QUATERNARY AMMONIUM SALTS AS INHIBITORS OF ACETYLCHOLINE ESTERASE

by

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In a number of recent publications[1-3] the structure of the active surface of acetylcholine esterase was studied by means of changes of activity under various p_H conditions and with the help of inhibitors. The following conclusions were reached:

1. Hydrolysis of the ester linkage is effected by an esteratic group, which undergoes reversible inactivation by either H or OH ions.

2. In the nearest neighborhood of this group two (or more) negative charges of unit magnitude create a negative field.

These findings lead to a natural classification of the known inhibitors as follows:

a. Inhibitors combining only with the esteratic group, *e.g.* DFP, TEPP.

b. Inhibitors combining exclusively with one or more of the negative sites, *e.g.* quaternary ammonium salts.

c. Inhibitors combining with the esteratic group and an anionic site simultaneously, *e.g.* eserine, prostigmine.

We now assume that unspecific esterases like *e.g.* liver esterase, are distinguished from acetylcholine esterase mainly by the lack of the negative sites, but otherwise possess the same active structure. Then it can be concluded that inhibitors of type b. should be ineffective for unspecific esterases and the effect of type c. inhibitors should be greatly diminished. On the other hand, inhibitors of type a. should behave in a similar way towards both types of esterases. Results reported in this paper verify this prediction.

The question now arises whether the esteratic group and the anionic sites are independent units or parts of a defined chemical structure, so that they could show mutual interaction. The present investigation aims at clarifying this problem.

MATERIALS AND METHODS

Dog's liver esterase was prepared as follows: One part of liver was homogenized with three parts of 3% ammonium sulfate and the mixture centrifuged at 10,000 rpm. The supernatant was brought to 15% by addition of a 50% ammonium sulfate solution. After centrifugation, the supernatant was adjusted to 25% ammonium sulfate, and the precipitate, after dissolution and dialysis, again fractionated with ammonium sulfate. The fraction obtained between 18 and 22% of ammonium sulfate showed the highest activity and was used for our enzymatic experiments. The final solution

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obtained was diluted 100 times to hydrolyze 3 μ equivalents/ml/hour, when $4.3 \cdot 10^{-1}$ M diacetine was used as substrate.

Acetylcholine esterase was prepared from the electric organs of *Torpedo marmorata** and *Electrophorus electricus*. The final solution obtained could be diluted 1800 times to hydrolyze 5 μmoles/ml/hour, when $3.3 \cdot 10^{-3}$ M acetylcholine was used as substrate.

Diacetine** was kept in a desiccator over solid KOH to reduce the amount of free acid in the commercial sample.

Activity measurements were carried out by the Warburg manometric method.

RESULTS

1. *Inhibition of* ACh *and liver esterases by different types of inhibitors*

In Table I we compare the three types of inhibitors by determining the concentration c_{50} required for 50% inhibition under standard conditions.

TABLE I

INHIBITION OF DIFFERENT ESTERASES BY THREE DIFFERENT TYPES OF INHIBITORS

Activity of ACh esterase was measured with $3.3 \cdot 10^{-3}$ M ACh as substrate and an enzyme dilution sufficient to hydrolyse 5 μmoles/ml/hour.

Inhibition of liver esterase was measured with $4.3 \cdot 10^{-1}$ M diacetine as substrate and an enzyme dilution that hydrolysed 3 μequivalents/ml/hour.

0.2 M bicarbonate buffer and a gas mixture of 5% CO_2 and 95% N_2 was used in all experiments.

	c_{50}	
	ACh esterase	*Liver esterase*
1. *Inhibitors of type b*		
Tetramethylammonium iodide	$1.5 \cdot 10^{-2}$ M	not measurable up to 10^{-1}
Tetraethylammonium bromide	$3 \cdot 10^{-3}$ M	not measurable up to $5 \cdot 10^{-1}$
Choline chloride	$4 \cdot 10^{-3}$ M	not measurable up to $5 \cdot 10^{-1}$
2. *Inhibitors of type c*		
Prostigmine bromide*	$4 \cdot 10^{-7}$ M	$6.4 \cdot 10^{-3}$ M
3. *Inhibitors of type a*		
Tetraethyl pyrophosphate	$4 \cdot 10^{-8}$ M	$2 \cdot 10^{-8}$ M

* This drug was made available to us through the courtesy of Dr FURTER of Hoffmann-La Roche, Nutley, N.J.

2. *Protection of* ACh *esterase by quaternary ammonium compounds against irreversible inhibition by TEPP.*

It has been shown by AUGUSTINSSON AND NACHMANSOHN[4] that reversible inhibitors of type c. protect the enzyme against the irreversible combination with organic phosphates. The analogous effect of tetraethylammonium ions is found in the experiment described in Table II. Considerable protection is obtained, although the two inhibitors, which compete for the active surface, attach themselves to entirely different groups. This result could be explained as a sterical hindrance of the approach to the esteratic site.

* We wish to thank Prof. BACCI of the Marine Biological Station of Naples, Italy, for the generous gift of these fish.

** We wish to thank the British Industrial Solvents Ltd. for the supply of this ester.

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TABLE II

PROTECTIVE EFFECT OF TETRAETHYLAMMONIUM BROMIDE AGAINST IRREVERSIBLE INHIBITION OF ACh ESTERASE BY TETRAETHYL PYROPHOSPHATE

The incubation mixture was composed as follows:

ACh esterase, 1800 times the final (standard) concentration	0.1 ml
Tetraethylammonium bromide, $7.2 \cdot 10^{-1}$ *M*	0.1 ml
Phosphate buffer, 0.5 *M*, pH 7.2	0.1 ml
After standing at room temperature (22°) for 45 min, there was added TEPP, $1.85 \cdot 10^{-6}$ *M*	0.1 ml

After 2 min contact, the mixture was diluted 450 times with bicarbonate buffer.

Three controls were run simultaneously:

a. as above, but tetraethylammonium replaced by phosphate buffer	0.1 ml
b. as above, but TEPP replaced by phosphate buffer,	0.1 ml
c. the enzyme alone, without any inhibitor.	

For measurement of activity 3 ml of the final dilution were placed in a Warburg vessel, with 0.1 ml of ACh, $3.3 \cdot 10^{-3}$ *M*, in the side bulb.

RESULTS:

Reaction mixture	*Rate of hydrolysis of ACh*
Enzyme alone	3.8 μmoles/ml/hour
Enzyme plus tetraethylammonium ion	3.8 μmoles/ml/hour
Enzyme plus TEPP	0.3 μmoles/ml/hour, *i.e.* 7.8% of control
Enzyme plus TEPP plus tetraethylammonium ion	3.0 μmoles/ml/hour, *i.e.* 78% of control

3. *Inhibition of the hydrolysis of an uncharged ester by quaternary ammonium salts*

If the foregoing explanation is correct, it could be expected that quaternary ammonium ions will inhibit the hydrolysis of neutral esters by ACh esterase. Such an effect could then be classified as non-competitive inhibition as defined by the applicability of equation (1):

$$\frac{v_o}{v_i} = 1 + \frac{I}{Ki} \qquad (1)$$

We first determined c_{50} in the system ACh esterase—diacetine for quaternary ammonium salts. The values represented in Table III show that the inhibitory power is of the same order of magnitude for both substrates. (Diacetine at the maximum concentration used, *viz.* $4.3 \cdot 10^{-1}$ *M*, was hydrolyzed by the standard concentration of enzyme at about one third the rate of ACh. Therefore the conditions of the enzymatic reactions with the two substrates are not strictly comparable).

TABLE III

COMPARISON OF C_{50} VALUES FOR THE INHIBITION OF HYDROLYSIS OF ACh AND DIACETINE CATALYZED BY ACh ESTERASE

Inhibitor	c_{50}	
	for ACh	*for diacetine*
Tetramethylammonium iodide	$1.5 \cdot 10^{-2}$ *M*	$4 \cdot 10^{-3}$ *M*
Tetraethylammonium bromide	$3.0 \cdot 10^{-3}$ *M*	$1.2 \cdot 10^{-3}$ *M*
Choline chloride	$4.0 \cdot 10^{-3}$ *M*	$1.7 \cdot 10^{-3}$ *M*

It is important that the number *n* of inhibitor molecules combining with the active surface is independent of the character of the substrate. Determination of *n* is carried

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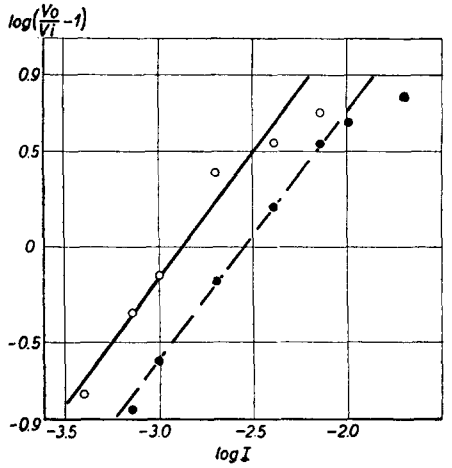

Fig. 1. Determination of number n of tetraethylammonium ions combining with ACh esterase. ACh esterase dilution used hydrolyzed 5 μmoles of ACh/ml/hour and 1.7 μequivalents/ml/hour, when diacetine served as substrate.

ACh: $3.3 \cdot 10^{-3}$ M (dashed curve)
Diacetine: $4.3 \cdot 10^{-1}$ M (solid curve)
I = inhibitor concentration
v_o = rate of uninhibited reaction
v_i = rate of inhibited reaction

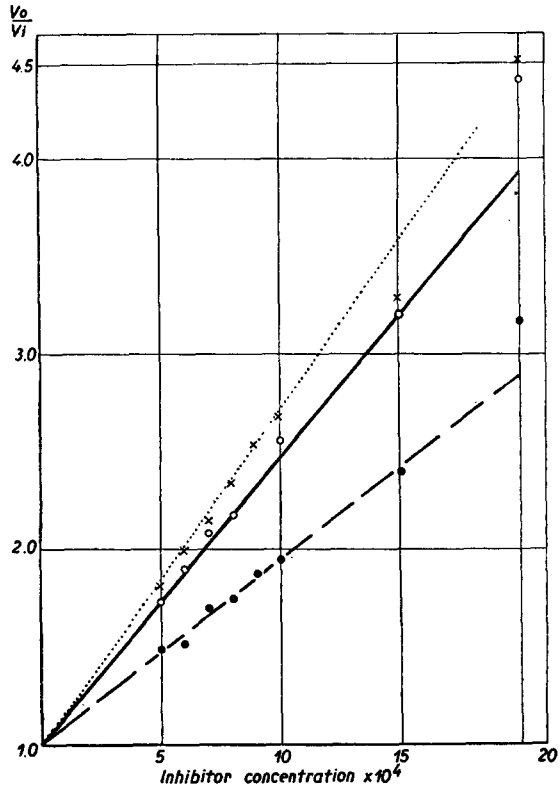

Fig. 2. Tetraethylammonium as competitive inhibitor of diacetine hydrolysis by ACh esterase.
Enzyme dilution: 1:900
..... $1.3 \cdot 10^{-1}$ M diacetine
——— $2.2 \cdot 10^{-1}$ M diacetine
– – – – $4.3 \cdot 10^{-1}$ M diacetine

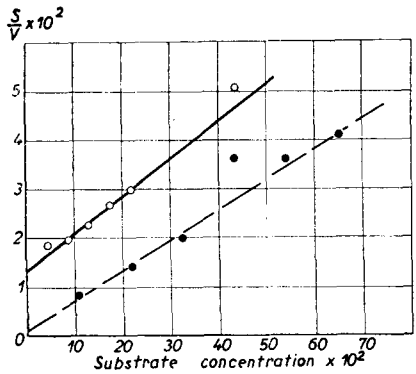

Fig. 3. Determination of the MICHAELIS-MENTEN constant for the systems diacetine – ACh esterase (solid line) and diacetine – liver esterase (dashed line)

ACh esterase diluted 1:900
Liver esterase di luted 1:60
Diacetine $4.3 \cdot 10^{-1} M$

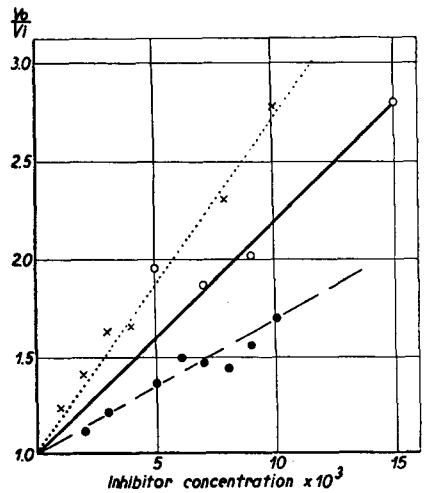

Fig. 4. Tetraethylammonium ion as competitive inhibitor of ethyl acetate hydrolysis by ACh esterase. Enzyme dilution: 1:240

..... $3.3 \cdot 10^{-1}$ M ethyl acetate ——— $5.6 \cdot 10^{-1}$ M ethyl acetate – – – – $8.5 \cdot 10^{-1}$ M ethyl acetate

out by application of equation (2). In Fig. 1 identical slopes are obtained for the inhibition by tetraethylammonium of the hydrolysis of ACh and diacetine.

$$\log\left(\frac{v_o}{v_i} - 1\right) = n \log I + C' \qquad (2)$$

To study the character of the inhibition for diacetine hydrolysis, the variation of the reaction rate as function of inhibitor concentration is shown in Fig. 2. It is evident that—contrary to our prediction—inhibition in this system is again competitive and equation (1) does not apply. Our measurements permit the evaluation of the MICHAELIS-MENTEN constant K_s for diacetine—ACh esterase (not taking into account the acid and base dissociation constant of the esteratic group[5]). The average from several measurements is $1.7 \cdot 10^{-1}$. The corresponding value for the system diacetine—liver esterase is $2 \cdot 10^{-2}$ (see Fig. 3).

An analogous series of experiments was carried out with ethyl acetate as substrate (Fig. 4). Inhibition by tetraethylammonium ion is again competitive.

DISCUSSION

The experiments reported in this investigation lend support to our hypothesis that the difference between ACh esterase and other "unspecific" esterases is due mainly to the presence of the anionic sites in the former. The inhibitory effect of quaternary ammonium salts against liver esterase is unmeasurably small.

The competitive inhibition of diacetine hydrolysis by tetraethylammonium ion could have been explained by the assumption that the free hydroxyl group of the glycerol ester forms a hydrogen bond with the negative site. However this explanation is excluded by the identical behavior of this inhibitor, when ethyl acetate is the substrate. We are thus faced with the paradox that two reagents compete for the active surface of an enzyme although they attach themselves to different chemical groups. One possible explanation of the situation would be that the anionic sites form part of the active "esteratic" group and thus participate in the splitting reaction. But this assumption would also require that the mechanism of hydrolysis be different for the two types of esterases.

However, a very curious fact emerges from our experiments, *viz.* tetramethylammonium ion is only one third to one fifth as active as the ethylated compound, although Coulombic forces are much bigger for the methyl derivative. It follows that other, more unspecific forces play a decisive role in the combination enzyme—quaternary salt. This problem will be dealt with in a forthcoming publication.

It is of interest that the inhibitory power of choline against choline esterase, $c_{50} = 4 \cdot 10^{-3}$ *M*, is sufficient to produce a significant slowdown when more than half of the optimum concentration of ACh has been saponified. Our value of c_{50} is considerably lower than the figure reported by ADAMS AND WHITTAKER[6], $2 \cdot 10^{-2}$ *M*.

An interesting technical observation was made during the use of diacetine as substrate for ACh esterase: Due to the high concentration of substrate required the latter remains practically constant throughout the experiment and gives therefore a constant rate over a long period, in contrast to ACh, where the sharp pS optimum and the inhibitory action of one of the hydrolytic products lead to a decline in the speed of hydrolysis.

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SUMMARY

Quaternary ammonium salts show practically no inhibition of the hydrolytic action of liver esterase. They protect ACh esterase against irreversible combination with TEPP, although the two types of inhibitors attach themselves to different points of the active surface.

Quaternary ammonium salts also inhibit the hydrolysis of uncharged esters (diacetine, ethyl acetate) by ACh esterase in a competitive way. Possible explanations for this paradox behavior are discussed.

RÉSUMÉ

Les sels d'ammonium quaternaires ne montrent pratiquement aucune inhibition de l'action hydrolytique de l'estérase de foie. Ils protègent l'ACh-estérase contre la combinaison irréversible avec le TEPP, bien que les deux types d'inhibiteurs se fixent à des points différents de la surface active.

Les sels d'ammonium quaternaires inhibent également d'une façon compétitive l'hydrolyse des esters non-chargés (diacétine, acétate d'éthyl) par l'ACh-estérase. Nous avons discuté des explications possibles de ce comportement paradoxal.

ZUSAMMENFASSUNG

Quaternäre Ammoniumsalze zeigen praktisch keine Hemmung der hydrolytischen Wirkung von Leberesterase. Sie schützen die ACh-Esterase gegen die irreversibele Verbindung mit TEPP, obwohl diese zwei Typen von Hemmstoffen an verschiedenen Stellen der aktiven Oberfläche gebunden werden.

Quaternäre Ammoniumsalze hemmen auch die Hydrolyse von ungeladenen Estern (Diacetin, Ethylacetat) durch ACh-Esterase in konkurrierender Weise. Mögliche Erklärungen dieses paradoxen Verhaltens werden erörtert.

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