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Inhibition of Acetylcholinesterase by *N*-Alkylpyridinium and *N*-Alkylpyridinium-2-aldoxime Salts

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The interaction of a series of *N*-alkylpyridinium and *N*-alkylpyridinium-2-aldoxime salts with bovine erythrocyte acetylcholinesterase was investigated for inhibition of the hydrolysis of the substrates acetylcholine and dimethylaminoethyl acetate.

The compounds cause a mixed inhibition of the acetylcholine hydrolysis which is interpreted as an interaction with the free enzyme (competitive component) and with the acetylenzyme (non-competitive component). The results suggest that the compounds have a higher affinity for the free enzyme than for the acetyl-enzyme. Enlargement of the alkyl-group increases the binding capacity to the free enzyme.

The aldoxime group hardly effects the binding to the free enzyme, but tends to increase the binding to the acetyl-enzyme.

Some results obtained with dimethylaminoethyl acetate support the mechanism of inhibition as proposed from acetylcholine hydrolysis inhibition. In contrast to this mechanism some compounds do not influence or even increase the maximum velocity of the dimethylaminoethyl acetate hydrolysis. It is suggested that a ternary complex of enzyme, substrate and pyridinium compound may be formed from which, in case of dimethylaminoethyl acetate, the enzyme is more rapidly acetylated.

INTRODUCTION

The toxicity of organophosphorus compounds is based on their ability to inactivate acetylcholinesterase by phosphorylating the enzyme. Potent reactivators, like the pyridinium oximes P2S* and TMB4, have been developed which are effective as antidotes. These compounds are able to displace the phosphoryl moiety from the enzyme by virtue of their high affinity for the enzyme and their powerful nucleophilic properties. Due to high affinity for the enzyme they are also inhibitors of the enzymatic substrate hydrolysis. A few studies on the mechanism of inhibition of substrate hydrolysis by pyridinium oximes have been reported[1–3].

In the present study we investigate the interaction of P2S and some *N*-alkyl analogues of P2S with acetylcholinesterase for their inhibition of the hydrolysis of the substrates acetylcholine and dimethylaminoethyl acetate. Cor-

* Abbreviations used: P2S, *N*-methylpyridinium-2-aldoxide methanesulphonate: TMB4 *N,N'*-trimethylenebis-(pyridinium-4-aldoxime)dibromide.

responding *N*-alkylpyridinium halides lacking an oxime moiety have been studied as inhibitors in order to evaluate the role of the oxime moiety in the binding to the enzyme.

MATERIALS AND METHODS

Materials

Bovine erythrocyte acetylcholinesterase (acetylcholine hydrolase EC 3.1.1.7) was obtained from Winthrop Laboratories Inc. and had a spec. act. of 25 nkat/mg protein at 25 °C in a 0.5 mM phosphate buffer, pH = 7.5, containing 3 mM acetylcholine chloride.

P2S was purchased from Dr. F. Raschig GmbH, W. Germany. The other pyridinium compounds were prepared in this laboratory by alkylating pyridine or pyridine-2-aldoxime with an alkyliodide or alkylbromide and had satisfactory elemental analysis. Dimethylaminoethyl acetate hydrochloride was prepared by reacting acetylchloride with dimethylaminoethanol m. p. 106 °C.

Anal. $C_6H_{14}NClO_2$ calc'd.: C 42.99; H 8.42; N 8.35; Cl 21.15%
found: C 42.92; H 8.31; N 8.38; Cl 21.02%

All other reagents were of analytical grade.

Methods

All enzyme activities were measured with a Radiometer pH-stat equipment modified for the dual-syringe technique. Titrant and substrate were simultaneously added to the assay mixture from two 0.5-ml syringes, one containing 50 mM NaOH and the other 50 mM substrate solution in water. By means of this technique it is possible to keep the substrate concentration constant during the assay. The determinations were carried out in an aqueous medium at 25 °C and pH 7.2 in an atmosphere of nitrogen. The reaction medium consisted of 45 ml substrate solution containing 0.1 M NaCl and 10 mM $MgCl_2$ to which 1 ml solution of a pyridinium compound and 1 ml enzyme solution were added. The enzyme concentrations were 1.25 mg/ml for acetylcholine and 3.13 mg/ml for dimethylaminoethyl acetate. All results were corrected for non-enzymatic hydrolysis.

The velocity of the substrate hydrolysis was measured in duplicate runs at six substrate concentrations ranging from 0.4—1.4 mM for acetylcholine and 1—3.5 mM for dimethylaminoethyl acetate. These experiments were repeated with different concentrations of the pyridinium compounds.

RESULTS

Inhibition of acetylcholine hydrolysis

The velocities of acetylcholine hydrolysis measured without and with added pyridinium compounds were fitted to the Michaelis-Menten equation according to the non-linear regression method of Wilkinson[4]. From these calculations the values of V (maximum velocity) and of K_m (the substrate concentration at which the velocity is $V/2$) were obtained with standard errors of about 8% and up to 4%, respectively. The K_m of acetylcholine measured in the absence of a pyridinium compound was found to be 136 ± 10 μM; no substrate inhibition was observed within the concentration range used. The pyridinium compounds affecting both, K_m and V, demonstrate a competitive and non-competitive inhibition of the acetylcholine hydrolysis. Krupka[5–8] showed that the interaction of quaternary ammonium compounds with substrate hydrolysis catalyzed by acetylcholinesterase can be described by

$$\begin{array}{ccccc} E + S \rightleftarrows E.S & \xrightarrow{k_2} & EA & \xrightarrow{k_3} & E \\ + & & + & & \\ M & & M & & \\ \downarrow\uparrow K_i & & \downarrow\uparrow K_i' & & \\ & \xleftarrow{ak_3} & & & \\ E.M & & EA.M & & \end{array} \qquad (1)$$

where E, S, M and EA stand for the enzyme, the substrate, the quaternary ammonium compound and the acylated enzyme, respectively. For this scheme it has been derived[6] that the equilibrium constant governing the competitive component of the inhibition (K_i) is given by

$$K_i = \frac{[M]}{\frac{K_m\,(+M)\;V\,(-M)}{V\,(+M)\;K_m\,(-M)} - 1} \qquad (2)$$

where $V\,(-M)$,$K_m\,(-M)$ and $V\,(+M)$, $K_m\,(+M)$ are values determined without and with added quaternary ammonium compounds, respectively. For the parameters of the noncompetitive component of the inhibition (K_i' and a) the following equation holds[7]

$$\frac{[M]\;V\,(+M)}{V\,(-M) - V\,(+M)} = \frac{K_i'\,(1 + k_3/k_2)}{1 - a} + \frac{a\,(1 + k_3/k_2)}{1 - a}\,[M] \qquad (3)$$

We adopted scheme (1) for the interpretation of our data. Values of K_i were obtained as an average of values calculated for the individual concentrations of the pyridinium compound used by means of Eqn. 2. The values of $a\,(1 + k_3/k_2)/(1 - a)$ and K_i'/a were determined as the slope and as the ratio of intercept/slope of plots of $[M]\;V\,(+M)/(V\,(+M) - V\,(+M))$ *vs.* [M], using the method of least-squares. For the *N*-dodecyl compounds values of $[M]\;V\,(+M)/(V\,(-M) - V\,(+M))$ were found to be independent of the concentration of the inhibitor indicating that $a = 0$. In that case Eqn. 3 becomes

$$\frac{[M]\;V\,(+M)}{V\,(-M) - V\,(+M)} = K_i'\,(1 + k_3/k_2) \qquad (4)$$

The value of $K_i'\,(1 + k_3/k_2)$ was obtained as the average of values calculated for the individual concentrations according to Eqn. 4. The results are summarized in Table I.

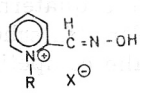
Inhibition of dimethylaminoethyl acetate hydrolysis

The values of V, K_m and K_i were evaluated as described for acetylcholine, the standard errors were about 7% for the K_m values and about 4% for the V values. $K_m\,(-M)$ was found to be 1.10 ± 0.06 mM; no substrate inhibition was observed. The results are given in Table II.

$V\,(+M)$ values corresponding to a small non-competitive inhibition were obtained with some pyridinium-2-aldoxime salts. With the other compounds $V\,(+M)$ values equal to or greater than $V\,(-M)$ were found. As shown before by

TABLE I.

Kinetic parameters for the interaction of N-alkylpyridinium-2-aldoxime salts (I) and N-alkylpyridinium salts (II) with acetylcholine hydrolysis catalyzed by acetylcholinesterase at 25 °C and pH = 7.2 in 0.1 M NaCl and 0.01 M $MgCl_2$.
Values are given with their standard errors.

compound		concn. range of pyridinium compound μM	K_i μM	K_i'/a μM	$a(1+k_3/k_2)/(1-a)$	$K_i'(1+k_3/k_2)$ μM
I 						
R	X					
CH_3	CH_3SO_3	200—600(4)[a]	152±3	570±40	0.83±0.04	
C_2H_5	I	100—500(5)	90±4	480±130	0.68±0.15	
C_3H_7	I	50—300(11)	76±3	470±80	0.34±0.06	
i-C_3H_7	I	100—500(5)	121±5	500±110	0.9 ±0.2	
C_7H_{14}	I	50—200(5)	28±1	220±40	0.35±0.05	
$C_{12}H_{25}$	I	0.2—10(5)	2.5±0.1	—	—	4.5±0.4
II 						
R	X					
CH_3	I	100—500(4)	96±4	410±70	1.07±0.14	
C_3H_7	Br	200—600(3)	152±8	2700±290	0.32±0.03	
C_7H_{15}	I	75—200(5)	42±2	1900±700	0.14±0.05	
$C_{12}H_{25}$	I	1—3(3)	2.0±0.1	—	—	10.3±1.0

[a] Values in parentheses give the number of inhibitor concentrations used.

Wilson[9] and Krupka[7] deacetylation is rate-limiting in the acetylcholine hydrolysis ($k_3 < k_2$) and acetylation is rate-limiting in the dimethylaminoethyl acetate hydrolysis ($k_2 < k_3$). Therefore, as a consequence of the relatively small concentration of acetyl-enzyme, only a small non-competitive inhibition of the latter substrate can be expected. More accurate parameters could not be evaluated from these small inhibiting effects. Only for *N*-dodecyl-pyridinium--2-aldoxime iodide $K_i'(1 + k_3/k_2)$ was estimated using Eqn. 4.

DISCUSSION

It is generally accepted that an esteratic site which is acylated during substrate hydrolysis and an anionic site to which ammonium ions are bound, are parts of the active center of acetylcholinesterase[10]. The inhibition of the acetylcholine hydrolysis by the pyridinium compounds can be described with a reaction scheme proposed for ammonium ions. Hence, it seems reasonable to assume that the inhibition by the pyridinium compounds is also due to binding of the ions to the anionic site.

TABLE II.

Kinetic parameters for the interaction of N-*alkylpyridinium-2-aldoxime salts (I) and* N-*alkylpyridinium salts (II) with dimethylaminoethyl acetate hydrolysis catalyzed by acetylcholinesterase at* 25 °C *and* pH = 7.2 *in* 0.1 M NaCl *and* 0.01 M $MgCl_2$.
Values are given with their standard errors.

compound		concn. range of pyridinium compound μM	K_i μM	$V(+M)/V(-M)$	$K_i'(1+k_3k_2)$ μ M
I					
R	X				
CH_3	CH_3SO_3	150—300(2)[a]	150 ± 10	≤1.04[c]	
C_2H_5	I	150—250(2)	120 ± 10	<1[b]	
C_3H_7	I	50—150(3)	80 ± 5	<1[b]	
i-C_3H_7	I	50—225(4)	130 ± 20	≤1.10[c]	
C_7H_{15}	I	25—150(4)	55 ± 5	<1[b]	
$C_{12}H_{25}$	I	2—4(3)	2.0 ± 0.2	<1[b]	9.5 ± 0.2
II					
R	X				
CH_3	I	100—200(2)	100 ± 10	≤1.12[c]	
C_3H_7	Br	200—300(2)	170 ± 10	≤1.05[c]	
C_7H_{15}	I	50—100(2)	44 ± 1	≤1.39[c]	
$C_{12}H_{25}$	I	3—4(2)	2.4 ± 0.1	≤1.04[c]	

[a] Values in parentheses give the number of inhibitor concentrations used.
[b] A small non-competitive inhibition was observed.
[c] The highest value found for the compound is given. Increasing the concentration of the pyridinium compound the value of $V(+M)$ increases for I, R=CH_3 and II, R=C_3H_7 and C_7H_{15}, decreases for II, R=CH_3, and has an optimum value for I, R=i—C_2H_7 and II, R=$C_{12}H_{25}$.

Pyridinium-2-aldoxime salts (I) and pyridinium salts (II) inhibit the acetylcholine hydrolysis in the same manner. The small differences in their competitive inhibition constants (K_i) indicate that the oxime group slightly contributes to the binding of the oximes to the free enzyme. Based on data given in Table I a direct comparison of the affinity of the compounds of the series I and II to the acetylated enzyme (K_i') is possible for the *N*-dodecyl compounds only. However, since the values of $a(1 + k_3/k_2)/(1 - a)$ and consequently the values of a are roughly the same for corresponding compounds of the two series, the large differences in K_i'/a values might be indicative of large differences in affinity to the acetylenzyme. So, except for the *N*-methyl compound, it may be derived from the results that the oxime group probably plays a role in binding to the acetylated enzyme.

The K_i value obtained for P2S roughly corresponds with values reported in literature[1-3]. Enlargement of the alkyl group increases the binding capacity of the pyridinium compounds. This finding supports the hypothesis of a hydrophobic area near the anionic binding site as proposed by Bergmann[11] and Belleau[12,13] from their results of inhibition experiments with *N*-alkyl trimethylammonium ions. Examination of the non-competitive component of the inhibition reveals that P2S does not block deacetylation completely ($a > 0$) in contrast with literature data. Tre value of a decreases with lengthening the alkyl group as can be seen from decreasing values of a $(1 + k_3/k_2)/(1 - a)$ and increases on branching of the alkyl group (*i*-propyl).

The parameters for the non-competitive inhibition, a and K_i', may be calculated separately from the data given in Table I, if k_3/k_2 is known. Wilson and Cabib[9] showed that this ratio is approximately 0.2 (pH = 7.0, 25 °C, 0.1 M NaCl). Adopting this value for our experimental conditions, the values of a and K_i' were calculated (Table III).

Comparison of K_i and K_i' values shows that acetylation of the enzyme decreases slightly its affinity for the pyridinium-2-aldoxime salts (I) and to

TABLE III.

Characteristics of N-*alkylpyridinium-2-aldoxime salts (I) and* N-*alkylpyridinium salts (II) for their non-competitive inhibition of the acetylcholine hydrolysis catalyzed by acetylcholinesterase*

compound		K_i' μM	a
I			
R	X		
CH_3	CH_3SO_3	230	0.41
C_2H_5	I	170	0.36
C_3H_7	I	100	0.22
i-C_3H_7	I	220	0.44
C_7H_{15}	I	50	0.23
$C_{12}H_{25}$	I	3.9	0
II			
R	X		
CH_3	I	190	0.47
C_3H_7	Br	570	0.21
C_7H_{15}	I	200	0.10
$C_{12}H_{25}$	I	8.8	0

a larger extent for the pyridinium salts (II). A comparison of the K_i' values for corresponding compounds of series I and II indicates, except for the *N*-methyl compound, that the oxime moiety contributes in the binding to the acetylated enzymes, as was already suggested from the kinetic parameters given in Table I.

Some results of the inhibition experiments with dimethylaminoethyl acetate (Table II) are consistent with the mechanism of inhibition as proposed from acetylcholine hydrolysis inhibition: the correspondence between K_i values obtained with the two substrates and the small non-competitive inhibition as should be expected for a substrate of which the hydrolysis is rate limited at acetylation. Another support for this mechanism can be obtained from a comparison of the V (—M) values for the two substrates with the values of the non-competitive inhibition constants determined for *N*-dodecylpyridinium--2-aldoxime iodide.

Since acetylcholine and dimethylaminoethyl acetate, being acetyl esters, will form an acetyl enzyme, k_3 will be the same for both substrates. So, the ratio of their V (—M) values (V (—M) = k_3 [E_o]/(1 + k_3/k_2)) equals

$$\frac{V\,(\text{—M})_{\text{acetylcholine}}}{V\,(\text{—M})_{\text{dimethylaminoethyl acetate}}} = \frac{1 + k_3/k_2^{\text{dimethylaminoethyl acetate}}}{1 + k_3/k_2^{\text{acetylcholine}}} \qquad (5)$$

assuming that the same enzyme concentration ([E_o]) is employed with both substrates. This ratio can also be derived from K_i' (1 + k_3/k_2) values determined with the two substrates. The present results show a close correspondence between the ratio of the V (—M) values, 2.1, and that of the constants for non-competitive inhibition by *N*-dodecylpyridinium-2-aldoxime iodide, 2.1.

However, in contrast with the given mechanism some of the pyridinium compounds do not influence or increase V of dimethylaminoethyl acetate hydrolysis. Since deacetylation is a common reaction of both acetylcholine and dimethylaminoethyl acetate hydrolysis, enhancement of V of the latter only might be caused by the formation of a ternary complex of enzyme, substrate and pyridinium compound from which acetylation proceeds more rapidly. From the present kinetic experiments it can not be concluded if a ternary complex is also formed with acetylcholine as a substrate. Interactions with enzyme--substrate complex will hardly be noticed since deacetylation is rate-limiting in the hydrolysis of this substrate. In this connection it is of interest that the acetylation rate considerably decreases on substitution of a dimethylamino group instead of the trimethylammonium group in acetylcholine. Krupka[14] explained this phenomenon by assuming a misfit of the dimethylamino group at the anionic site resulting in a misorientation of the substrate to the esteratic site. In view of this hypothesis it appears reasonable to assume that in the supposed ternary complex this misorientation might be reduced. Since the substrate must occupy the active center, a second binding site, besides the anionic site, has to be assumed for the pyridinium compounds. These results afford a supporting indication for an allosteric or peripheral anionic site as suggested by various investigators[15–17].

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DISCUSSION

E. Reiner:

Is the kinetic model you presented for inhibition by oximes valid also at high substrate concentrations?

L. P. A. de Jong:

I do not know. We only used substrate concentrations in the range of 0.4—1.4 mM acetylcholine and 1—3.5 mM dimethylaminoethyl acetate.

H. Kuhnen:

I think it agrees with your scheme that the action of pyridinium compounds depends on the substrate and antagonist concentration, resulting in either an activation or inhibition of the enzyme. Did you observe this?

L. P. A. de Jong:

What we observed at different substrate and inhibitor concentrations could be described except for one observation, by the given reaction scheme. That means that the pyridinium compounds bind both, to the free enzyme and to the acetyl--enzyme, and block deacetylation partly or completely. So far, only inhibition was observed. In the presence of some compounds, however, we did not find a small non--competitive inhibition of the hydrolysis of dimethylaminoethyl acetate, as you would expect, but the V_{max} values equaled to or were greater than the V_{max} values measured in the absence of a pyridinium compound. We concluded that there might be a ternary complex of enzyme, substrate and pyridinium compound present which has a faster rate of acetylation. We explain this phenomenon by assuming a better orientation of this substrate on the active center rather than by assuming an activation of the enzyme by these compounds.

SAŽETAK

Inhibicija acetilkolinesteraze solima N-alkilpiridinija i N-alkilpiridinijum-2-aldoksima

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Interakcija niza *N*-alkilpiridinijevih i *N*-alkilpiridinij-2-aldoksimskih soli s acetilkolinesterazom goveđih eritrocita istraživana je putem inhibicije hidrolize acetilkolina i dimetilaminoetil-acetata.

Istraživani spojevi uzrokuju mješovitu inhibiciju, koja je protumačena kao interakcija sa slobodnim enzimom (kompetitivna komponenta) i acetiliranim enzimom (nekompetitivna komponenta). Rezultati upućuju na to da ti spojevi imaju jači

afinitet za slobodni nego li za acetilirani enzim. Povećanje alkilne skupine povećava kapacitet vezivanja za slobodni enzim.

Aldoksimska skupina gotovo da i nema učinka na vezivanje za slobodni enzim, ali utječe na povećanje vezivanja za acetilirani enzim.

Neki rezultati dobiveni s dimetilaminoetil-acetatom, govore u prilog mehanizma inhibicije postuliranog na osnovi inhibicije hidrolize acetilkolina. Nasuprot tomu, neki spojevi nemaju učinka ili čak ni ne povećavaju maksimalnu brzinu hidrolize dimetilaminoetil-acetata. Pretpostavlja se da je moguće nastajanje ternarnog kompleksa enzima, supstrata i piridinijeva spoja, iz kojega se u slučaju dimetilaminoetil-acetata enzim može brže acetilirati.

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Effects of Natural Polyamines on Membrane-bound Solubilized Acetylcholinesterase of Human Red Cells

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The effects of the polyamines on membrane-bound and solubilized acetylcholinesterase of human erythrocytes were studied at low ionic strength, using acetylcholine at pH = 8 as the substrate for the former and acetylthiocholine at pH = 6 as the substrate for the latter. Evidence is obtained that within the concentration range studied (putrescine: 0.01 mM—2 mM; spermidine: 0.01 mM —1 mM; spermine: 1 μM —0.5 mM) two molecules of putrescine and spermidine and three molecules of spermine are sequentially bound to a molecule of acetylcholinesterase in both states. Kinetic analysis suggests that thereby, at least within a certain concentration range of the amines, the affinity of acetylcholinesterase to both substrates is lowered, while the rate of product formation is increased: a substrate concentration-dependent shift from inhibition to activation occurs which cannot be explained merely by the amines preventing blockade of deacylation by excess substrate. Augustinsson-Hofsteet plots of the data show curvatures which are accentuated by increasing polyamine concentrations; at pH = 6, also the control plot is clearly curved. Since erythrocyte acetylcholinesterase is likely to be an oligomeric protein, this curvature is concluded to be due to negative cooperativity caused by subunit interactions, rather than to substrate activation as suggested by other authors. A treatment on the basis of Koshland's sequential model is proposed.