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INFLUENCE OF ALKANE-BIS-ONIUM COMPOUNDS UPON THE ACTIVITY OF THE AChE AND UPON ITS INHIBITION BY DFP

H. LÜLLMANN, F. K. OHNESORGE, H. D. TONNER, O. WASSERMANN and A. ZIEGLER

Dept. of Pharmacology, Christian-Albrechts-University, Kiel, W. Germany

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Abstract—Some alkane-bis-onium-derivatives display a dual action on acetylcholinesterases: They are reversible inhibitors and delay the irreversible inhibition by DFP. The kinetic analysis of the inhibitory action of the compounds revealed a competitive, a non-competitive or a mixed type of mechanism depending upon the chemical structure. Derivatives with larger substituents act mainly or entirely non-competitively as shown by their α-values.

Calculations of the binding energy indicate that all C-atoms of the interionic chain as well as of the substituents at the N-atoms add to the binding via hydrophobic and van der Waal's forces.

The non-competitive acting alkane-bis-onium compounds have to be bound to anionic site receptors and adjacent surface areas. An interference with the primary substrate binding does not occur. The acylation, however, of the esteratic site becomes impaired, thus leading to an inhibition of the enzymatic activity as well as a retardation of the phosphorylation by DFP.

SOME newly synthesized alkane-bis-onium-compounds are able to protect mice to a certain degree against an intoxication by diisopropylphosphorofluoridate (DFP). This effect can only be observed if the mice are simultaneously treated by atropine.[1] The alkane-bis-onium compounds should most probably act either upon the acetylcholine receptors or upon the acetylcholinesterase (AChE) or on both sites. In experiments with isolated guinea pig atria, an antiacetylcholine-activity of the alkane-bis-onium compounds could be demonstrated, which belongs to the noncompetitive, "allosteric" type of antagonism.[2] In preliminary experiments with AChE the alkane-bis-onium compounds displayed two actions: (1) the degree of the inhibition of the AChE-activity by organophosphates was diminished and (2) the AChE-activity was impaired. Both effects were dose-dependent and depended upon the chemical structure of the compounds involved. The present paper deals with a more detailed analysis of the protective and inhibitory action of the alkane-bis-onium compounds. Out of the great number of substances only a few typical compounds have been selected for the study. For sake of comparison a bis-tertiary compound was included, which was protonized at the pH used for the present experiments.

METHODS

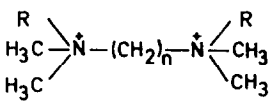
All experiments were performed using partially purified acetylcholinesterase (EC 3.1.1.7) of *Electrophorus electricus* of a specific activity of 100 U/mg protein

(Schwarz Bioresearch, Orangeburg, New York, U.S.A.). The enzymatic hydrolysis of acetylcholine was continuously titrated by means of the pH-stat-method (Radiometer, Copenhagen, Denmark) at pH 7·5 and 25°.

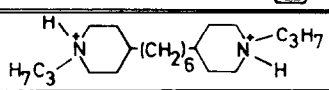
Acetylcholine-iodide (Fluka AG., Buchs, Switzerland) was used as substrate throughout the experiments in the concentration range 10^{-4}–10^{-2}M. The salt concentration of the incubation medium was always 0·6 M NaCl. The relatively high ionic strength was chosen to avoid aggregation of the monomers of AChE.[3,4]

The seven compounds investigated are summarized in Table 1. With the exception of compound VII the compounds are quaternary. The most simple one is hexamethonium, a well known ganglionic blocking agent. The drug was included in our investigation since it has been looked upon as the first member of the homologous series the newly synthesized compounds belong to.

TABLE 1



General structure

Nr.	n	R	X	Compound
I	6	$-CH_3$	$2J^-$	hexane-1,6-bis-(trimethyl-ammonium)
II	6	$-CH_2-CH_2-CH_3$	$2Br^-$	hexane-1,6-bis-(N,N-dimethyl-N-propyl-ammonium)
III	8	$-CH_3$	$2J^-$	octane-1,8-bis-(trimethyl-ammonium)
IV	8	$-CH_2-CH_2-CH_3$	$2Br^-$	octane-1,8-bis-(N,N-dimethyl-N-propyl-ammonium)
V	6	$-CH_2-CH_2-CH(CH_3)(CH_3)$	$2J^-$	hexane-1,6-bis-(N,N-dimethyl-N-(3'-methyl-butyl)-ammonium)
VI	6	$-CH_2-CH_2-CH_2-C_6H_5$	$2J^-$	hexane-1,6-bis-(N,N-dimethyl-N-3'-phenyl-propyl-ammonium)
VII			$2Cl^-$	hexane-1,6-bis-(4'-N-propyl-piperidyl)

The experimental data were evaluated according to Webb,[5] whose procedure is based upon the theory of Michaelis and Menten.[6] The method was applicable in our case, since the compounds investigated immediately equilibrated with the enzyme, and the inhibition was completely and instantaneously reversible upon dilution.

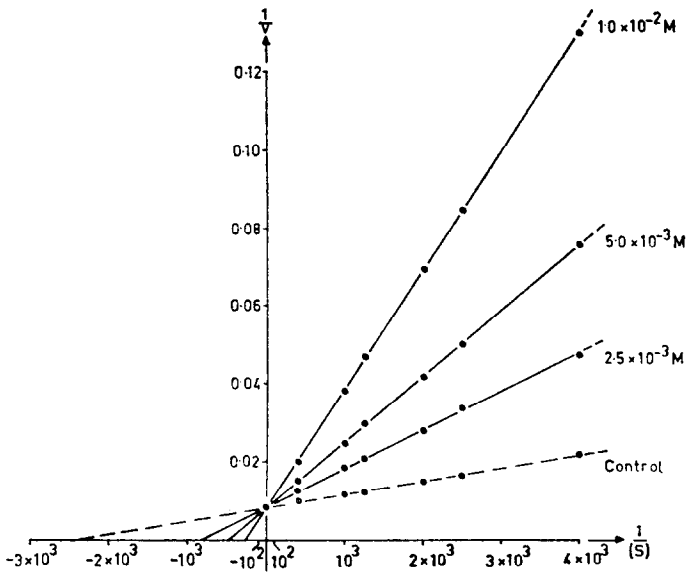
RESULTS

Under our experimental conditions the hydrolysis of ACh by the AChE of electric eel yielded the following kinetic parameters: $K_m = 4{\cdot}25 \times 10^{-4}$M, $V_{max} = 124\%$ of $v_{opt.}$, $K'_s = 3 \times 10^{-2}$ and $[S]_{opt.} = 3{\cdot}6 \times 10^{-3}$M.

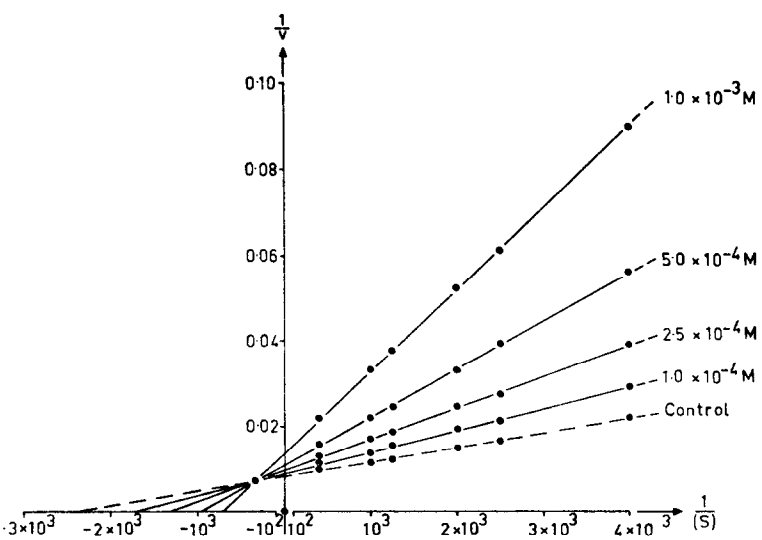
(1) *Inhibition by alkane-bis-onium compounds of the AChE*

The compounds were investigated in the appropriate dose range, and the data plotted according to Lineweaver and Burk.[7] The inhibitory action of three com-

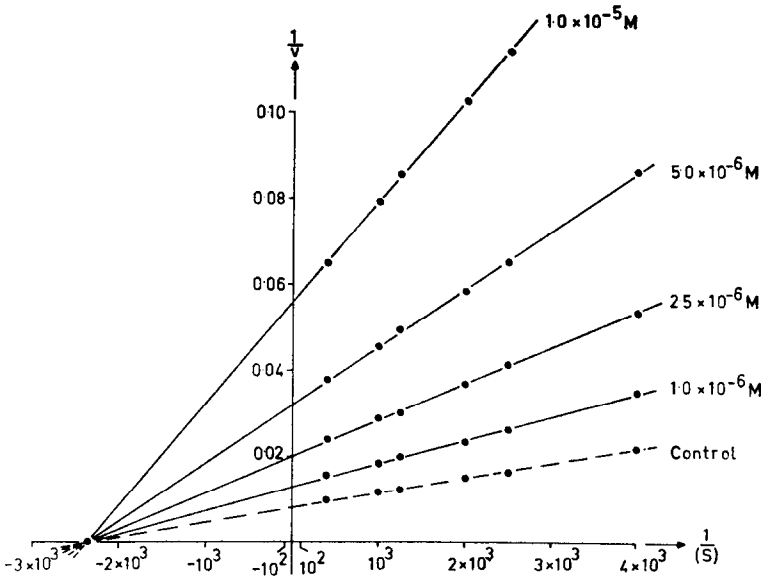
pounds is depicted in Fig. 1. Hexamethonium displayed a low affinity and the characteristics of a competitive inhibitor of the AChE-activity. The affinity of compound VII was about 1000-fold as high as that of hexamethonium. The type of inhibition was clearly non-competitive. Compound II took an intermediate position. The K_i values obtained by plotting I/v_i versus $[I]$ according to Dixon are listed for all compounds in Table 2. The replacement of a methyl group at each nitrogen atom by larger substituents or an increase in the interionic distance enhanced the affinity. The most



(a)



(b)



(c)

FIG. 1. Inhibition of the AChE activity by alkane-bis-onium compounds I, II, and VII plotted according to Lineweaver–Burk. The concentrations of the alkane-bis-onium compounds are indicated at each curve. (a) compound I, (b) compound II and (c) compound VII.

active compound of the series was compound VI, in which two methyl groups of hexamethonium are replaced by 3-phenyl-propyl groups. This change increased the K_i from $1{\cdot}2 \times 10^{-3}$ to $6{\cdot}6 \times 10^{-6}$M.

If the point of intersection of the straight lines of a Lineweaver–Burk plot lies neither on the x- nor on the y-axis, the antagonism in question belongs to the mixed competitive–non-competitive type. According to Webb[5] the coordinates of the point of intersection make it possible to calculate α, which provides an estimation of the proportion of the two types of antagonism for a particular compound:

$$\frac{1}{[S]} = -\frac{1}{\alpha \cdot K_s}, \quad \alpha = -\frac{[S]}{K_s}$$

$$\frac{I}{v_i} = \frac{1}{V_{max}} \cdot \left(1 - \frac{1}{\alpha}\right), \quad \alpha = \frac{1}{1 - \frac{V_{max}}{v_i}}.$$

The numerical value of α is infinite in the case of a pure competitive antagonism and approaches, 1 as the antagonism becomes purely non-competitive. The α values of the investigated alkane-bis-onium compounds are listed in Table 2. Most of the compounds show α-values close to 1 indicating a preponderance of the non-competitive type of antagonism. The competitive component is almost negligible for compounds IV and V and entirely absent in case of the tertiary compound VII.

TABLE 2. INHIBITION OF AChE BY ALKANE-BIS-ONIUM COMPOUNDS (K_i) AND TYPE OF INHIBITION (α)

Compound no.	Inhibition-constant K_i	α	Type of inhibition
I	$1{\cdot}2 \times 10^{-3}$	∞	competitive
II	$2{\cdot}2 \times 10^{-4}$	6·74	
III	$3{\cdot}7 \times 10^{-4}$	2·20	mixed
IV	$3{\cdot}5 \times 10^{-5}$	1·60	competitive–
V	$1{\cdot}2 \times 10^{-5}$	1·61	non-competitive
VI	$6{\cdot}6 \times 10^{-6}$	2·50	
VII	$1{\cdot}7 \times 10^{-6}$	1·00	non-competitive

(2) *Influence of alkane-bis-onium compounds upon the AChE-inhibition by DFP*

If the concentration of organophosphates greatly exceeds that of the AChE, the time course of the inhibition can be described by a pseudo-unimolecular reaction. Since this was the case in our experiments, the curves of inhibition yielded straight lines in a semilogarithmic plot and could be characterized by the half life time resp. the "bimolecular" rate constant

$$k_2 \left(\frac{\ln 2}{t_{\frac{1}{2}} \cdot [\text{DFP}]}\right).$$

Out of the seven compounds three have been investigated in more detail. The time courses of the AChE inhibition by DFP (2×10^{-6}M) in the presence of different concentrations of compound II are demonstrated in Fig. 2. Increasing concentrations of compound II prolonged the half life of the inhibition (but also reduced the AChE activity by its own. The curves do not start any more at 100 per cent activity = log

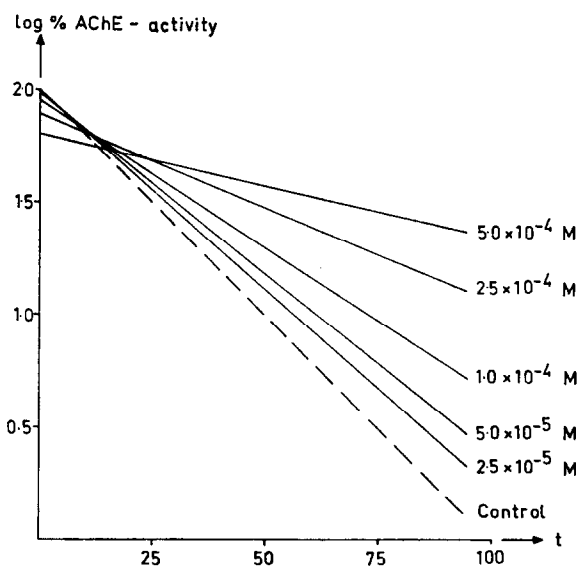


FIG. 2. Time course of the inactivation by DFP of the AChE activity in the presence of different concentrations of compound II as marked at each curve. ACh 3×10^{-3} M, DFP 2×10^{-6}M.

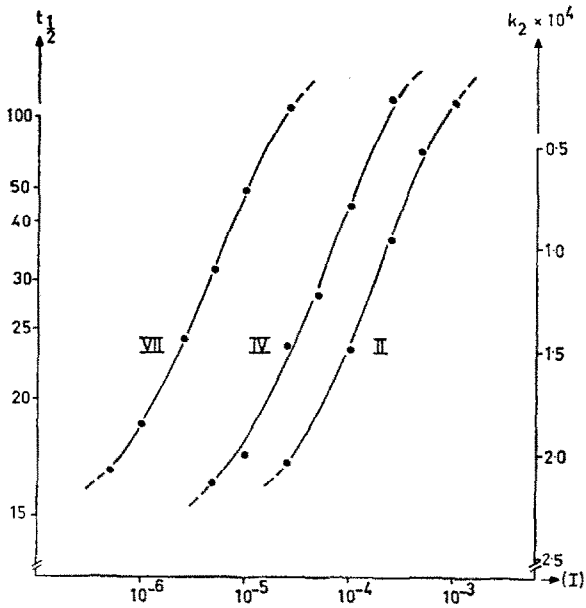


FIG. 3. Bimolecular rate constants, k_2 (mole^{-1} × min^{-1}) resp. half life times, $t_{\frac{1}{2}}$ (min) of the inacti vation by DFP of AChE plotted versus the concentration of alkane-bis-onium compounds II, IV and VII.

2·00). By looking at a particular time the delay of the inactivation process can be interpreted as a protective action of the compound against the inhibition by DFP. The "protective" effect of the alkane-bis-onium compounds was dose dependent as shown in Fig. 3, in which the changes of the half-lifes resp. of the k_2 values have been plotted against the concentration of compounds II, IV and VII. The curves obtained ran parallel; compound VII was the most active one. A further analysis of the curves shows that the $t_{\frac{1}{2}}$ values are directly correlated to the concentration of the "protective" compounds over a range of one and a half decades according to the equation

$$t_{\frac{1}{2}} = a \cdot [C] + 15$$

where a is $8{\cdot}6 \times 10^4$; $2{\cdot}9 \times 10^5$ resp. $3{\cdot}4 \times 10^6$ for compound II, IV resp. VII, $[C]$ gives the concentration of the respective compound and the term 15 stand for the half life (in minutes) of the control (inhibition by DFP 2×10^{-6}M).

DISCUSSION

The alkane-bis-onium compounds investigated are reversible inhibitors of the AChE and delay the irreversible inhibition of the AChE by DFP. The question arises to which sites of the AChE the compounds are bound to exert their effects.

Except compound I (hexamethonium) the derivatives are mainly or entirely non-competitive acting. Due to their chemical structure the compounds cannot lead to an acylation of the esteratic site as it has been shown for protective drugs like carbamates and cholinesters.[8–11] Also a binding to the (classical) anionic site of AChE is highly improbable, since an impairment of the substrate binding does not occur according to the non-competitive character of the inhibition.

To obtain some information concerning the mode of bindings of the alkane-bis-onium compounds by AChE, the free binding energy has been calculated.[5] Since the investigated compounds are very closely related the relative energy can be obtained by

$$\Delta\ (\Delta F) = \Delta F_1 - \Delta F_2 = 1{\cdot}368 \cdot \log. \frac{K_{i1}}{K_{i2}} \text{ (at } T = 25°).$$

To take into account the different types of inhibition the K_i-values were corrected as follows:

$$K_i \text{ comp.} = [I]_{0{\cdot}5} \cdot \frac{1}{1 + \frac{[S]}{K_s}}$$

$$K_i \text{ non-comp.} = [I]_{0{\cdot}5} \text{ and}$$

$$K_i \text{ mixed} = [I]_{0{\cdot}5} \cdot \frac{1 + \frac{[S]}{\alpha \cdot K_2}}{1 + \frac{[S]}{K_s}}$$

where $[I]_{0{\cdot}5}$ equals the inhibitor concentration, that reduces the AChE activity by 50 per cent. The values for $\Delta(\Delta F)$ in kcal/mole for different pairs of compounds are listed in Table 3. The binding energies per methylen-resp. methylgroup lie in the range of 0·25–0·55 and that of the phenylring by about 2 kcal/mole and agree well with

TABLE 3. DIFFERENCES IN BINDING ENERGIES PER METHYL-RESP. METHYLEN GROUP AND PER PHENYLRING (MARKED BY ASTERISK) EXPRESSED AS $\Delta\ (\Delta F)$ FOR DIFFERENT PAIRS OF COMPOUNDS

Comparison of compounds	$\Delta\ (\Delta F)$ (kcal/mole)
I–III	0·35
II–IV	0·55
I–II	0·25
III–IV	0·35
I–V	0·34
II–V	0·43
I–VI	2·09*
II–VI	2·09*

* Numbering of compounds see Table 1.

determination by others.[5] The figures thus obtained demonstrate the importance of van der Waals and hydrophobic bonds for the interaction of the alkane-bis-onium compounds with the AChE molecule. They indicate, furthermore, that all C-atoms independent of their position either in the interionic chain or in the substituent add to the binding. As proposed by Smith and Williams[12] the orientation of the inhibitor molecule will be brought about by the far reaching electrostatic forces whereas the final binding at the surface of the enzyme molecule is mainly governed by van der Waals and hydrophobic bonds.

Although we have only investigated seven compounds a few tentative conclusions

concerning the structure–activity relationship may be drawn. An increase of the interionic chain length and of the length of the two substituents at each nitrogen atom enhance the affinity and the non-competitive component of the inhibitory action. A substitution by branched aliphatic chains or by aromatic moieties increases the parameter still more.

The conclusions are in good agreement with the findings by Gerlach *et al.*,[13] who investigated the anti-acetylcholine activity of some 30 alkane-bis-onium compounds upon the muscarinic receptor.

As far as the mode of action of alkane-bis-onium compounds is concerned, we can state how they probably do not act, namely by a direct interference with the two classical active sites of the AChE. More recent publications by Koshland,[14,17] Belleau[15] and Wilson[16] have promoted the hypothesis, that the function of the active sites can be influenced by drugs bound to the neighbourhood (or side receptors). Accordingly it is tempting for us to look at the newly synthesized compounds as conformational modifiers. The non-competitively acting derivatives become bound to anionic side receptors and to the surface adjacent to the active sites. The reversible binding does not interfere with the primary substrate binding but renders the acylation resp. the deacylation more difficult thus decreasing the enzymatic activity. The same mode of action would explain the "protective" effect. The alkane-bis-onium derivatives would interfere with the second step of the interaction of AChE and DFP, namely the phosphorylation of the esteratic site. Since the enzymatic hydrolysis of ACh and the inhibition of the AChE by organophosphates have the acylation of the esteratic site in common, we think it most probable that the alkane-bis-onium compounds impair the acetylation resp. phosphorylation of the esteratic center thus leading to a reduction of the enzymatic activity as well as to a diminished inhibition by DFP.

The acylation, however, by different acylating agents (acetic- or phosphoric acid) may be influenced to a different degree and rate by certain compounds as demonstrated by the present results.

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