvarternih piridinijevih spojeva na acetilkolinesterazu (AChE), vezanu na membrane govedih eritrocita, istražena su *in vitro*. Djelotvornost istraživanih spojeva zavisi prvenstveno o njihovoj kvarternoj strukturi a utjecaj supstituenata na piridinskom dijelu od manjeg je značaja. Učinak tih spojeva na hidrolizu acetyl- β -metilkolina (MeCh) različit je od učinka na hidrolizu acetilkolina (ACh). Reakcije enzima s toksogoninom različite su za enzim vezan na strukturu i enzim topiv u vodi; za razliku od vezanog enzima, topivi enzim nije bio aktiviran.

Vrijednosti V_m određene su metodom po Hofstee-u. Hillovi dijagrami dali su nekoliko pravaca. Postulirano je da acetilkolinesteraza postoji u nekoliko stanja kooperativnosti (negativna kooperativnost, pozitivna kooperativnost i nekooperativnost). Pretpostavlja se da su različita stanja kooperativnosti u korelaciji s različitim alosteričkim konformacijama proteina.

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