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Conference Paper

Allosteric Reactions of Horse Serum Cholinesterase

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The reactions of serum cholinesterase with organic cations, such as decamethonium and atropine, as well as with inorganic cations, such as Sc^{3+}, Eu^{3+}, and Yb^{3+}, have been studied. Graphical analysis of the inhibition of serum cholinesterase by decamethonium and lanthanide ions revealed a linear mixed inhibition. Atropine inhibited competitively serum cholinesterase with butyrylcholine or acetylcholine as a substrate, whereas with benzoylcholine as a substrate atropine increased the enzyme activity. The acceleration of benzoylcholine hydrolysis by the simultaneous action of atropine and $EuCl_3$ was more pronounced than with atropine alone. The concentration of $EuCl_3$ which practically did not affect the enzyme activity, decreased the inhibition of serum cholinesterase by D-tubocurarine. It was suggested that the mixed type of inhibition of serum cholinesterase by decamethonium and the lanthanide ions, as well as the effect of the combined action of organic cations and lanthanides on the enzyme activity, might reflect the allosteric properties of serum cholinesterase.

INTRODUCTION

There are many similarities and differences between serum cholinesterase (serum ChE) and acetylcholinesterase (AChE). Since serum ChE is in its native form soluble, it is less affected by various preparation procedures than membrane bound AChE. The catalytic sites of the two enzymes are considered to be similar, consisting of one anionic and one esteratic subsite, the difference being in the hydrophobic areas[1,2]. Kinetically the two enzymes differ in many respects, especially in the steric requirements for substrates and inhibitors[3,4]. The reactions of serum ChE with acylating agents were more intensively studied than those with noncovalently acting agents[5]. However, reactions of serum ChE with organic cations, such as mono- and bis-quaternary ammonium ions, proved the existence of an anionic subsite in the catalytic site of serum ChE[6]. The organic cation D-tubocurarine (TC), which is a cholinoreceptor blocking agent, was an important tool in the study of AChE reactions as a model for cholinoreceptor reactions. Recently it has been shown that the inhibition of serum ChE by TC is of the mixed type; this was interpreted as pointing to an allosteric binding of TC to the enzyme protein[9]. One aim of the present work was to investigate the influence of organic cations decamethonium (DM) and atropine (Atr) on serum ChE activity. These two compounds interfere with the cholinoreceptor and the AChE reactions[7,8].

Bivalent inorganic cations influence the activity of serum ChE, but the mechanism of this effect is not clear. Van der Meer[10] obtained 10 to 25%

activation of serum ChE by Ca^{2+} with acetylcholine (ACh) as a substrate, while with the uncharged substrate butylacetate, the activation was of the order of 100%. Hofstee[11] found that bivalent metal ions greatly increase the rate of hydrolysis of the phenothiazine derivatives catalyzed by serum ChE and interpreted his finding in the sense that in addition to the catalytic site an auxiliary group was present on the enzyme molecule. Recently, lanthanide ions were suggested as isomorphus substitutes for Ca^{2+} binding sites in proteins[12]. Thus the second aim of this work was to find whether and how scandium, europium, and ytterbium ions interact with serum ChE and how they influence the interactions of serum ChE with some organic cations.

MATERIALS AND METHODS

The two serum ChE preparations were: 1) horse serum, diluted 30-times in the assay sample; and 2) a filter cake obtained from the fifth stage on the serum ChE purification according to Strelitz (250 enzyme units per mg of protein); the concentration in the assay sample was 10 μg per ml. Substrates: butyrylcholine iodide (BuCh) in concentrations from 0.5 mM to 20 mM, butyrylthiocholine iodide (ButhCh) from 0.02 mM to 20 mM, ACh iodide from 1 mM to 20 mM, and benzoylcholine chloride (BzCh) from 1 mM to 10 mM. Organic salts: DM iodide from 0.05 mM to 5 mM, Atr (as sulfate) from 0.05 mM to 10 mM (with regard to Atr), TC chloride in concentrations 0.1 mM and 1 mM, and 0.01 μM eserine (as sulfate; concentration with regard to eserine). All substrates, as well as organic and inorganic salts, were obtained from Koch-Light. Molar solutions of lanthanide chlorides were prepared in 10 mM HCl and diluted and neutralized by 10 mM NaOH before use.

The experiment with exhausting dialysis was performed at 4 °C against physiological solution buffered with veronal-Na to pH=7.0. When the experiment required 24 h incubation time the enzyme-inhibitor solution and the normal sample were buffered with veronal-Na to pH = 7.0 and kept at 4 °C.

The enzyme activity was measured with a Radiometer automatic titrator (Type TTTlc titrator, ABUlb automatic buret, TTA3 titration assembly, and SBR2c recorder). To the enzyme preparation (in saline solution without buffer adjusted to and kept at pH=7.0 with NaOH solution) one of the organic or inorganic salts, or a mixture of the two and the substrate, were added. In the experiments with the purified enzyme preparation, a gelatine solution was added to the saline solution in order to obtain a final gelatine concentration of 0.02%. In one series of the assays with a lanthanide and an organic salt the former was added before the latter, whereas in another series the order was reversed. The measurements were carried out at an ionic strength of 0.17 M and 25 °C, in at least three parallel runs. The 10 mM solution of NaOH was prepared every day in a nitrogen atmosphere and bubbled through with nitrogen during measurements. A flow of nitrogen was also maintained over the surface of the reaction solution. The value of the non-enzymatic hydrolysis rate of the substrate was measured and subtracted from the value of the corresponding enzyme hydrolysis rate.

The dependence of the initial hydrolysis rates on the concentration of organic or lanthanide salts at different substrate concentrations was graphically analyzed according to Dixon and Cornish-Bowden[13]. In some experiments the inhibition was analyzed by the Lineweaver-Burk plot.

RESULTS AND DISCUSSION

The graphical analysis according to Cornish-Bowden[13] of the inhibition of serum ChE by DM with BuCh as a substrate revealed that the inhibition was of the mixed type (Fig. 1); the constant for the noncompetitive component of inhibition (K_i') was 0.90 mM, whereas the constant for the competitive inhibition determined by the Dixon plot was 75 μM. Practically the same results were obtained with diluted horse serum and with the partially purified enzyme preparation. Linear mixed inhibition occurs if an inhibitor and a homogeneous enzyme protein form the EIS complex or if an inhibitor and an

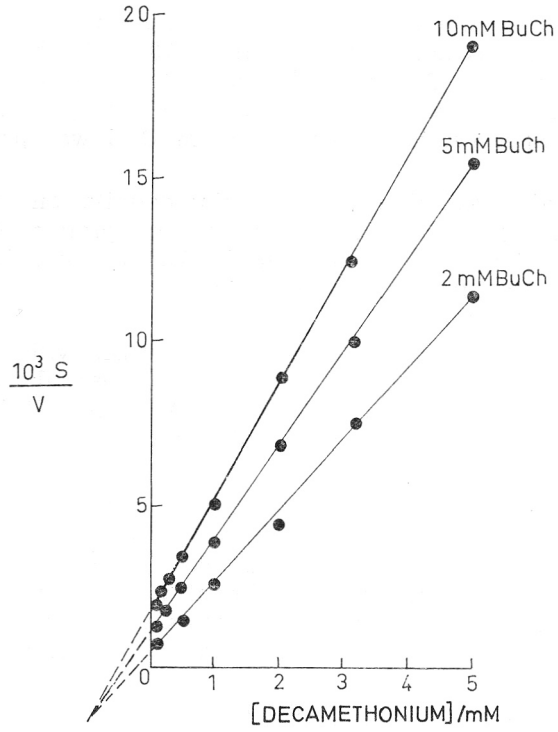


Fig. 1. Inhibition of horse serum cholinesterase by decamethonium; substrate butyrylcholine (Cornish-Bowden plot); titrimetric data at pH = 7.0 and 25 °C.

acylated enzyme form a binary complex. These two types of complexes point to an important difference in the nature of the interaction between an inhibitor and an enzyme protein. In the case of the complex EIS, the allosteric properties of an enzyme protein are implied, whereas the binding of an inhibitor to an acylated enzyme can induce a localized conformational change only in the active site; this phenomenon might manifest in the form of mixed inhibition. The formation of a complex inhibitor—acylated enzyme has been suggested for the interaction of some organic cations with AChE[14]. If the inhibition of serum ChE by DM is studied, the possibility that acylated serum ChE might form a complex with the inhibitor can not be excluded. But, in the view of the fact that the tetraethylammonium ion only slightly activates serum ChE at lower pH values[15], and that the acceleration by tetraethylammonium ion of the inhibition of serum ChE by methanesulfonyl fluoride is around ten times lower than in the case of AChE[16], it can be assumed that conformational changes induced by the binding of an inhibitor to the acylated serum affect the activity of the esteratic subsite of serum ChE in a lesser degree than in the case of AChE.

Further, the possibility that serum ChE is not a single enzyme but a mixture of isoenzymes should also be considered. The existence of isoenzymes of serum ChE was demonstrated by several authors[17–19]. Recently, four isoenzymes of serum ChE with different values of K_m were obtained by means of

polyacrylamide gel electrophoresis. The value of K_m for ButhCh of the predominant isoenzyme was found[20] to be (2.46 ± 0.73) mM. The value of K_m for ButhCh of both our serum ChE preparations (diluted horse serum and partially purified serum ChE) was around 0.27 mM. These data permit the conclusion that under our experimental conditions serum ChE was practically homogeneous.

On the basis of formal kinetic data nothing definite can be said about the nature of the binding of an inhibitor to an enzyme protein. We suggest only that the mixed inhibition of serum ChE by DM is a manifestation of the existence of a ternary EIS complex rather than of the binary complex of DM with the acylated enzyme.

It is generally considered that the interaction of DM with AChE is isosteric and allosteric[7,8,21,22]. Thus, it seems that in this respect the reaction of DM with serum ChE resembles the reaction of DM with AChE. An analogous similarity was found for the interaction of serum ChE with TC and that of AChE with TC[9].

Atropine is the oldest known and the most important antagonist of the ACh action at the muscarinic cholinoreceptor[23]. The fact that after 24 hours the inhibition of serum ChE by Atr was no weaker than after a few minutes, permits the assumption that Atr, in spite of its carbonyl group, is not a substrate for serum ChE. From the experiments with Atr and eserine acting simultaneously on serum ChE (Table I) it might be assumed that Atr does

TABLE I.

The influence of atropine (Atr) *and eserine* (E) *on the rate (V) of serum cholinesterase-catalyzed hydrolysis of benzoylcholine* (BzCh). *Titrimetric data at* pH = 7.0 *and* 25 °C.

Agent	[BzCh] mM	[Atr] mM	[E] μM	V enz. unit
BzCh	2	—	—	0.56
BzCh + Atr	2	1	—	1.20
BzCh + E	2	—	0.01	0.10
BzCH + Atr +E	2	1	0.07	1.15

not bind to the esteratic subsite of serum ChE. The Dixon plot (BuCh as substrate) and the Lineweaver—Burk (ACh as substrate) revealed that the inhibition of serum ChE by Atr was competitive (Figs. 2 and 3), whereas with BzCh as a substrate Atr activated serum ChE. The acceleration of serum ChE-catalyzed hydrolysis of BzCh by Atr depends on the concentration ratio between Atr and BzCh[24] and on pH; at pH = 7.0 the highest acceleration was about 120%, whereas at pH = 5.5 it was about 500% (Fig. 4). Acceleration effects of Atr on benzoylcholine hydrolysis might be due to a conformational change induced by the binding of Atr to the anionic subsite of the benzoylated serum ChE. However, Atr does not practically accelerate the inhibition of serum ChE by methanesulfonyl fluoride (Pavlič, personal communication). Competitive inhibition might point to isosteric binding only if the structure of the inhibitor and that of the substrate closely resemble each other. A

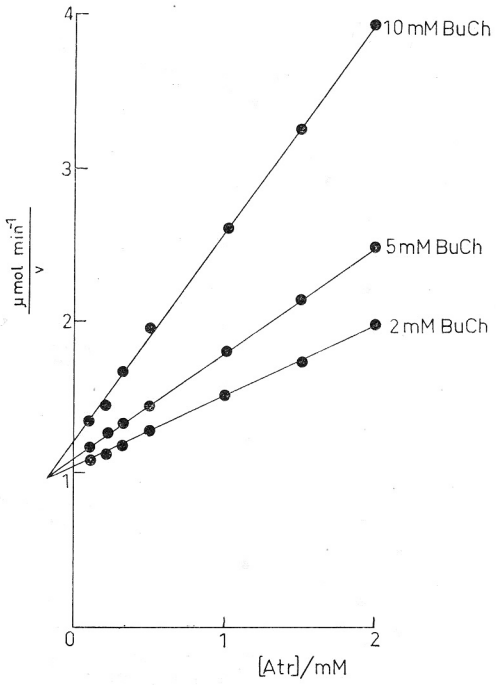
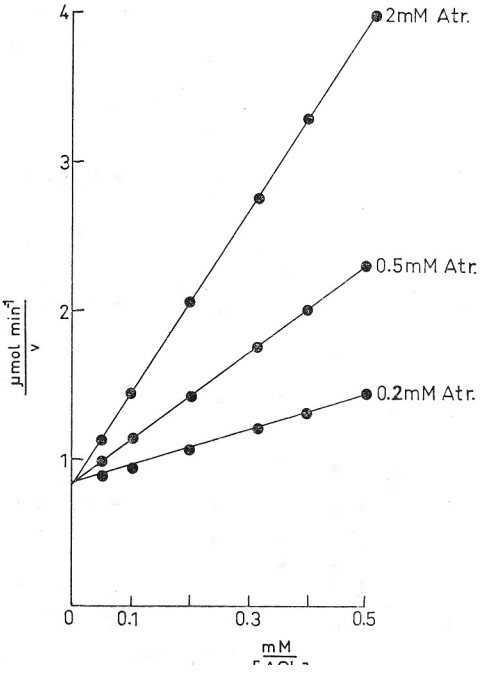


Fig. 2. Inhibition of horse serum cholinesterase by atropine; substrate butyrylcholine; titrimetric data at pH = 7.0 and 25 °C.



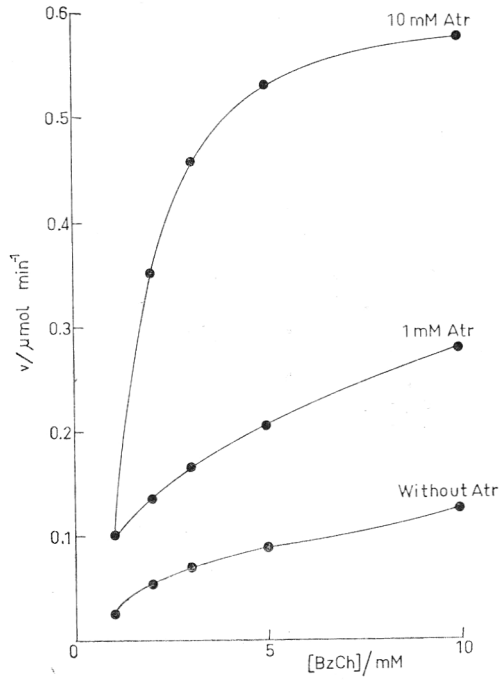


Fig. 4. The influence of atropine on the activity of serum cholinesterase with benzoylcholine as substrate. pH = 5.5, 25 °C.

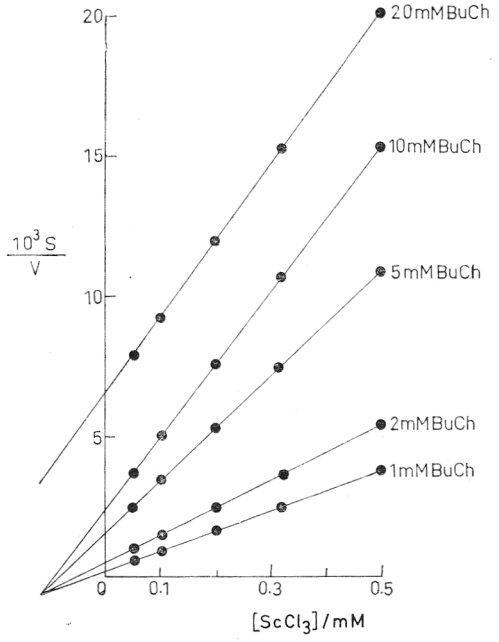


Fig. 5. Inhibition of serum cholinesterase by $ScCl_3$; substrate butyrylcholine (Cornish-Bowden plot); titrimetric data at pH = 7.0 and 25 °C.

structural relationship between Atr and ACh is not obvious at first sight. Then, in such a case competitive inhibition might be due to a steric hindrance. Some years ago allosteric binding of Atr to AChE was shown by means of n. m. r. spectroscopy even though the inhibition of AChE by Atr with ACh at suboptimal concentrations is competitive[26,27].

The hydrolysis of lanthanide ions limits the pH range over which measurements can be done without observable precipitation of the metal ions. Thus 7.0 was the highest pH value at which the inhibition of serum ChE by lanthanide ions was studied. The inhibition of serum ChE (ACh or BuCh as substrates) by lanthanide ions was of the mixed type (Figs. 5 and 6). $ScCl_3$ inhibited serum ChE most strongly; 3 mM $ScCl_3$ completely inhibited serum ChE, but practically all enzyme activity was recovered by exhaustive dialysis. The inhibition of serum ChE by $YbCl_3$ was weaker, whereas $EuCl_3$ in concentrations up to 1 mM did practically not inhibit the enzyme activity. The inhibition of serum ChE by $EuCl_3$ at higher concentrations was likewise of the mixed type. The hydrogen ion interfered with the inhibition of serum ChE by $ScCl_3$ noncompetitively (Fig. 7). It seems that Sc ions can block the

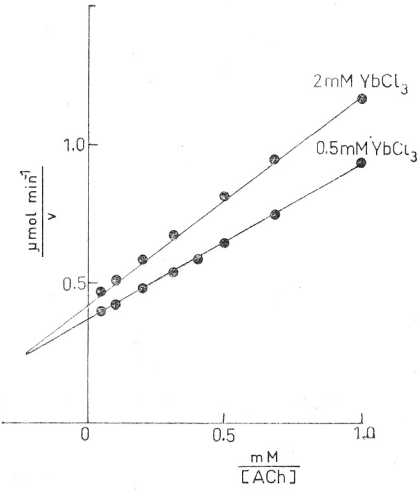


Fig. 6. Inhibition of serum cholinesterase by $YbCl_3$; substrate acetylcholine; titrimetric data at pH = 7.0 and 25 °C.

sites in the enzyme protein for which hydrogen and substrate ions compete; as a consequence of this competition the pH dependence of the enzyme activity in the presence of lanthanide ions is similar to the pH dependence in the case of an uncharged substrate[28].

Comparing the effect of lanthanide ions and that of Ca^{2+} on serum ChE activity one can see that Ca^{2+} in concentrations up to 50 mM increases K_m as well as V_m, thus acting as an activator[29]. However the activating effect of a metal ion defines neither its structural association with an enzyme nor its physiological function[30]. The inhibitory effect of a metal ion might be more helpful for the study of a metal ion with an enzyme protein. The inhibitory effect of $ScCl_3$ is comparable to that of organic cations, but $EuCl_3$ is a very poor inhibitor. However, at concentrations which do not affect the enzyme

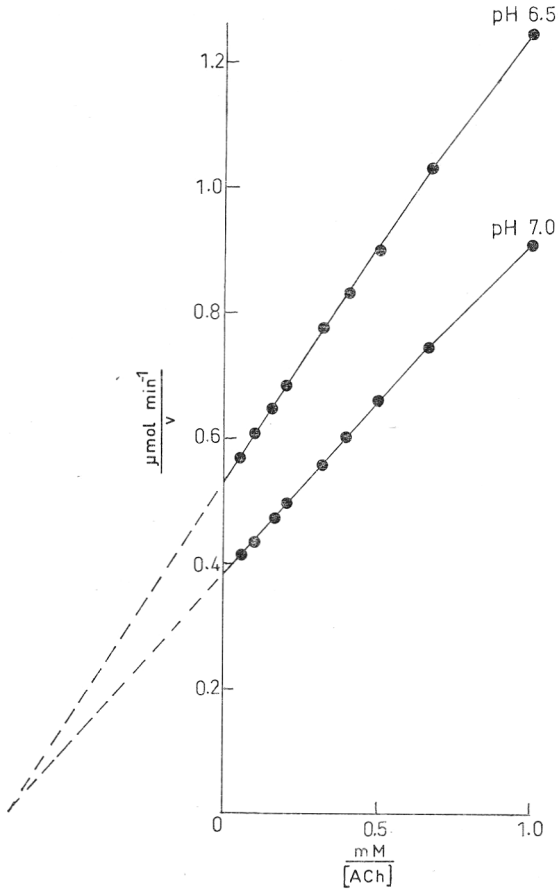


Fig. 7. The influence of pH on the inhibition of serum cholinesterase by 0.5 mM $ScCl_3$; substrate acetylcholine; titrimetric data at pH = 6.5 and 7.0, 25 °C.

activity, $EuCl_3$ decreases the inhibition of serum ChE by TC (Table II) and by DM (Table III). $EuCl_3$ accelerated the hydrolysis of BzCh, but this accelaration was less pronounced than with Atr. However, when Atr and $EuCl_3$ simul-

TABLE II.

The effect of D-tubocurarine (TC) *and* $EuCl_3$ *on the activity of serum cholinesterase. Titrimetric data with 10 mM butyrylcholine,* pH = 7.0, 25 °C.

Inhibitor	Activity (%)
0.1 mM TC	40
1 mM TC	16
1 mM $EuCl_3$	96
0.1 mM TC + 1 mM $EuCl_3$	65
1 mM TC + 1 mM $EuCl_3$	30

TABLE III.

The effect of decamethonium (DM) and lanthanide chlorides on the activity of serum cholinesterase; titrimetric data at pH = 7.0 *and* 25 °C. *Substrate:* 10 mM *butyrylcholine.*

Inhibitor	Activity (%)
1 mM DM	40
1 mM $ScCl_3$	32
1 mM $EuCl_3$	97
1 mM $YbCl_3$	68
1 mM DM + 1 mM $ScCl_3$	5
1 mM DM + 1 mM $EuCl_3$	62
1 mM DM + 1 mM $YbCl_3$	48

TABLE IV.

The effect of atropine (Atr) and lanthanide chlorides on the activity of serum cholinesterase. Titrimetric data at pH = 7.0 *and* 25 °C. *Substrate:* 10 mM *benzoylcholine.*

Activator or inhibitor	Activity (%)
1 mM Atr	165
1 mM $ScCl_3$	25
1 mM $EuCl_3$	138
1 mM $YbCl_3$	75
1 mM Atr + 1 mM $ScCl_3$	39
1 mM Atr + 1 mM $EuCl_3$	197
1 mM Atr + 1 mM $YbCl_3$	147

taneously acted on serum ChE the acceleration of BzCh hydrolysis was about 20% greater than in the case of Atr alone (Table IV). The same result was obtained regardless of the order of addition of the activators.

Regarding the magnetic properties of lanthanide ions (all but Sc, Sm, and La are paramagnetic) and their effects on serum ChE reactions, it might be assumed that lanthanide ions can be good spectroscopic probes of metal ion binding sites in serum ChE.

The mixed type of inhibition of serum cholinesterase by decamethonium and lanthanide ions as well as the effect of the combined action of organic cations and lanthanides on the enzyme activity permits the assumption that these reactions reflect allosteric properties of serum ChE. If this assumption is correct then the question arises of the physiological or pharmacological significance of this allosterism. The possible role of the allosterism of serum ChE might be that the binding properties and binding capacity of serum ChE are involved in the control of the concentration of various physiologically or pharmacologically active cations, to which membrane bound cholinesterases are more sensitive than serum ChE.

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DISCUSSION

M. E. Eldefrawi:

Europium and terbium solutions are highly fluorescent, and we find that the ACh-receptor protein binds these ions, and when bound their fluorescence intensity increases several fold. Have you observed anything similar with butyrylcholinesterase and these ions?

Lj. Kamarić:

I have not measured the fluorescence intensity. I only studied the effect of scandium, europium and ytterbium ions on the overall reaction of horse serum cholinesterase.

TABLE III.

The effect of decamethonium (DM) and lanthanide chlorides on the activity of serum cholinesterase; titrimetric data at pH = 7.0 *and* 25 °C. *Substrate:* 10 mM *butyrylcholine.*

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DISCUSSION

M. E. Eldefrawi:

Europium and terbium solutions are highly fluorescent, and we find that the ACh-receptor protein binds these ions, and when bound their fluorescence intensity increases several fold. Have you observed anything similar with butyrylcholinesterase and these ions?

Lj. Kamarić:

I have not measured the fluorescence intensity. I only studied the effect of scandium, europium and ytterbium ions on the overall reaction of horse serum cholinesterase.

E. Heilbronn:

Do you happen to know if any atropinesterase activity is present in your serum preparations?

Lj. Kamarić:

Atropine was not hydrolysed either by diluted horse serum or by purified serum cholinesterase preparations.

IZVLEČEK

Alosterične reakcije serumske holinesteraze

Lj. Kamarić

Pri proučevanju reakcij serumske holinesteraze z organskimi kationi, kot sta dekametonij in atropin, ter anorganskimi, kot so ioni Sc, Eu in Yb, se je pokazalo, da je inhibicija serumske holinesteraze z dekametonijem in z lantanidi linearne mešane vrste. Atropin je pri butirilholinu ali acetilholinu kot substratu inhibiral serumsko holinesterazo kompetitivno, medtem ko je pri bencoilholinu kot substratu zvečal encimsko aktivnost. Atropin in $EuCl_3$ sta skupaj bolj kot sam atropin pospešila hidrolizo bencoilholina. V koncentraciji, ki praktično ne vpliva na encimsko aktivnost, je $EuCl_3$ zmanjšal inhibicijo serumske holinesteraze z D-tubokurarinom. Domneva se, da bi mešana inhibicija serumske holinesteraze z dekametonijem in lantanidi kot tudi učinek skupnega delovanja organskih kationov in lantanidov na encimsko aktivnost mogla odsevati alosterične lastnosti serumske holinesteraze.

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