Influence of Ions and Chelating Agents on the Haemolymphacetylcholinesterase of *Mytilus edulis*

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By use of different inhibitors as well as atomic absorption spectrophotometry it has been shown that the haemolymph-acetylcholinesterase (E. c. 3.1.1.7) of the sea mussel *Mytilus edulis* is a metalloprotein containing 2,95 Fe^{2+}-ions per subunit. All inhibitors used (1,10-phenanthroline, salicylic aldehyde, 2,2′-dipyridyl, 8-hydroxyquinoline) showed a non-competitive inhibition, which was not pH-dependent. Some divalent cations caused a marked increase of the enzyme activity, some heavy metals inhibited the enzyme almost completely; monovalent inorganic cations did not influence the enzyme at all. Besides NaF and Na_2SiF_6, which showed a non-competitive inhibition comparable to the inhibition observed with the chelating agents, and NaN_3, whose mode of action was not identifiable, no inhibition by different mono- and divalent inorganic anions was to be observed. Ammonium ions caused no enzyme inhibition, but length the inhibition power of substituted ammonium ions increased with an increasing C-chain. The influence of some organic solvents on the enzyme activity is demonstrated.

Introduction

The interaction of metal ions with enzymes, which is of interest from structural, mechanistic or regulatory points of view, has been extensively studied in the case of acetylcholinesterase [1 − 3]. From all acetylcholinesterases the membrane-bound esterases from erythrocytes [4] and electric organs [1, 5, 6] are most frequently investigated, but the findings are sometimes contradictory. Kossorotow *et al.* [4] report no different kinetic properties of membrane-bound acetylcholinesterases from nervous tissue and from erythrocyte "ghosts", but Massoulié *et al.* [7] showed that a change in the molecular properties of this enzyme is observed if, after tryptic treatment of electric organ tissue from the electric eel, the enzyme was released from the innervated surface of the electroplax membrane. We present data on interactions of various cations and anions as well as chelating agents with a soluble acetylcholinesterase, whose isolation from the haemolymph of the sea mussel *Mytilus edulis* has been described elsewhere [8, 9]; by use of this soluble enzyme problems whould be avoided which may occur when using the solubilized membrane-bound acetylcholinesterases. The interaction of ions and chelating agents might give information about ions necessary for the specific action of the enzyme or about the conformation or structure of the enzyme in its active centre.

Materials and Methods

Acetylcholinesterase was prepared by $(NH_4)_2SO_4$-precipitation followed by Ultrogel Ac 22-gelfiltration and DEAE-Biogel ion exchange chromatography from the haemolymph of the common edible sea mussel *Mytilus edulis* as described earlier [8, 9]. The enzyme proved to be homogeneous by gelfiltration, electrophoresis and end group determination and had a specific activity of 1670 nkat per mg protein.

1,10-phenanthroline hydrochloride, 2,2′-dipyridyl, 8-hydroxyquinoline and salicylic aldehyde were from Fluka AG (Neu-Ulm), all other chemicals used were of "Analytical grade" from Merck AG (Darmstadt). In all analytical procedures quartz-distilled water was used.

Enzyme assays

Assay 1 according to Ellman *et al.* [10]

In a total volume of 2.5 ml the assay mixture contained 0.02 M sodiumphosphate (pH 7.6), 0.113 mM 5,5′-dithiobis(2-nitro-benzoic acid), varying concentrations of inhibitor and/or metal ions and

33.3 nkat acetylcholinesterase. After starting the reaction by addition of varying amounts of acetyl-thiocholine iodide the mixture was incubated at 25 °C for 15 min before the reaction was stopped by adding 100 µl of 10^{-2} M eserine salicylate. Absorbance was then read at 412 nm using a Zeiss PMQ III spectrophotometer.

Assay 2 according to Michel [11]

To 3.0 ml buffer (0.02 M sodium barbital and 0.004 M KH_2PO_4, pH 8.1 at 25 °C) 33.3 nkat acetylcholinesterase, the inhibitor and/or a cation or an anion were added, brought to 25 °C, stirred and pH_1 was measured. Then 0.4 ml of acetylcholine bromide (0.09 M) were added, the solution brought to 4.4 ml by addition of distilled water and well mixed. Forty-five min later pH_2 was measured. The difference in pH-values ($\Delta pH = pH_2 - pH_1$), which was corrected by subtracting the ΔpH of a blank test containing the same composition besides acetylcholinesterase, indicates the residual activity of the acetylcholinesterase.

Inhibition experiments

Results of inhibition experiments are presented as Lineweaver-Burk plots [12]; apparent K_i-values were determined according to Dixon [13] or Cleland [14].

Atomic absorption spectrophotometry

To a frieze-dried probe of acetylcholinesterase (1 – 2 mg, dialyzed against quartz-distilled water) 60% HNO_3 was added, the teflon vial was sealed and heated at 170 °C for 2 hours. The probe was diluted with quartz-distilled water (checked beforehand by atomic absorption spectrophotometry) and analyzed for metal ions with a Beckman atomic absorption spectrophotometer model 1223.

Results

Fig. 1 shows the influence of solvents commonly used to dissolve organic compounds, on the acetylcholinesterase. A marked increase of enzymatic
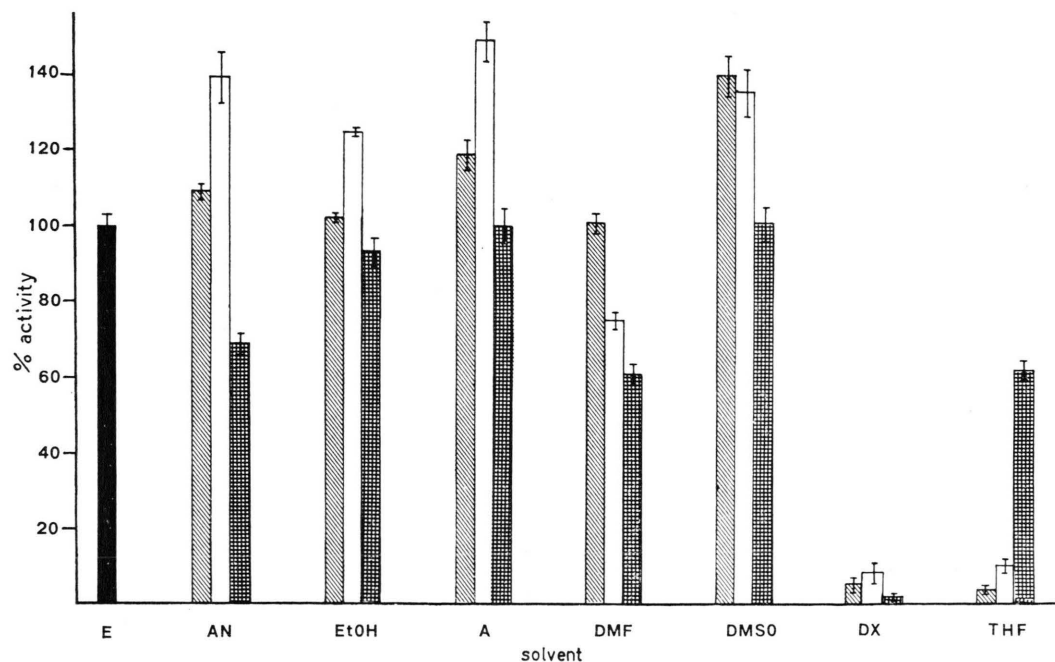
Fig. 1. The picture shows the influence of some organic solvents on acetylcholinesterase; the bars indicate the range of activity.
E, initial enzyme activity (100%); AN, acetonitrile CH_3CN; EtOH, ethanol; A, acetone; DMF, dimethylformamide $HCON(CH_3)_2$; DMSO, dimethylsulphoxide $(CH_3)_2SO$ oder Me_2SO; DX, dioxane; THF, tetrahydrofurane;

▨ activity at a solvent concentration of 20 ml/l;
▢ activity at a solvent concentration of 40 ml/l;
▩ activity at a solvent concentration of 80 ml/l.

activity is observed after adding 20 – 40 ml solvent per 1 buffer besides dimethylformamide, dioxane and tetrahydrofurane. The further addition of the solvent, however, leads to a decrease of the enzyme activity; only in the case of tetrahydrofurane does the activity increase when adding the solvent up to 80 ml per 1. In further studies acetonitrile was used because of its ability to dissolve the organic chelating compounds better than all solvents tested and to keep them in solution even at higher concentrations. Elevated enzyme activities caused by the solvent are taken into account when giving percentage values.

Different types of chelating agents have been tested with regard to their ability to influence the acetylcholinesterase activity. Whereas EDTA and EGTA produced no esterase inhibition even at concentrations up to 40 mM, other aromatic chelating agents, e. g. 1,10-phenanthroline, 2,2′-dipyridyl, 8-hydroxyquinoline and salicylic aldehyde, inhibited the enzymatic hydrolysis of acetylcholine at concentrations in the millimolar level (Fig. 2). In Fig. 3 to 5 the rates of acetylthiocholine hydrolysis at substrate concentrations between 0.42 and 4.2 mM in the presence of varying concentrations of inhibitor are shown. In these determinations all double-reciprocal plots were linear and showed non-competitive inhibiton. Drawing the intercepts or the slopes with the ordinate in the Lineweaver-Burk plots as a function of inhibitor concentration different types of inhibition were recorded. With 1,10-phenanthroline intercept-hyperbolic-slope-linear inhibition patterns were found (insets in Fig.
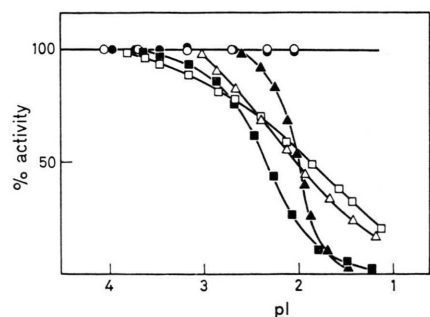

Fig. 2. The inhibition of acetylcholinesterase by different chelating agents depending on their concentration is shown. pI=neg. decad. log of the inhibitor concentration
$-\triangle-\triangle-$ 2,2′-dipyridyl;
$-\blacktriangle-\blacktriangle-$ salicylic aldehyde;
$-\square-\square-$ 8-hydroxyquinoline;
$-\blacksquare-\blacksquare-$ 1,10-phenanthroline;
$-\bullet-\bullet-$ EDTA;
$-\bigcirc-\bigcirc-$ EGTA.

3), whereas with 2,2′-dipyridyl both intercept and slope were parabolic functions (insets in Fig. 4). Intercept-parabolic-slope-linear inhibition patterns were obtained with salicylic aldehyde, and for 8-hydroxyquinoline, Na_2SiF_6 and NaF the intercepts were linear and slopes were hyperbolic functions of the inhibitor concentrations (insets in Fig. 5). Fig. 5 represents the inhibition caused by 8-hydroxyquinoline; by use of Na_2SiF_6 and NaF higher concentrations had been necessary to produce the same inhibition pattern. The results of these inhibition experiments with the corresponding K_i-values are shown in Table I.

These results might suggest that metal cations play an essential role in the hydrolysis of acetylcholine. Vallee [15], however, cautioned that the identification as a metalloenzyme should not solely based on the inhibition by metal-complexing agents. If this agent reacts specifically with a metal ion of an enzyme, inhibition should be prevented by occupying the chelation site of the agent with a metal ion prior to exposure of the enzyme [15]. Further when a mixed metalloenzyme-inhibitor complex has formed, a critical excess of metal ions should reverse the inhibition by competing with the metalloenzyme for the inhibitor bound to its metal. Removal of the inhibitor from the metalloenzyme by mass action should restore the activity. As shown in Table II, significant differences in the inhibition of acetylcholinesterase were observed using 1,10-phenanthroline as chelating agent. In all but two cases activity was not fully restored after adding equimolar contents of metal ions to the inhibitor-enzyme solution. But whereas after adding equal amounts of Fe^{2+}-ions full activity was observed, moves the activity of the enzyme up and down after adding differing amounts of Ca^{2+}-ions. Additional experiments showed that the inhibition of the acetylcholinesterase by chelating agents was a time-dependent irreversible inactivation which can often be observed with metalloenzymes [16].

To make sure that the enzyme contains the supposed metal ion a probe was subjected to atomic absorption spectrophotometry which revealed Fe^{2+}-ions as the only ions in the protein with a ratio of 17.2 Fe^{2+}-ions per molecule (mol weight 240 000) or 2.95 Fe^{2+}-ions per subunit (mol weight 39 000, [9]).

Fig. 6 and Table II show the influence of different mono- and divalent metal cations and inorganic
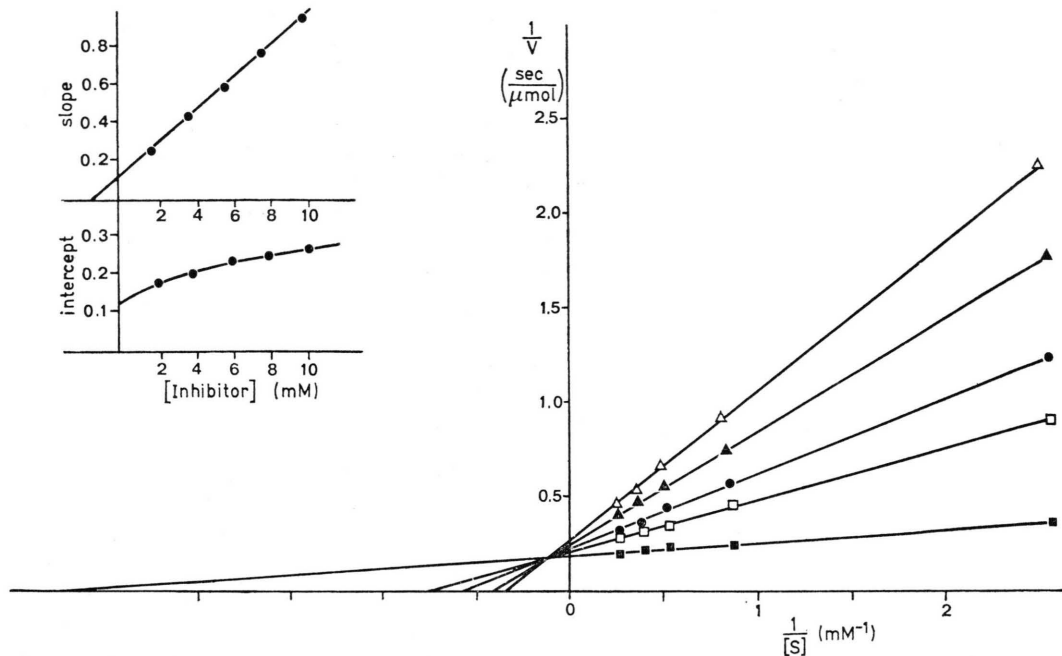
Fig. 3. Lineweaver-Burk plot of acetylcholinesterase inhibited by 1,10-phenanthroline. Acetylcholinesterase activity was determined using assay 1 (see materials and methods). Inhibitor concentrations were
—■—■— 2 mM;   —▲—▲— 8 mM;
—□—□— 4 mM;   —△—△— 10 mM.
—●—●— 6 mM;
The insets show secondary plots of slopes and intercepts versus inhibitor concentration.

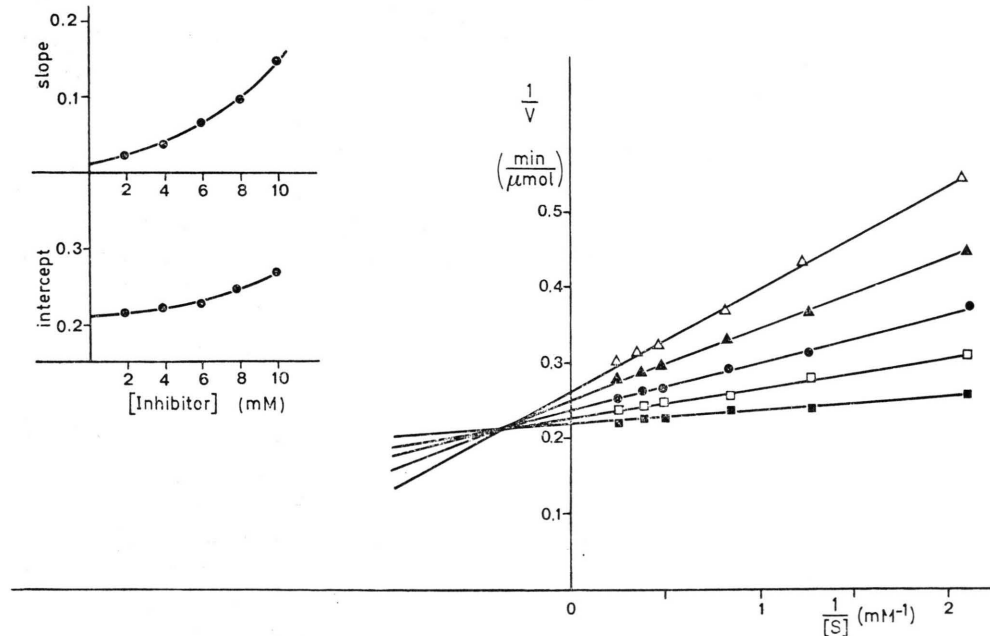Fig. 4. Lineweaver-Burk plot of acetylcholinesterase inhibited by 2,2′-dipyridyl. The conditions are the same as described for 1,10-phenanthroline (see Fig. 3). Inhibitor concentrations were
—■—■— 2 mM;   —▲—▲— 8 mM;
—□—□— 4 mM;   —△—△— 10 mM.
—●—●— 6 mM;
The insets show secondary plots of intercepts and slopes versus inhibitor concentration.
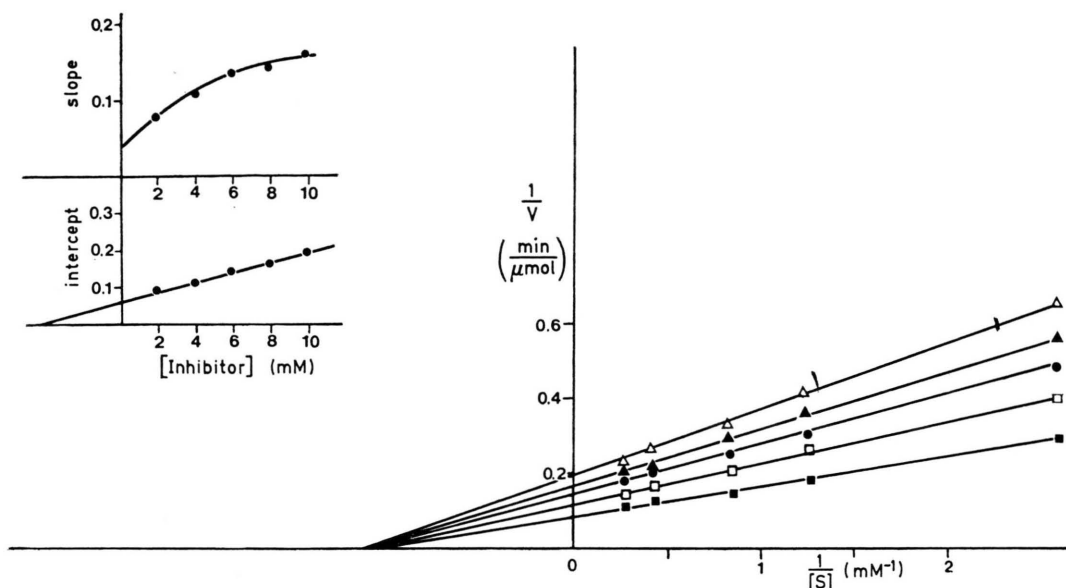
Fig. 5. Lineweaver-Burk plot of acetylcholinesterase inhibited by 8-hydroxyquinoline; the conditions are described in the legend to Fig. 3. Inhibitor concentrations were

— ■ — ■ — 2 mM;   — ▲ — ▲ — 8 mM;
— □ — □ — 4 mM;   — △ — △ — 10 mM;
— ● — ● — 6 mM;

The insets show secondary plots of slopes and intercepts versus inhibitor concentration.

By use of NaF or Na_2SiF_6 the inhibitor concentrations were

— ■ — ■ — 1 mM;   — ▲ — ▲ — 30 mM;
— □ — □ — 5 mM;   — △ — △ — 50 mM.
— ● — ● — 10 mM;

The secondary plots of slopes and intercepts versus inhibitor concentrations gae the same picture as in the case of 8-hydroxyquinoline, but other K_i-values resulted (see Table I).

Table I. Inhibition of acetylcholinesterase by chelating agents.

Inhibitor	Type of inhibition			Inhibition constant from	
	1/v vs 1/[s]	intercept vs [I]	slope vs [I]	intercept vs [I] [mM]	slope vs [I] [mM]
1,10-phenanthroline	NC	H	L	n. d.	1.4
2,2'-dipyridyl	NC	P	P	n. d.	n. d.
8-hydroxyquinoline	NC	L	H	4.8	n. d.
salicylic aldehyde	NC	P	L	n. d.	1.5
Na_2SiF_6	NC	L	H	12.4	n. d.
NaF	NC	L	H	39.5	n. d.

L=linear; H=hyperbolic; P=parabolic; NC=non-competitive; n. d.= not determinable. The inhibition constant was determined from secondary plots of slopes and intercepts versus [I] according to Cleland [14].

anions on the haemolymph-AChE. There is generally an increased activity to be observed besides for some heavy metals, but the increase is not as marked as reported for red cell AChE [5] or serum cholinesterase [3]. Monovalent metal ions do not influence the enzyme at all.

A decrease of AChE activity by use of different mono- and divalent inorganic anions is, besides NaF, Na_2SiF_6 and NaN_3, only marked at concentrations higher than 1 M, indicating that only the high ionic strength is responsible for this inactivation (Fig. 6); the initial enzyme activity can be

Table II. Effects of metal ions on the inhibition of acetyl-cholinesterase by complexing with 1,10-phenanthroline.

Metal ions	no inhibitor * (%)	metal ion : 1,10-phenanthroline-ratio			
		1:3	2:3	1:1	3:2
Control	100%	—	—	100%**	—
MgCl$_2$	110.2	111.9	108.5	88.1	78.0
CaCl$_2$	157.4	93.6	85.7	97.6	45.8
CoCl$_2$	187.5	98.2	18.5	11.6	16.2
NiCl$_2$	106.8	16.4	10.3	12.0	17.1
FeCl$_2$	135.6	17.1	68.3	99.5	108.0
FeCl$_3$	61.0	12.1	21.2	35.4	45.8
MnCl$_2$	154.2	76.2	32.2	12.4	5.2
ZnCl$_2$	106.8	23.7	21.2	11.9	10.2
CuSO$_4$	0	18.1	10.3	4.5	0
HgCl$_2$	7.1	1.4	2.3	3.2	6.4
Pb(Ac)$_2$	18.6	27.1	19.1	11.0	9.3
Cd(Ac)$_2$	140.7	15.4	11.4	5.4	7.1

* In all experiments 33.34 nkat acetylcholinesterase have been used and the enzyme activity was determined using assay 2 (see material and methods); to the mixture varying amounts of metal ions were added to 1,10-phenanthroline and the reactions started by adding acetylcholine. The concentration of the inhibitor 1,10-phenanthroline was 6.0 mM; salt concentrations in absence of the inhibitor were 6.0 mM.

** The concentration of the chelating agent was adjusted to give 50% enzyme activity in absence of inhibitor and is set as 100% when referring to metal ions added.
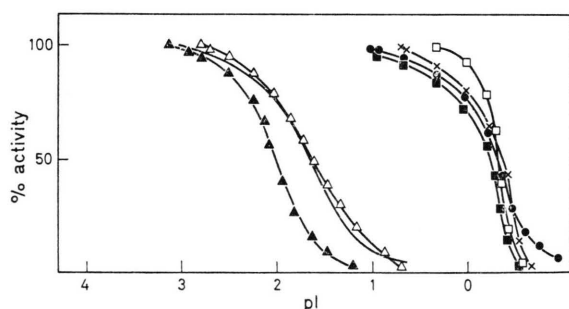

Fig. 6. This figure shows the influence of different mono- and divalent anions on acetylcholinesterase. Assay 1 (see materials and methods) has been used.
The anions (as sodium salts) are represented by the symbols:

— ▲ — ▲ —   SiF$_6^{2-}$;
— △ — △ —   F$^-$;
———————   N$_3^-$;
— × — × —   Cl$^-$;
— ● — ● —   Br$^-$;
— ▨ — ▨ —   J$^-$;
— ☐ — ☐ —   SO$_4^{2-}$.

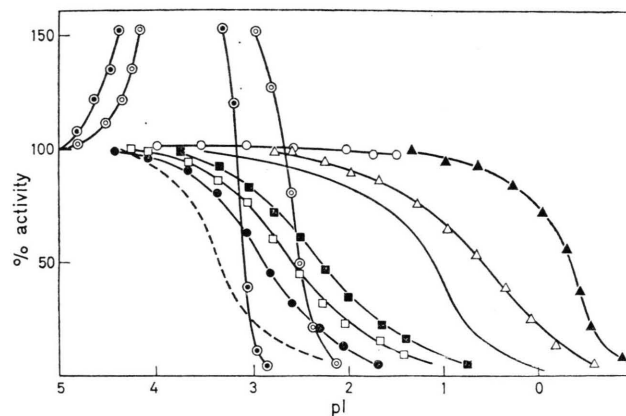PO$_4^{3-}$ and HCO$_3^-$ are not listed because they showed no influence on the enzyme activity upto 0.2 M.

Fig. 7. The drawing shows the influence of quaternary ammoniumions as well as SDS on acetylcholinesterase. Assay 1 (see materials and methods) has been used in these experiments. Symbols:

— ▲ — ▲ —   (NH$_4$)$_2$SO$_4$;
— △ — △ —   NH$_4$Cl;
———————   Choline;
— ▨ — ▨ —   tetramethylammonium chloride;
— ☐ — ☐ —   tetraethylammonium chloride;
— ● — ● —   trimethylhexylammonium bromide;
— ○ — ○ —   Hexamethonium;
— — — —   Decamethonium;
◉ — ●   SDS;
◎ — ○   trimethylcetylammonium bromide.

The highest activity obtained by use of SDS was 340% (not shown on the picture) and the highest activity obtained by trimethylcetylammonium bromide was 288%.

(Fig. 5; Table I), but the type of inhibition by use of NaN$_3$ could not be discovered.

Fig. 7 represents the inhibition of AChE by different ammonium- and substituted ammonium ions. It is shown that ammonium ions do not act as inhibitors, but by use of substituted ammonium ions with an increasing C-chain length an increase of the AChE-inhibition is to be observed. Hexamethonium produces an inhibition only at very high concentrations, but Decamethonium showed a more pronounced inhibition. N-trimethyl-N-cetyl ammonium bromide and the opposite charged SDS produce at low concentrations an increase of the enzyme activity, but this is probably due to an unfolding of the protein chain to a more active state resulting in an increased activity.

Discussion

An enzyme is called "acetylcholinesterase" when it almost only splits acetylcholine and when its activity is inhibited by selective inhibitors which only act on this enzyme. Investigating the hae-

restored after removal of the high ionic concentration by short dialysis. NaF and Na$_2$SiF$_6$, however, cause a non-competitive inhibition of the AChE comparable to that of 8-hydroxyquinoline

molymph of the sea mussel *Mytilus edulis* we detected four cholinesterases which were isolated and identified. Besides of three unspecific cholinesterases (E.C. 3.1.1.8) [22] we found one enzyme which has been undoubtedly characterized to be a specific acetylcholinesterase (E.C. 3.1.1.7) [8, 9]. The enzyme hydrolyzes only acetylcholine, acetyl-β-metylcholine and the corresponding thiocholine esters up to about 3×10^{-3} M; at higher concentrations occurs an enzyme inhibition by these substrates. Substrates for cholinesterases like propionyl-, benzoylor butyrylcholine are scarcely attacked. Selective inhibitors, *e. g.* 1,5-bis(N-dimethylallylammoniophenyl)-pentan-3-one (284 C 51) proved the enzyme to be acetylcholinesterase, and cholinesterase inhibitors like isoOMPA did not influence the enzyme activity at all. The enzyme, purified by fractional $(NH_4)_2SO_4$-precipitation, gelfiltration and ion-exchange chromatography, consists as a hexamer with subunits of about 40,000, as revealed by gelfiltration, SDS-electrophoresis and end group analysis [9]; these results have been confirmed by affinity chromatography using N-trimethyl-hexyl- and N-trimethyl-phenyl-derivatives as ligands, and preparative isoelectric focusing (Engels and v. Wachtendonk, unpublished). To get more informations concerning the kinetic and physicochemical properties of this haemolymph enzyme the present studies were undertaken, especially to see whether these results are in agreement with the properties of other known acetylcholinesterases.

In accordance with the literature [5] an increase of the AChE-inhibition is shown by substituted ammonium ions with an increasing C-chain length and no inhibition was observed caused by bromine, as reported for substituted ammonium bromides by Bockendahl *et al.* [17], but contrary to the findings obtained by Bergman and Segal [18] hexamethonium produces a very low inhibition in contrast to decamethonium. As previously shown [9] the haemolymph-AChE consists of 6 subunits with a molecular weigth of 40,000, which might be situated close together. Assuming this, decamethonium might inhibit the anionic sites of two subunits in contrast to hexamethonium, which can only interact with one anionic site. These findings are confirmed by the observation, that succinylbischoline is split by the AChE — even though only at a low rate and remaining constant at 5×10^{-3} M — but succinylmonocholine remains completely un-

affected. This would indicate a distance of about 16 Å between two anionic sites in this esterase [9].

The influence of organic solvents — necessary to dissolve some chelating agents — has been studied, for Mendoza and Wales [25] demonstrated that certain solvents applied with pesticides to various enzyme solutions would increase or decrease the inhibition or activate or deactivate the enzymes. It was therefore necessary to define and control conditions when solvents are being used in inhibition experiments carried out with enzymes. Organic solvents showing a high dipole moment (acetonitrile, acetone) caused an increase of the enzyme activity, solvents with a low dipole moment (dioxane) decrease the activity; this was taken into account when calculating the residual enzyme activity.

In contrast to AChE from the electric tissue of *Electrophorus electricus* [6], the haemolymph-AChE of *Mytilus edulis* is a metalloprotein. This is confirmed by the following observations: Inhibition by chelating agents was not feversible upon dilution and a time-dependent irreversible inactivation could be observed. Furthermore, a reversal of the inhibition could only be seen when excess Fe^{2+}-ions were added to the enzyme-inhibitor complex and, finally, atomic absorption spectrophotometry showed the enzyme to contain three Fe^{2+}-ions per subunit. The inhibition by use of 1,10-phenanthroline or 8-hydroxyquinoline was not pH-dependent; this refutes the hypothesis that protonation of a tertiary nitrogen atom might be responsible for the observed inhibition by interaction of the positively charged ammonium group with the anionic site of the enzyme [6]. In the case of the observed non-competitive inhibition (Table I) there must be separate sites on the AChE molecule to accomodate substrate and inhibitor. Substrate and inhibitor do not influence each other's interaction with the enzyme molecule, but the rate of hydrolysis is decreased, because the enzyme molecules which have combined with inhibitors are not available for substrate hydrolysis, although they may by combined with substrate as well.

It must be mentioned that all inhibitor concentrations besides the complexing agents, had to be a hundred to a thousand times higher to produce inhibition rates comparable to inhibition rates reported for acetylcholinesterases from vertebrate sources.

The influence of metal ions on AChE-activity is quite dubious; for the same ion, *e. g.* Ca^{2+}-ions, increasing and decreasing enzyme activities are reported [2, 3, 19]. The effects of these metal ions added to the enzyme without chelating agents, is difficult to explain, especially in the case of added Mg^{2+}- or Ca^{2+}-ions, as after adding EDTA or EGTA to the test solution no change in the activity is observed. These findings are in accordance with the results of Nachmansohn [20] or Friess *et al.* [21] who suppose that the effect of some bivalent metal ions in raising the activity to a constant value is due to a displacement of an inhibitor and not to direct activation. The nature and action of such an inhibitor for the haemolymph-AChE is in doubt, as in contrast to the other haemolymph-cholinesterases [22] no such inhibitor could be found. In the case of membrane-bound AChE (from *Electrophorus electricus*) Ca^{2+}-ions, which have been used for solubilizing the enzyme, lead to a marked decrease in the enzyme's activity which could be restored after adding EDTA [1]. Effects of this nature are explained by Myers [23] as due to a competitive inhibition.

An activation by monovalent inorganic cations was not found, in contrast to Nachmansohn's findings on eel acetylcholinesterase [20], but in accordance to Pyttel and Robinson [19] who also showed bovine red cell AChE to be insensitive to the nature and valency of the added cations. The influence of the ionic strength is not so pronounced with haemolymph esterase as described for acetylcholinesterases [5], no significant changes in the activity are seen using 0.001 til 0.2 M phosphate buffers. Furthermore, no decrease in the enzyme activity is observed using mono- and divalent inorganic anions besides NaN_3, NaF and Na_2SiF_6; the latter two cause a non-competitive inhibition as observed on the chelating agents. Similar observations have been reported by Knappworst and Westendorf [24], but using bovine erythrocyte AChE NaF produced there a competitive inhibition; it was mentioned by the authors that NaF might form ferrocomplexes in the presence of Fe^{2+}-ions. This possibility is a further evidence that the haemolymph-AChE of *Mytilus edulis* is a metalloenzyme containing Fe^{2+}-ions.

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