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# Hydrolysis of Carboxylic Acid Esters of Thiocholine and its Analogues

## 3. Hydrolysis Catalysed by Acetylcholine Esterase and Butyrylcholine Esterase

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The hydrolysis of thiocholine esters, α-methylthiocholine esters and β-methylthiocholine esters, catalysed by the enzymes acetylcholine esterase and butyrylcholine esterase, was studied with an automatic recording titrator at pH 8.00 and 25°C. Michaelis-Menten constants, maximum velocities and reaction rates are given. It was shown that acetylthiocholine is easier split by butyrylcholine esterase than acetylcholine. Both are split at the same rate by acetylcholine esterase.

The influence of some reversible and irreversible inhibitors on the hydrolysis of acetylcholine and acetylthiocholine, catalysed by acetylcholine esterase and butyrylcholine esterase, was studied at pH 8.00 and 25°C. It was shown that the substrates are about equally good protectors for acetylcholine esterase, but that the thioester is the better protector for butyrylcholine esterase.

The hydrolytic effect of imidazole and cysteine on the hydrolysis of the thioesters was shown. Hydrolysis was enhanced about 4 times with imidazole and about 50 times with cysteine.

The bonds between the enzymes and the substrates are discussed on the basis of the results obtained.

Choline esters are known to be of interest *in vivo* for their potent pharmacodynamic effects and *in vitro* as substrates for choline esterases. Some of their thiocholine analogues have been prepared during the 1930's and were investigated for their pharmacodynamic actions [1]. Acetylthiocholine was used by Koelle [2] and others in histochemical methods for the determination of choline esterase activity, and by Zajiceck [3] and others in a diver technique method for determination of choline esterase activity in single cells. Thiocholine esters were also used in a few studies on the structure of choline esterases by, *e.g.*, Wilson [4] and Bergmann [5].

In two preceding papers the acid [6] and alkaline [7] hydrolysis of thiocholine esters and analogues has been described. This paper reports studies on the hydrolysis, catalysed by acetylcholine esterase (AChE) and butyrylcholine esterase (BuChE), of the thiocholine esters and their methylsubstituted analogues, acetylhomothiocholine and acetylcholine. The work was done in order to gain more knowledge about the reaction of this type of ester with choline esterases. Earlier studies [7] had demonstrated the catalytic effect of some buffer anions, e.g. phosphate, on the hydrolysis of the thioesters. To avoid this effect an automatic recording titrator [8], allowing fairly exact studies of hydrolysis, was used for the study of the enzymic hydrolysis. Besides the measurements on the enzymic hydrolysis a few experiments were carried out in order to show that the thiocholine esters and analogues interact with the same groups in the enzymes as the choline esters do. The protective effect of the thioesters against some reversible and irreversible inhibitors was studied, and the catalytic effect of some organic compounds, proposed in literature [9,10] to be related to the active centre in choline esterases, on the hydrolysis of acetyl-$\beta$-methylthiocholine and acetylthiocholine, was confirmed.

## EXPERIMENTAL

### Materials

*Substrates.* The same esters as described earlier [6,7] were used. Acetylhomothiocholine was prepared by Hansen according to the same method as was used for the other thiocholine esters [11,12].

*Enzymes.* The source for *AChE* was freeze-dried electric organs from *Torpedo oscellata*. *BuChE* was obtained from fraction IV-6-3, prepared from retroplacentar serum (AB Kabi, Stockholm).

*Catalysts.* Imidazole was from Schuchardt GMBH, L-cysteine hydrochloride and DL-serine were from Merck & Co. 2-Methyloxazoline was synthetized according to Wenker [13].

*Inhibitors.* Sarin was prepared by Fagerlind [14], eserine salicylate was from Smith Ltd, London, choline iodide and thiocholine iodide were prepared by Hansen.

### Methods

*pS-Activity curves.* Reaction velocities were determined with an automatic recording titrator [8] at 25°C and pH 8.00. A 0.100 M solution of sodium hydroxide was added. For reasons explained later reaction velocities are expressed as recorder units, proportional to the amount of sodium hydroxide consumed by the hydrolysed ester, per unit of time (relative activities). The values used for calculations of reaction rates refer to hydrolysis from 1 to 10 min after addition of the substrate.

The activities of the enzyme solutions were checked each day with acetylthiocholine at $10^{-2}$ M substrate concentration. All activity values were corrected to the activity obtained the first day. The activity of BuChE solutions was constant during about 14 days when the solutions were kept in a refrigerator. The solutions of AChE were less stable.

*Enzyme solutions.* AChE: 500 mg of freeze-dried electric organs were homogenised in 100 ml of 0.10 M potassium chloride with a Potter-Elvehjem homogenizer. The solution was filtered through filter paper Munktell No. 3 and diluted 1:4 with 0.10 M potassium chloride. BuChE: 25 mg of serum fraction IV-6-3 were dissolved in 100 ml of 0.10 M potassium chloride.

*Substrate solutions.* The final concentration of the substrate was in all pS-activity experiments 0.100 M, 0.050 M, 0.010 M, 0.005 M, 0.001 M and 0.0005 M. The substrates were dissolved in 0.10 M potassium chloride.

*Procedure and reaction mixture.* The reaction mixture without enzyme was titrated to pH 8.00 with 0.100 M sodium hydroxide. The enzyme solution was added and the pH immediately again corrected to pH 8.00. The following reaction mixtures were used: enzyme solution 1 ml (AChE) or 3 ml (BuChE), substrate solution 30 ml, 0.10 M potassium chloride to make a total volume of 40 ml.

*Rate of hydrolysis.* In these experiments the automatic recording titrator [9] was used to keep pH constant at 8.00, and the amount of unhydrolysed ester was determined as iron-hydroxamate complex as described earlier [15]. The concentration of the esters was 0.001 M, enzyme concentration as described for pS-activity determinations. The temperature was 25°C.

*Spontaneous hydrolysis and catalytic effects of some organic compounds.* The spontaneous hydrolysis of each thioester was determined for 0.050 M and 0.010 M solutions. In these experiments the enzyme solution was replaced by 0.10 M potassium chloride. From the values obtained the spontaneous hydrolysis for the other substrate concentrations was calculated. Enzymic hydrolysis was calculated according to eqn (2) below. The spontaneous hydrolysis of acetylcholine was calculated from values found in this laboratory and published earlier [7]. The following equations were used for the corrections of the observed hydrolysis of acetylcholine:

$$k1_{\text{spont.}} \times C_S = k0_{\text{spont.}} \quad (1)$$

$$k0_{\text{obs.}} - k0_{\text{spont.}} = k0_{\text{enz.}} \quad (2)$$

All enzyme activity values were corrected for spontaneous hydrolysis.

In experiments with imidazole, 2-methyloxazoline, cysteine and serine the final concentration of the catalyst was the same as the concentration of the substrate, 0.001 M. The substance was dissolved in 0.10 M potassium chloride and pH adjusted to 8.00. The solution was then added to the reaction mixture. The total volume was 40 ml and the temperature 25°C.

*Inhibition studies.* An electrometric apparatus [16] was used for all experiments, except those where the substrate was used to protect the enzymes against inhibition by sarin. In these experiments the automatic recording titrator [8] was used.

*Electrometric method. Enzyme solutions and procedure.* AChE: 50 mg of freeze-dried electric organs were homogenised in 20 ml of Michels' buffer, run through filter-paper Munktell No. 3 and diluted 1:20 with Michels' buffer. BuChE: 50 mg of serum fraction IV-6-3 were dissolved in 200 ml of Michels' buffer. The reaction mixtures were incubated at 25°C. The final concentration of inhibitor was the same in all experiments, $10^{-7}$ M eserine (experiments with BuChE) or $10^{-2}$ M butyrylcholine (experiments with AChE). The concentration of the substrate was varied between $10^{-1}$ M and $10^{-3}$ M.

In experiments with sarin, thiocholine or choline the inhibitor concentration causing 50 % inhibition of enzyme activity was determined for the different pairs of substrates and inhibitors. The concentration of the substrates was $8.0 \times 10^{-3}$ M. Incubation was allowed to proceed for 30 min before the addition of the substrate.

*Automatic recording titrator. Procedure.* Reaction velocities for inhibition with sarin after protection of the enzymes by substrate were determined with $3.9 \times 10^{-7}$ M (BuChE) or $1.2 \times 10^{-6}$ M (AChE) sarin and the substrates acetylcholine or acetylthiocholine, $4 \times 10^{-3}$ (BuChE) or $5 \times 10^{-3}$ M (AChE). The method and enzyme solutions were the same as described for pS-activity curves, but 2 ml of sarin were added after about 4 min. The concentrations refer to the reaction mixture. Calculations were done analogously to Guggenheim's [17] method in all cases where the spontaneous hydrolysis of substrates and inhibitors could be neglected, but otherwise as described by Tammelin [18].

*Ionic Strength. Procedure and reaction mixture.* Enzyme solutions were as described for pS-activity curves. By addition of the necessary amounts of potassium chloride the ionic strength was varied between 0.10 – 0.25 M. The following reaction mixture was used: 38 ml of 0.10 M potassium chloride + 1 ml of 0.40 M acetylthiocholine bromide + 1 ml of enzyme solution + varying amounts of solid potassium chloride.

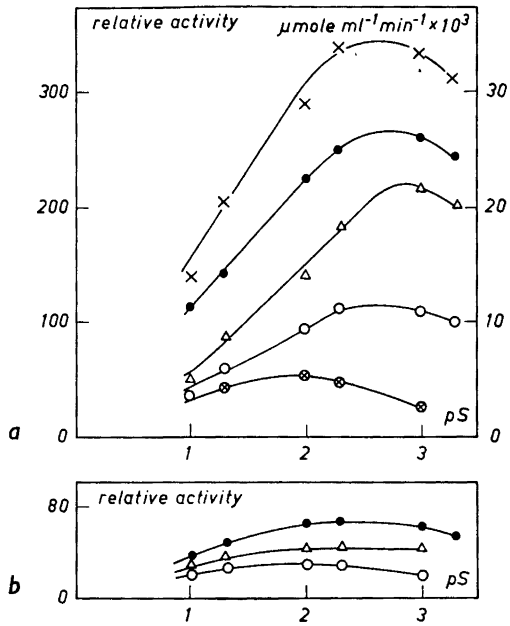


*Fig. 1.* Acetylcholine esterase: a. pS-Activity curves for the acetyl esters of thiocholine (●), α-methylthiocholine (○), β-methylthiocholine (△), homothiocholine (⊗) and choline (×) at pH 8.00 and 25°C. Only acetylcholine refers to the y-axis on the right hand side. b. pS-Activity curves for the propionyl esters of thiocholine (●), α-methylthiocholine (○) and β-methylthiocholine (△).

## RESULTS

### pS-Activity curves

Relative activities and values of maximum velocity given here are only comparable if the alcohol group in the esters is the same.

*AChE.* Fig. 1 shows the pS-activity curves for acetylcholine esterase and the acetyl and propionyl esters of thiocholine, α-methylthiocholine and β-methylthiocholine, and acetylcholine. Michaelis-Menten constants were not calculated, as the enzyme is inhibited by excess of substrate as seen by the decreasing activity at higher substrate concentrations. Only the right limb of each curve would have allowed calculations of the constants, and this was not obtained over a large enough substrate range. The activity values are expressed as relative activities, except for acetylcholine, as the $pK_a$-values for some of the products of hydrolysis, α-methylthiocholine and β-methylthiocholine, could not be determined, because these compounds were not available. Studies on the spontaneous hydrolysis of the thioesters [7] also show that secondary reactions occur with some of the products of hydrolysis. Therefore it was impossible to calculate the amount of sodium hydroxide equivalent to 1 mole of hydrolysed ester.

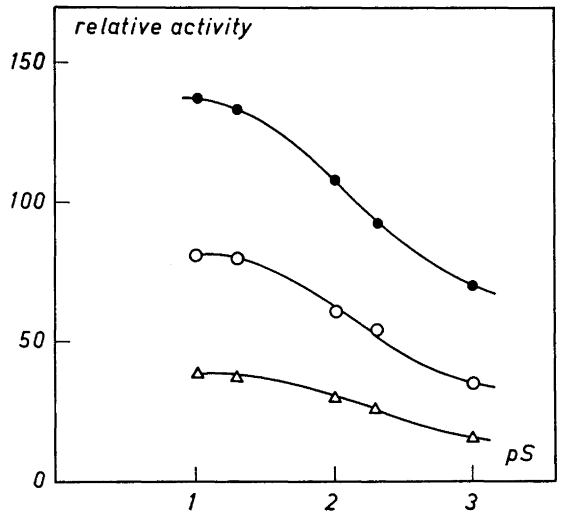


*Fig. 2.* Butyrylcholine esterase. pS-Activity curves for the acetyl esters of thiocholine (●), α-methylthiocholine (△) and β-methylthiocholine (○) at pH 8.00 and 25°C.

The experiments show that the catalytic effect of AChE resembles the one demonstrated for hydroxide ions [7], but decreases more rapidly with elongation of the acyl group in the ester. All acetyl esters are split easily but propionyl esters are split at a lower rate. The hydrolysis of *n*-butyryl, *iso*-butyryl, valeryl and benzoyl esters is extremely slow and not measurable with the method used. As in hydroxide-ion-catalysed hydrolysis the decreasing rate of hydrolysis with the length of the acyl group in the ester may be explained by combined steric and inductive effects of this group. The more rapid decrease in AChE-catalysed hydrolysis compared with hydroxide-ion-catalysed hydrolysis is probably caused by the importance of steric hindrance in enzyme-catalysed hydrolysis. The effect of the acyl group in the esters on the rate of AChE-catalysed hydrolysis is well known from choline esters [19]. The substrate maximum with thioesters was obtained at about the same concentration of ester as with acetylcholine, as seen in Fig. 1. The effect of substitution in the alcohol group in the esters upon the AChE-catalysed hydrolysis was also the same as observed with hydroxide ions as catalysts. All three types of esters are readily split and show the same type of curve. Homothiocholine shows a very slow hydrolysis and a somewhat different substrate curve.

*BuChE:* Fig. 2 shows curves obtained with butyrylcholine esterase and thioesters. When the enzyme velocity ($v$) is plotted against $v/[S]$ according to the Michaelis-Menten equation, expressed as

$$v = -\frac{v}{[S]} \cdot K_M + V_{max} \qquad (3)$$

the lines are not straight, just as has earlier been shown with the substrate butyrylcholine [20]. This deviation from straight lines in the Michaelis-Menten plot was explained as a sign of a possible existance of two enzymes able to split thiocholine esters as well as choline esters. One of the enzymes seems to have a fairly low substrate maximum [20] and to have reached its maximum velocity ($V_{max_2}$) below the substrate concentration of $10^{-3}$ M. Michaelis-Menten constants ($K_M$) and maximum velocities ($V_{max}$) for the BuChE-catalysed hydrolysis of the thioesters were therefore calculated from the determinations at the four highest substrate concentrations and are seen in Table 1. If there are two enzymes, then the values of the Michaelis-Menten constant are still correct for the enzyme with the highest $V_{max}$ ($V_{max_1}$), because $V_{max_1}$ is reached at the substrate concentrations used for the calculations. A subtraction of $V_{max}$ with $V_{max_2}$ would only cause a parallel shift of the curve obtained for the enzyme with $V_{max_1}$. The values of maximum velocity given in Table 1 would then be too high. It is seen from Table 1 that $K_M$

*Table 1.* Michaelis-Menten constants and maximum velocities for the reaction of BuChE with some carboxylic acid esters of thiocholine, α-methylthiocholine and β-methylthiocholine at pH 8.00 and 25°C.

| $R_2$ | $-CH_2-CH_2-\overset{+}{N}(CH_3)_3$ | | $-CH_2-\underset{\displaystyle CH_3}{\underset{\vert}{CH}}-\overset{+}{N}(CH_3)_3$ | | $-\underset{\displaystyle CH_3}{\underset{\vert}{CH}}-CH_2-\overset{+}{N}(CH_3)_3$ | |
|---|---|---|---|---|---|---|
| $R_1$ | $V_{max}$ relative activity | $K_M$ mole $\times l^{-1} \times 10^3$ | $V_{max}$ relative activity | $K_M$ mole $\times l^{-1} \times 10^3$ | $V_{max}$ relative activity | $K_M$ mole $\times l^{-1} \times 10^3$ |
| $CH_3-$ | 140 | 2.6 | — | — | 82 | 2.6 |
| $CH_3CH_2-$ | 287 | 2.3 | — | — | 146 | 2.3 |
| $CH_3CH_2CH_2-$ | 308 | 0.9 | 31 | 1.2 | 144 | 1.1 |
| $\begin{matrix} CH_3 \\ CH_3 \end{matrix}\!\!>\!CH_2-$ | 54 | 3.1 | — | — | — | — |
| $CH_3CH_2CH_2CH_2-$ | 271 | 0.8 | 29 | 0.8 | 144 | 0.4 |

becomes smaller with elongation of the acyl group of the thioesters. Branching of the acyl group gives a larger constant. The benzoyl ester was not split to any measurable extent. $V_{max}$ has its highest value for the butyryl esters and falls again before $K_M$ becomes larger. The dependence of $V_{max}$ from the length and shape of the carbon chain of the acyl group in the esters confirms the result obtained by, e.g., Whittaker[19] with choline esters. Elongation of the acyl group has thus the reverse effect in BuChE-catalysed hydrolysis from that in hydroxide-ion and AChE-catalysed hydrolysis, but the steric effect of the acyl group seems to be the same.

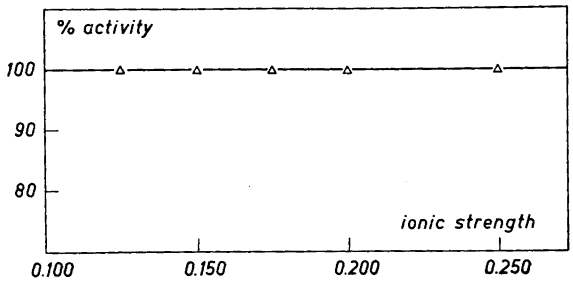


*Fig. 3.* Acetylcholine esterase. Dependence of enzyme activity at pH 8.00, 25°C and substrate concentration $10^{-3}$ M (acetylcholine) on ionic strength.

It has been observed [21] that the catalytic effect of BuChE on acetyl-β-methylthiocholine is different from its effect upon the analogous oxygen ester. It is shown here that this difference exists for all the esters examined. The $K_M$-values for thiocholine esters and β-methylthiocholine esters, seen in Table 1, are the same in contrast to those for the corresponding choline esters, where the β-methyl compound is known to be split at a nonmeasurable rate by BuChE. Catalysis by hydroxide ions was shown [7] to have the same effect. Only a few values for α-methylthiocholine esters are given because in the other cases it was difficult to reproduce the values. Acetylhomothiocholine was split at a nonmeasurable rate. The Michaelis-Menten constant for reaction with acetylcholine was measured for comparison and found to be $3{,}7 \times 10^{-3}$ moles $\times$ $l^{-1}$. The thiocholine ester is thus more easily split.

*Influence of ionic strength.* Varying the ionic strength between 0.10 and 0.25 M was shown to have no influence on AChE-catalysed reactions, see Fig. 3. The effect on the BuChE-catalysed hydrolysis has been demonstrated earlier [20].

## Rate of hydrolysis

In a few experiments the rate of enzymic hydrolysis of the acetyl esters was measured at constant pH by determination of the amount of unhydro-

*le 2.* First order rate constants for the enzymic hydrolysis of acetylcholine, acetylthiocholine, acety hylthiocholine and acetyl-β-methylthiocholine at pH 8.00 and 25°C. Enzymes are AChE and BuC

| Ester / yme | $-O-CH_2-CH_2-\overset{+}{N}(CH_3)_3$ | $-S-CH_2-CH_2-\overset{+}{N}(CH_3)_3$ | $-S-CH_2-\underset{CH_3}{CH}-\overset{+}{N}(CH_3)_3$ | $-S-\underset{CH_3}{CH}-CH_2-\overset{+}{N}(CH_3)_3$ |
|---|---|---|---|---|
| hE $min^{-1}$ | $1.7 \times 10^{-2}$ | $1.6 \times 10^{-2}$ | $1.2 \times 10^{-2}$ | $1.4 \times 10^{-2}$ |
| ChE $min^{-1}$ | $1.1 \times 10^{-2}$ | $1.8 \times 10^{-2}$ | $6.6 \times 10^{-3}$ | $9.9 \times 10^{-3}$ |

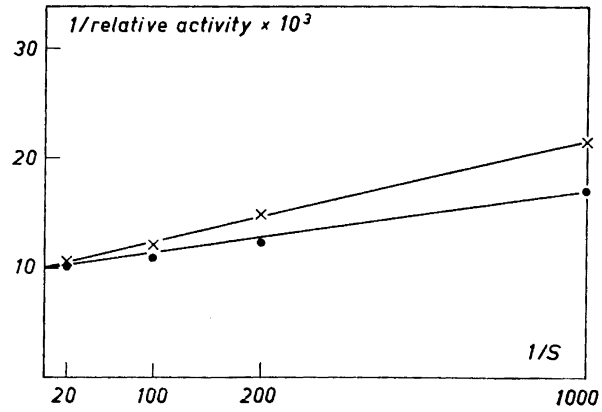


*Fig. 4.* Butyrylcholine esterase. Enzyme activity in presence (×) or absence (●) of eserine. pH 8 and 25°C. Substrate acetyl-β-methylthiocholine.

lysed ester. The curves obtained show a first order reaction with respect to ester concentration. The pseudo-first order rate constants are seen in Table 2. It is seen that AChE catalyses the hydrolysis of acetylcholine and acetylthiocholine at practically the same rate at the substrate concentration used, while BuChE is a better catalyst for acetylthiocholine.

## Effect of inhibitors

*Eserine and butyrylcholine.* Fig. 4 shows the curve obtained with the system BuChE — acetyl-β-methylthiocholine in the presence of, or without, the inhibitor eserine, which is competitive with choline esters. These curves are representative for a competitive reaction. As expected the thioesters react with at least some of the same active groups in the enzyme as the inhibitor does. An experiment to demonstrate this with acetyl-β-methylcholine as inhibitor did not work. The affinity of this compound for BuChE was too low to show any inhibitory effect even at a high concentration ($5 \times 10^{-2}$ M). For AChE, butyrylcholine was used as inhibitor to demonstrate the competitive effect.

*Choline and thiocholine.* Table 3 shows the negative logarithm of the inhibitor concentration causing 50 % inhibition after incubation of the enzymes with choline for 30 min at 25°C and pH 8. Substrates were acetylcholine and acetylthiocholine, respectively. The higher concentration of choline necessary to inhibit the enzymes to 50 % when acetylthiocholine is used as substrate shows that thioesters have a greater affinity for BuChE than the oxygen analogues. In the case of AChE the difference in $pI_{50}$ is very small. From the equations according to Briggs-Haldane [22] for the rate of enzyme reaction in the presence of, (eqn 4), or without, (eqn 5), a competitive reversible inhibitor

*Table 3.* $pI_{50}$- values for the inhibition of AChE and BuChE by choline or thiocholine in the presence of acetylcholine or acetylthiocholine and equilibrium constants for the inhibition of BuChE. Concentration of substrates $8 \times 10^{-3}$ M. 25°C and pH 8. Incubation time 30 min.

| | Enzyme | | | | | |
|---|---|---|---|---|---|---|
| | AChE | | | BuChE | | |
| Inhibitor | choline | | thiocholine | choline | | thiocholine |
| Substrate | acetyl-choline | acetyl-thiocholine | acetyl-choline | acetyl-choline | acetyl-thiocholine | acetyl-choline |
| $pI_{50}$ | 1.46 | 1.38 | 1.92 | 1.36 | 0.80 | 2.45 |
| $K_I$, moles $\times l^{-1} \times 10^2$ | – | – | – | 1.4 | 3.9 | 0.11 |

$$v = \frac{V}{1 + \frac{K_M}{[S]}\left(1 + \frac{[I]}{K_I}\right)} \quad (4)$$

$$v = \frac{V}{1 + \frac{K_M}{[S]}} \quad (5)$$

the values of the dissociation constants for the enzyme-inhibitor complex $K_I$ could be calculated for the systems BuChE-acetylcholine-choline, BuChE-acetylthiocholine-choline and BuChE-acetylcholine-thiocholine. They are seen in Table 3.

*Sarin.* It was shown that after inhibition by an irreversible phosphorylating inhibitor, such as sarin, the active groups of the enzyme are blocked in such a way that no difference in the enzymic hydrolysis of oxygen esters and thioesters after inhibition can be demonstrated. This is seen from the values of $pI_{50}$ in Table 4. When, however, the esters were used to protect the enzymes against inhibition, it could be demonstrated that the thioester was the better protector for BuChE. Acetylcholine and acetylthiocholine were found to be about equally good protectors for AChE. The results are seen in Table 5.

## Effect of some organic compounds

The effect of imidazole, serine, cysteine and 2-methyloxazoline upon the hydrolysis of acetylcholine, acetyl-β-methylcholine, acetyl-β-methylthiocholine and acetylthiocholine was studied with the automatic recording titrator at pH 8.00 and 25°C. None of the compounds had any

*Table 4.* Concentration of sarin, expressed as the negative logarithm, causing a 50 % inhibition of the hydrolysis of the acetyl esters of choline, thiocholine, $\alpha$-methylthiocholine, $\beta$-methylthiocholine and homothiocholine by the enzymes AChE and BuChE at pH 8, $t = 25°C$. Incubation time 30 min.

| Substrate \ Enzyme | AChE | BuChE IV-6-3 |
|---|---|---|
| Acetylcholine | 8.80 ± 0.01 | 8.29 ± 0.01 |
| Acetylthiocholine | 8.93 ± 0.01 | 8.36 ± 0.01 |
| Acetyl-$\alpha$-methylthiocholine | 8.92 ± 0.01 | 8.24 ± 0.005 |
| Acetyl-$\beta$-methylthiocholine | 8.92 ± 0.05 | 8.29 ± 0.01 |
| Acetylhomothiocholine | 8.85 ± 0.05 | — |

observable effect upon the hydrolysis of the choline esters, nor could any effect be noted on the hydrolysis of the thioesters by serine and methyloxazoline. Imidazole enhanced the hydrolysis of the thiocompounds about 4 times and cysteine about 50 times. The rate constants obtained with cysteine were found to be 0.144 $min^{-1}$ for acetylthiocholine and 0.139 $min^{-1}$ for acetyl-$\beta$-methylthiocholine. The effect of the above mentioned compounds seems to be related to the $pK_a$ of the substances, the compound with the higher $pK_a$ having the better hydrolytic effect. It has been pointed out by Larsson [23], that the basicity of a reagent seems to be a significant factor for its reactivity.

*Table 5.* Second order velocity constants for reaction of sarin with AChE and BuChE in the presence of acetylcholine iodide or acetylthiocholine iodide at pH 8.00 and 25°C.

| | Enzyme | | | |
|---|---|---|---|---|
| | AChE | | BuChE | |
| Type and concentration of inhibitor, moles × $l^{-1}$ | sarin $1.2 \times 10^{-8}$ | | sarin $3.9 \times 10^{-7}$ | |
| Type and concentration of substrate, moles × $l^{-1}$ | acetylcholine iodide $5.0 \times 10^{-3}$ | acetylthiocholine iodide $5.0 \times 10^{-3}$ | acetylcholine iodide $4.0 \times 10^{-3}$ | acetylthiocholine iodide $4.0 \times 10^{-3}$ |
| Inhibition of enzyme, $k_2 \times l \times mole^{-1} \times min^{-1}$ | $(2.4 \pm 0.2) \times 10^5$ | $(2.9 \pm 0.2) \times 10^5$ | $(9.3 \pm 0.1) \times 10^5$ | $(5.2 \pm 0.1) \times 10^5$ |

## DISCUSSION

The catalytic activity of esterases [24,25] is generally described as one of bifunctional type, a basic and an acid group taking part in the reaction. There are reasons to believe that the choline esterases have at least two sites in their active center [24,25], called the anionic and the esteratic sites. The anionic site seems to bind and direct substrates or inhibitors with quaternary nitrogen by means of an ionic bond. There is some evidence [24,25] that the anionic site is a carboxyl group. The hydrolytic activity of the enzyme seems to originate from the esteratic site, which seems to consist of a nucleophilic part, maybe a nitrogen atom in an imidazole ring (the basic group) and an electrophilic part, for instance the hydrogen atom in an amino group or a phenolic hydroxyl group (the acid group). The basic group in the enzyme is thought to form a covalent bond with the carbonyl carbon of a substrate, resulting in an addition complex. With the aid of the acid group in the esteratic site, which may form a hydrogen bond to the oxygen in the substrate, the combined acid-base attack upon the substrate results in the formation of an acylated enzyme.

There are several theories about the nature of the nucleophilic group. There are reasons to believe that it is an imidazole ring in histidine [10], but degradation experiments with choline esterases and other esterases show that, after inhibition with organophosphorus compounds, phosphorylated serine is always found, and no imidazole is found near the phosphorylated serine [25,26]. These findings caused a revival of a theory first suggested by M. Bergmann,

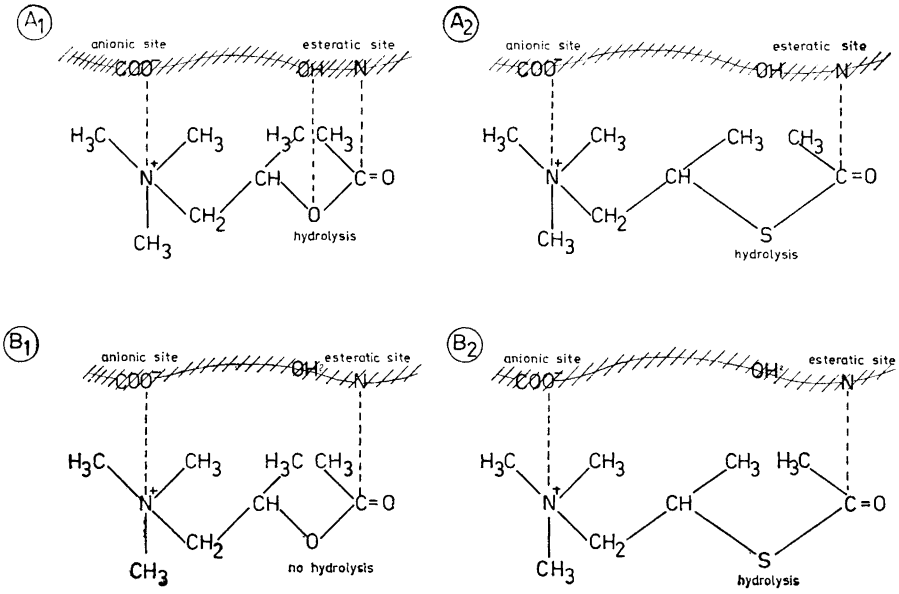


*Fig. 5.* Models showing attraction of choline esters (1) and thiocholine esters (2) to the surface of acetylcholine esterase (A) and butyrylcholine esterase (B). Dotted lines show points of attraction between enzymes and esters.

about the existence of an oxazoline ring formed from the aminoacid sequence aspargyl-serine [9]. However there is the possibility that the catalytically active imidazole is working near the phosphorylated serine, but situated in another part of the $\alpha$-helix of the protein and therefore lost in degradation experiments.

The experiments here described confirm for *AChE* the picture of a combined acid-base attack at the enzyme and the picture of the esteratic and anionic sites and their importance and limiting effect in the orientation and binding of the substrate. The difference in radius of oxygen and sulphur and the difference in their influence upon the electrophilic properties of the carbonyl carbon seem not very important as seen from the small differences in the rates of hydrolysis and in the protection effects of the two types of esters.

Bergmann *et al.*[5] have published values for the rate of the AChE-catalysed hydrolysis of acetylcholine and acetylthiocholine at pH 7.5 and 28°C. They found that acetylthiocholine at the substrate concentration used ($10^{-3}$ M) was split 5 times faster than acetylcholine. We were not able to confirm this result but obtained the same rate for the AChE-catalysed hydrolysis of acetylcholine and acetylthiocholine (Table 2). Our measurements were done at pH 8.0 and 25°C. To get the right value for the hydrolysis of acetylthiocholine we had to use the Hestrin method and to keep the pH constant with the automatic recording titrator, otherwise we would have obtained catalysis by buffer ions and difficulties in keeping the pH constant because of the acid properties of thiocholine ($pK_a \sim 7.7$) [7], one of the products of hydrolysis. Thiocholine would also have consumed sodium hydroxide, if the titration method had been used, or evolved $CO_2$, if the Warburg method had been used. Of course this latter effect would not have increased 5 times the value obtained for the rate of hydrolysis.

It may be worthwhile to discuss the shape of the pS-activity curves for AChE and substrates. The same type of curve was obtained with thioesters as substrate as with acetylcholine. The shape of the acetylcholine curve was found by some authors [5,27] to be a rather narrow bell showing maximum activity at pS around 2.25. These curves were mostly determined at pH 7.4 with the Warburg method. From our laboratory already in 1954 curves were published [28] showing a less narrow bell and an activity which still was near its maximum value at pS 3. Maximum activity was around pS 2.75 and this pS-value increased with the pH. It was observed that this type of curve was obtained as soon as the enzyme activity was measured from near the starting point of the reaction. It was clearly seen during the experiments that the activity in each determination decreased very soon if low substrate concentrations were used. The curves were at that time measured with the electrometric method at pH 8. In the meantime we have used the automatic recording titrator, which allows measurements of enzyme activity from the starting point of the reaction and the use of a second syringe [20,23] containing substrate solution to keep the concentration of the substrate constant. pS-Activity curves were measured at pH 7.5 [18] and now at 8.0 and showed the same shape as published earlier by us.

*BuChE* is known to be a less "specific" enzyme than AChE. The rate of hydrolysis of oxygen esters and thioesters increases, up to a certain limit,

with the length of the acyl group in the ester in spite of the inductive effects of the acyl group, when BuChE catalyses the reaction. The limit may depend on steric hindrance from the long acyl group in the formation of the enzyme-substrate addition complex. Furthermore the probably somewhat less electrophilic sulphur ester is more easily split than the oxygen analogue and is a better protector aginst inhibitors. Both hydroxide-ion- and BuChE-catalysed hydrolysis of thioesters was shown to be independent of a shielding methyl group near the ester sulphur, the hydrolysis possibly, therefore, taking place without an attack on the sulphur. It may be that BuChE is less ready to attack a substrate with the aid of an acid group than AChE and therefore able to split the thioester but not the oxygen ester. The shape of the pH-activity curve for the enzyme with acetylcholine gives no definite information concerning an acid group, because the decreasing activity at high pH [29] seems to be the result of more general protein denaturation as well as the dissociation of a functional acid group. The catalytic effect of imidazole on thioesters, but not on oxygen esters, demonstrated by Bergmann *et al.*[5] for acetylthiocholine and here for acetyl-$\beta$-methylthiocholine, demonstrates the ability of sulphur esters to split on attack of this nucleophilic group alone. That the thioesters are less susceptible to an attack by hydrogen ions than oxygen esters was demonstrated by their slower hydrolysis in acid medium.

The enzymes AChE and BuChE differ in some important way in the reaction with their substrates. Summarizing the results obtained in this and two preceding papers [6,7] the following points are found:

The thiocholine esters and their methylsubstituted analogues hydrolyse slower in acid medium than their oxygen ester analogues.

The hydrolysis of the thioesters is affected by some buffer ions, e.g. phosphate. Secondary reactions with the products of hydrolysis seem to occur in alkaline medium at least with the methylsubstituted thioesters.

The hydrolysis of the thioesters at pH 8.00 is catalysed by imidazole and enhanced by cysteine in contrast to their oxygen analogues.

The activation energies in hydroxide-ion-catalysed hydrolysis are about equal for acetylthiocholine, acetyl-$\beta$-methylthiocholine and acetylcholine, but about 33 % higher for acetyl-$\beta$-methylcholine.

Acetyl-$\beta$-methylthiocholine is split by BuChE in contrast to its oxygen analogues.

The reaction rates between AChE and acetylcholine or acetylthiocholine are about equal, but BuChE reacts faster with acetylthiocholine than with acetylcholine.

Choline and thiocholine are about equally good inhibitors for AChE, but thiocholine is a better inhibitor for BuChE.

Acetylcholine and acetylthiocholine protect AChE about equally well against sarin, but acetylthiocholine is the better protector for BuChE.

On the basis of these results one way to explain the difference between the two enzymes and their reaction with the substrates is shown in Fig. 6. A hydrogen bond is thought to exist between the enzyme and the ether oxygen to explain the shielding effect of the $\beta$-methyl group in hydroxide-ion- and enzyme-catalysed hydrolysis [7] of $\beta$-methylcholine esters. For perhaps steric reasons, BuChE is not able to create this hydrogen bond between its acid group and

the ether oxygen in $\beta$-methylcholine esters. Competition with water may occur and be unfortunate for the enzyme. The thioesters seem to be able to split without this hydrogen bond and are therefore split also by BuChE.

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