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Carboxylic Acid Esters as Substrates of Cholinesterases

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Data on the kinetics of the hydrolysis of various carboxylic acid esters by two main types of cholinesterases—acetylcholinesterase from human erythrocytes and buryrylcholinesterase from horse blood serum—are surveyed. It is shown that the rate of enzyme hydrolysis depends significantly on the structure of the acyl part of the ester molecule, the nature of the ester heteroatom, the structure of the alcohol component, and particularly the structure of the onium group. Esters based on natural products are of special interest as specific substrates of these enzymes. The role of the productive and non-productive sorption of the substrates in enzyme catalysis is demonstrated.
The bibliography includes 81 references.

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I. INTRODUCTION

Cholinesterases (CE) are enzymes which hydrolyse the choline esters of carboxylic acids at a higher rate than non-choline esters. A characteristic feature of these enzymes is the inhibition of their catalytic activity by low concentrations of tetra-alkylammonium compounds, certain organophosphorus compounds, and esters of substituted carbamic acids. The sensitivity to eserine at concentrations of 10^{-5} M and below is believed to be one of the most reliable criteria for the classification of an enzyme as a cholinesterase.

Two main types of cholinesterases have been most thoroughly investigated: acetylcholinesterase (ACE) or acetylcholine acyl-hydrolase (EC 3.1.1.7) and butyrylcholinesterase (BuCE)—a variety of acylcholine acyl-hydrolases (EC 3.1.1.8), which differ in their substrate specificity and sensitivity to various inhibitors. ACE hydrolyses acetylcholine (AC) at the highest rate, propionylcholine (PrC) and acetyl-β-methylcholine (MeC) somewhat more slowly, and has almost no influence on the rate of hydrolysis of butyrylcholine (BuC) and benzoylcholine (BzC). On the other hand, butyrylcholinesterase hydrolyses BuC at the highest rate, PrC, AC, and BzC somewhat more slowly, and does not influence the rate of hydrolysis of MeC. Furthermore, in contrast to BuCE, the catalytic activity of ACE is inhibited by high substrate concentrations.

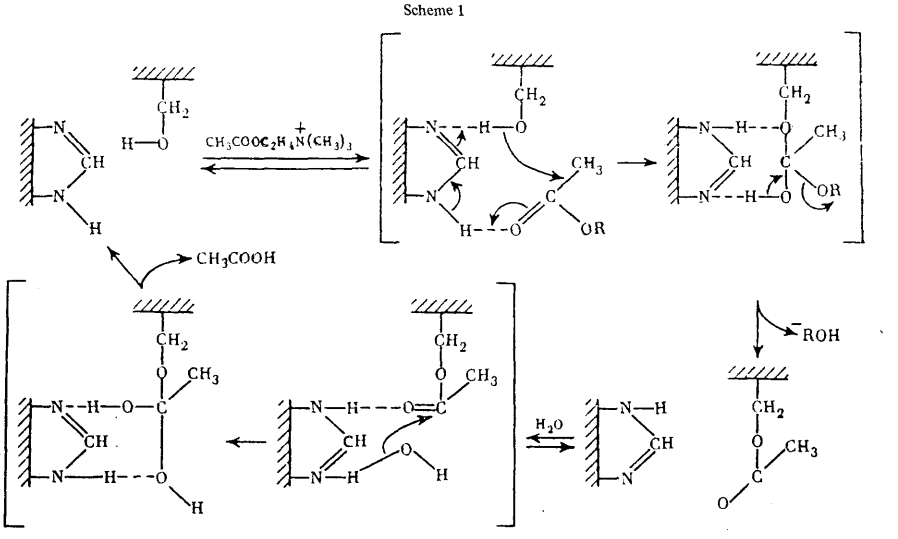
ACE of nerve tissue is directly involved in the transmission of nerve excitation from the nerve to the innervated cell. The biological role of BuCE as well as that of the ACE contained in the erythrocytes of many animals has not been elucidated.

Cholinesterases are the target of the inhibiting activity of certain medicinal preparations, organophosphorus and carbamate insectoacaricides, and organophosphorus poisons.

There exists an extensive literature on cholinesterases, which has been comprehensively surveyed in monographs[1-5] and a number of reviews[6-8].

The extremely high catalytic activity of cholinesterases is due to the unique structure of their active surface. Apart from the esterase site, where the ester linkage is ruptured, at a distance of 4–5 Å from it on the surface there is an anionic site which is responsible for the specific binding of the ammonium group of AC and promotes a favourable orientation of the substrate molecule[2-4]. Apart from Coulombic interaction of the ammonium group with the anionic site of the enzyme, the orientation of substrates and inhibitors is

Scheme 1

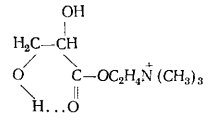


greatly influenced by the hydrophobic interaction of their hydrocarbon groups with the hydrophobic sections located in the region of both the anionic and esterase sites[8].

It is suggested that the anionic site consists of the carboxylate anion derived from the dibasic glutamic or aspartic acid incorporated in the polypeptide chain of the enzyme. The esterase site on the active surface of the enzyme has a more complex structure. The serine hydroxyl, which is acylated on interaction with substrates and phosphorylated on interaction with organophosphorus inhibitors, plays the main role in it. According to modern ideas[9,10], this chemically inert hydroxyl is activated with the aid of the imidazole group of histidine located in its immediate vicinity. On interaction with the substrate, the activation process begins with formation of a hydrogen bond between its carbonyl oxygen and the imino-nitrogen of imidazole, which increases the basicity of the azole nitrogen and leads to the formation of a hydrogen bond between this nitrogen and the serine hydroxyl[9] (Scheme 1).

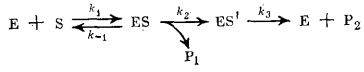
There is then a sharp increase of the nucleophilicity of the hydroxyl oxygen and its interaction with the central carbon atom is facilitated. The redistribution of electron density in the eight-membered ring of the enzyme–substrate complex formed results in the formation of a covalent bond between the serine oxygen and the acid group of the substrate and in the elimination of the alcohol group. The subsequent reaction of the acylated enzyme with water proceeds analogously, forming an intermediate acyl-enzyme–hydrate complex, which then decomposes to the initial enzyme and an acid. Thus the reactive form of the serine hydroxyl is produced at the instant of the oriented binding of the substrate molecule and does not precede it.

The mechanism of cholinesterase hydrolysis presented here is confirmed by a number of facts[4]. Cholinesterases do not catalyse the hydrolysis of choline esters containing thione sulphur (C=S) instead of carbonyl oxygen (C=O), since the sulphur atom is incapable of forming a strong hydrogen bond, which hinders the activation of the serine hydroxyl. For the same reason, organophosphorus compounds containing thione sulphur (P=S) do not phosphorylate cholinesterase, in contrast to their oxygen analogues. Glyceroylcholine, the cyclic structure of the acid group of which has been demonstrated by infrared spectroscopy[11], is not hydrolysed by ACE, because the carbonyl oxygen is already linked by a hydrogen bond to the hydroxy-group of the ester itself:



The hydrolysis of substrates (S) by an enzyme (E) is usually described by Scheme 2[4,12]:

Scheme 2



where ES is the Michaelis complex, ES′ the acylated enzyme, P_1 and P_2 are the alcohol and the acid respectively, and k_1, k_{-1}, k_2, and k_3 are the rate constants for the individual reaction states. Evidently, apart from the complexes ES and ES′, other intermediate complexes, for example, those occurring in Scheme 1, are also formed, but these additional complexes do not, generally speaking, vitiate the kinetic conclusions which follow from the three-stage scheme presented[12].

When the concentration of the substrate [S] in the reaction mixture is many times higher than the initial enzyme concentration $[E]_0$, it is believed that the enzyme hydrolysis proceeds under steady-state conditions where the concentrations of the complexes [ES] and [ES′] are established at almost constant levels. Under these conditions, the scheme leads to the familiar Michaelis–Menten equation

$$v=\frac{a_c[E]_0[S]}{K_M+[S]}=\frac{V[S]}{K_M+[S]}\ , \qquad (1)$$

where v is the rate of hydrolysis of the substrate by the enzyme, V is the maximum velocity of the reaction when the enzyme is fully saturated by the substrate, i.e. for $[S] \gg K_M$, whereupon Eqn.(1) becomes

$$v=V=a_c[E]_0'\ , \qquad (1a)$$

a_C is the activity of the catalytic centre of the enzyme linked to the rate constants of the individual reaction stages by the formula

$$a_c=\frac{k_2k_3}{k_2+k_3} \qquad (1b)$$

and K_M is the Michaelis constant expressed by the formula

$$K_M=\frac{[E][S]}{[ES]+[E']S}=\frac{(k_{-1}+k_2)\,k_3}{k_1(k_2+k_3)}\ . \qquad (1c)$$

When $[S] = K_M$, Eqn.(1) becomes

$$v=\frac{V}{2}\ , \qquad (1d)$$

and it follows from Eqn.(1c) that

$$\frac{K_M}{[S]}=\frac{[E]}{[ES]+[ES']}=1\ , \qquad (1e)$$

where [E] is the concentration of the free enzyme.

Hence it follows that K_M is numerically equal to the substrate concentration for which the rate of the enzyme reaction is smaller by a factor of two than the maximum rate defined by Eqn.(1d) and when half the enzyme is bound in the enzyme–substrate complexes [Eqn.(1e)].

The susceptibility of cholinesterases to irreversible inhibition by organophosphorus inhibitors (OPI) as a result of the phosphorylation of the active centres has been used to determine $[E]_0$ in the enzyme solution investigated[13]; $[E]_0$ is found from the minimum amount of a highly active OPI which fully inhibits the catalytic activity; $[E]_0$ makes it possible to calculate easily the activity of the catalytic enzyme centres a_C from Eqn.(1a): $a_C = V/[E]_0$.

The knowledge of a_C and V for one substrate ($a_{C,1}$ and V_1), for example, for AC, makes it possible to find a_C for another substrate ($a_{C,2}$) from the maximum rate of its enzyme hydrolysis V_2 for the same amount of the preparation of the given enzyme, i.e. for $[E]_{0,1} = [E]_{0,2}$:

$$a_{c,1}/a_{c,2}=V_1/V_2$$

It follows from Eqn.(1a) that a_C characterises the catalytic activity when the enzyme is fully saturated by the substrate, i.e. it does not take into account the first binding stage which has a significant influence on the ability of the enzyme to hydrolyse the substrate at low concentrations. To a first approximation, this stage is assessed in terms of the ratio

$$a_c/K_M : a_c/K_M=k_1k_2/(k_1+k_2)$$

because $k_2/(k_{-1} + k_2) < 1$, so that $a_C/K_M < k_1$.

The ratio a_C/K_M is an important kinetic parameter characterising the catalytic activity of the enzyme in relation to the given substrate when $[S] < K_M$, since under this condition Eqn.(1) becomes

$$v=\frac{a_c[E_0][S]}{K_M}\ . \qquad (1f)$$

Thus at high substrate concentrations the rate of enzyme hydrolysis is determined by the quantity a_C and at low concentrations by the quantity a_C/K_M.

Eqn.(1) is valid for the hydrolysis of the choline esters of carboxylic acids by ACE only at low concentrations of the esters, since at high concentrations the catalytic activity of the enzyme is inhibited. This, the so-called substrate inhibition is taken into account in the Haldane equation[14]

$$v = \frac{V[S]}{K_M + [S] + K_{SS}[S]^2} = \frac{V[S]}{K_M + [S] + (K_M/[S]^2_{opt})[S]^2} \quad (2)$$

where K_{SS} is the substrate inhibition constant.

The quantity K_{SS} is calculated from the formula

$$K_{SS} = K_M/[S]^2_{opt} \quad (2a)$$

where $[S]_{opt}$ is the substrate concentration for which the reaction rate has a maximum, i.e. the substrate concentration corresponding to the maximum on the $v-[S]$ curve[14].

Haldane[14] believes that substrate inhibition is associated with the fact that at high concentrations [S] a second inactive enzyme–substrate sorption complex ES_2 is formed in addition to the ES complexes. At high electrolyte concentrations in the reaction mixture, substrate inhibition of ACE vanishes[15], which is difficult to reconcile with Haldane's hypothesis. Substrate inhibition is probably associated with the interaction of the substrate with an allosteric centre[15].

There is at present no reliable method for determination of the rate constants for the individual stages of the cholinesterase hydrolysis. The study of the kinetics of this reaction is restricted to the determination of K_M and V. One of the commonest procedures for the determination of these kinetic parameters is the Lineweaver–Burk method[16].

Table 1. The cholinesterase hydrolysis of the choline esters of carboxylic acids $RC(O)OCH_2CH_2\overset{+}{N}(CH_3)_3$ (the rate of hydrolysis of AC was assumed to be 100)[4].

Compound	R	BuCE	ACE	Compound	R	BuCE	ACE
(I)	H	180	66	(XI)	$CH_2=CH$	100	12
(II)	CH_3(AX)	100	100	(XII)	$CH_3CH=CH$	20	2
(III)	C_2H_5	180	70	(XIII)	$CH_2=CH-CH_2$	186	11
(IV)	C_3H_7	240	2	(XIV)	$C_2H_5CH=CH$	10	0
(V)	C_4H_9	180	1	(XV)	$CH_2=CHCH_2CH_2$	220	0
(VI)	C_5H_{11}	110	—	(XVI)	$CH_2=C(CH_3)$	47	20
(VII)	C_6H_{13}	120	—	(XVII)	$CH_3CH=C(CH_3)$	11	0
(VIII)	$(CH_3)_2CH$	108	40	(XVIII)	$(CH_3)_2C=CH$	0	0
(IX)	$C_2H_5(CH_3)CH$	13	1	(XIX)	$CH_3CH=C(C_2H_5)$	9	0
(X)	$(C_2H_5)_2CH$	16	0				

II. DIFFERENT ANALOGUES OF ACETYLCHOLINE

The study of the kinetic parameters of the hydrolysis of various carboxylic acid esters by cholinesterases is necessary for the determination of the specificity of their catalytic activity and also for the elucidation of the mechanism of the cholinesterase hydrolysis and of the structure of the active surfaces of these enzymes.

The cholinesterase hydrolysis of AC analogues, differing from the latter either by the structure of the acyl group $[R-C(O)OCH_2CH_2\overset{+}{N}(CH_3)_3]$, or the nature of the ester linkage $[R-C(O)XCH_2CH_2\overset{+}{N}(CH_3)_3]$, or the structure of the group between the ester oxygen and the onium nitrogen atom $[R-C(O)O-A-\overset{+}{N}(CH_3)_3]$, or the structure of the onium group $[CH_3C(O)-OCH_2CH_2\overset{+}{N}R^1R^2R^3]$, is discussed below.

1. Substrates Having the General Formula $RC(O)-OCH_2CH_2.\overset{+}{N}(CH_3)_3$

The enzyme hydrolysis of choline esters is known[2–4] to depend significantly on the structure of the acyl residue (Table 1). Among the choline esters of saturated straight-chain carboxylic acids ranging from formic (I) to oenanthic (VII), only the first three esters are hydrolysed by ACE, AC being hydrolysed at the maximum rate, while BuCE hydrolyses all seven esters, the rate of hydrolysis of BuC (IV) being highest.

In contrast to BuC, isobutyrylcholine (VIII), in which the length of the hydrocarbon group in the acyl component is the same as in PrC, is readily hydrolysed by ACE and not less effectively than PrC. It is striking that BuCE hydrolyses isobutyrylcholine less effectively than PrC and BuC, i.e. the branching of the hydrocarbon group impairs the susceptibility of the choline esters to hydrolysis by this enzyme. This is confirmed also by data for the enzyme hydrolysis of the choline esters of ethylmethylacetic (IX) and diethylacetic (X) acids which are hardly hydrolysed by BuCE, while the choline esters of the isomeric straight-chain acids—valeric (V) and caproic (VI)—are hydrolysed by this enzyme at a high rate.

The presence of a double bond in the acyl component reduces the susceptibility of choline esters to cholinesterase hydrolysis. Thus acryloylcholine (XI) is hydrolysed by BuCE and ACE more slowly by factors of almost two and five respectively than its saturated analogue (III). It has been shown in relation to the choline esters of crotonic acid that the influence of the double bond depends greatly on its position. The presence of the double bond in the α-position in compound (XII) reduces sharply the susceptibility of this ester to hydrolysis by BuCE, while the double bond in the β-position in compound (XIII) reduces the rate of hydrolysis of the ester by this enzyme only by a factor of 1.3 compared with BuC. Analogous data have been obtained in experiments with the choline esters of unsaturated analogues of valeric acid [compounds (XIV) and (XV)][4].

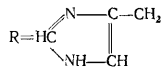
Unusual results have been obtained for the choline esters of aminoacids in which $R = NH_2(CH_2)_n$.[17] Under the influence of BuCE, the rates of hydrolysis of esters with $n = 3–4$ were lower by a factor of 15–20 than the rate of hydrolysis of AC and for $n = 6$ and 7 these esters were hydrolysed at the same rate as AC. The conversion of NH_2 into $\overset{+}{N}(CH_3)_3$ leads in all cases to a sharp reduction of the rate of enzyme hydrolysis[17].

It has been shown[18] that carbamoylcholine ($R = NH_2$) is not hydrolysed at all by BuCE.

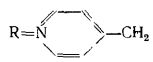
Interesting data have been obtained[19,20] for the cholinesterase hydrolysis of fluoro-, chloro-, bromo-, and iodo-substituted AC derivatives. All these derivatives were hydrolysed faster by BuCE than AC (by a factor greater than 2.5), the bromo-derivative being hydrolysed at the maximum rate. The inhibition of catalytic activity by high substrate concentrations, which is atypical for BuCE, was observed for all the derivatives. The fluoro-derivative was hydrolysed by ACE like AC, while the remaining derivatives were hydrolysed more slowly by a factor of two, despite the fact that in all of them the strength of the ester linkage is much lower than in AC. Obviously the observed effect of the substitution of a hydrogen atom by a halogen is associated with the increase of the size of the acid group. This hypothesis is confirmed by the fact that the dichloro-derivative of AC is hydrolysed by ACE five times more slowly than AC, while the trichloro-derivative is not hydrolysed at all. It is surprising that BuCE, for which the steric factor should not play a

significant role in the given instance, does not hydrolyse these two esters.

The introduction into the acyl part of the AC molecule of the imidazole group[21]

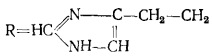


and the pyridine group[22]



reduced the rate of hydrolysis by BuCE, while ACE altogether failed to hydrolyse these derivatives.

It is striking that imidazolylpropionylcholine



is hydrolysed by BuCE as effectively as BuC, i.e. 2.5 times faster than AC[21].

At high concentrations phenylpropionylcholine ($R=C_6H_5CH_2.CH_2$) is hydrolysed by BuCE almost twice as fast as AC and BzC, while at low concentrations it is hydrolysed faster by a factor greater than 40 owing to the lower value of K_M; it is not hydrolysed by ACE[23]. As for the imidazole derivatives, an increase of the distance between the carbonyl carbon and the phenyl group leads to an appreciable increase of the rate of enzyme hydrolysis.

The stereospecificity of cholinesterases has been investigated in experiments with lactoylcholine. The L(+)- isomer was hydrolysed by ACE faster than AC by a factor of 1.5, while the rate of hydrolysis of the D(-)-isomer was five times lower; the rate of hydrolysis of the racemate was 70% of that of AC[11]; BuCE did not exhibit stereospecificity: both isomers were hydrolysed by this enzyme 3.5 times faster than AC[24].

Thus the data examined in this section show that ACE is more sensitive than BuCE to changes in the structure of the acid components of the molecules of choline esters.

2. Substrates Having the General Formula $RC(O)XC_2H_4\overset{+}{N}(CH_3)_3$

Analogues of AC are known in which the ester oxygen has been replaced by S, Se, and NH. Among them, the greatest interest attaches to the thiocholine esters, whose study was begun as early as 1939[18]. Acetylthiocholine (ATC) and butyrylthiocholine (BuTC) have already been used for a long time in histological investigations in order to discover and locate cholinesterases in various tissues. These esters as well as propionylthiocholine (PrTC) and acetyl-β-methylthiocholine (AMTC) are also widely used in biochemistry in the study of the catalytic activities of cholinesterases; the thiocholine formed on their hydrolysis can be readily determined colorimetrically with the aid of the Ellman chromogen reagent[25]. This method for the determination of the cholinesterase activity is more sensitive than the potentiometric method.

Data according to which the rate of hydrolysis of thiocholine esters by cholinesterases is several times higher than that of the choline analogues have been presented in a series of reviews and monographs[2,3,5,6]. It has been demonstrated convincingly[26,27] that the available literature data are much too high, since the investigators who used the potentiometric titration method did not take into account the acid properties of the thiocholine formed on hydrolysis of these substrates.

Table 2 presents the results of a comparative kinetic study of the enzyme hydrolysis of choline and thiocholine esters by ACE and BuCE[27].

All the relations established for the oxygen analogues are valid for the cholinesterase hydrolysis of the thiocholine esters. Thus ATC is hydrolysed fastest by ACE, PrTC is hydrolysed more slowly, and BuTC is hardly hydrolysed at all. The rate of hydrolysis by BuCE decreases in the sequence BuTC > PrTC > ATC. The inhibition of enzyme activity by high concentrations of thiocholine esters is manifested only in experiments with ACE. There are also similarities between other substrate properties of thiocholine and choline esters—an S optimum and a pH optimum. It is striking that at high substrate concentrations ACE hydrolyses AC at a higher rate (in terms of a_C), while at low concentrations the rate of hydrolysis of ATC is higher (in terms of a_C/K_M). Butyrylcholinesterase hydrolyses ATC faster than AC at both high and low concentrations.

In contrast to its oxygen analogue, AMTC is hydrolysed comparatively effectively by BuCE and cannot therefore be used as a specific substrate of ACE[26,28].

Table 2. Kinetic parameters of the hydrolysis of choline and thiocholine esters by BuCE and ACE[27].

Substrate	BuCE			ACE		
	a_c, min^{-1}	K_M, mol $litre^{-1}$	a_c/K_M, litre mol^{-1} min^{-1}	a_c, min^{-1}	K_M, mol $litre^{-1}$	a_c/K_M, litre mol^{-1} min^{-1}
AC	$7.6\cdot10^4$	$9.1\cdot10^{-4}$	$8.4\cdot10^7$	$2.8\cdot10^5$	$1.9\cdot10^{-4}$	$1.5\cdot10^9$
PrC	$1.2\cdot10^5$	$5.5\cdot10^{-4}$	$2.2\cdot10^8$	$2.2\cdot10^5$	$2.8\cdot10^{-4}$	$8.0\cdot10^8$
BuC	$2.1\cdot10^5$	$7.7\cdot10^{-4}$	$2.7\cdot10^8$	0	—	—
ATC	$9.5\cdot10^4$	$5.4\cdot10^{-4}$	$1.8\cdot10^8$	$2.2\cdot10^5$	$1.0\cdot10^{-4}$	$2.2\cdot10^9$
PrTC	$1.2\cdot10^5$	$5.1\cdot10^{-4}$	$2.4\cdot10^8$	$1.2\cdot10^5$	$1.9\cdot10^{-4}$	$6.0\cdot10^8$
BuTC	$1.6\cdot10^5$	$4.3\cdot10^{-4}$	$3.8\cdot10^8$	0	—	—

The observed differences between the abilities of ACE and BuCE to hydrolyse choline and thiocholine esters are due to different causes: the different amounts of anhydride character in the ester linkages, the different configurations of the choline and thiocholine esters, and the different radii of the heteroatoms.

The selenium analogue of AC was hydrolysed by ACE at a lower rate than ATC[29]. It was believed that the amide analogue of AC (X = NH) cannot be hydrolysed by cholinesterases[4]. However, according to the latest data[30], this substrate is hydrolysed by ACE. In the study of the rate of hydrolysis of this amide one must bear in mind that the trimethylaminoethylammonium cation $(CH_3)_3\overset{+}{N}CH_2CH_2NH_2$ has pronounced basic properties and the traditional potentiometric titration method is not suitable for its determination.

3. Substrates Having the General Formula $RC(O)OAN(CH_3)_3$

The susceptibility of choline esters to hydrolysis by cholinesterases is greatly influenced by the structure of the group A between the ester oxygen and the onium nitrogen atom. Several AC derivatives in which the hydrogen atoms in the ethylene chain have been substituted by various hydrocarbon groups ($A = -CHR^1CHR^2$) have been synthesised. The β-methyl derivative of AC, which is relatively easily hydrolysed by ACE, is of greatest interest: the rate of its hydrolysis is smaller by a factor of three than that of AC[31] and it is hardly hydrolysed by BuCE (at least 20 times more slowly than AC[32]). Until recently, acetyl-β-methylcholine was therefore regarded as the only specific substrate

of ACE. The substitution of the hydrogen in the β-position by more complex hydrocarbon groups ranging from C_2H_5 to C_6H_{13} increases only slightly the rate of hydrolysis of esters by BuCE[18]. Unfortunately the hydrolysis of these AC derivatives by ACE has not been investigated.

The specificity of ACE to the acyl residue of the β-methylcholine esters is the same as for choline esters: the propionate hydrolyses more slowly than the acetate, the rate of hydrolysis of the isobutyrate is lower still, and trimethylacetate is not hydrolysed at all[33].

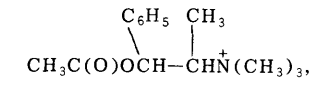
Experiments with the optical isomers of acetyl-β-methylcholine established that the L-isomer is hydrolysed by ACE more slowly by a factor of two than AC, while the D-isomer proved to be a reversible inhibitor and not a substrate. As mentioned above, the racemate is hydrolysed by this enzyme approximately three times more slowly than AC.

In experiments with butyryl-β-methylcholine it was shown that stereospecificity is characteristic also of BuCE: the L-isomer is hydrolysed by this enzyme 10 times more slowly than AC, while the D-isomer is not hydrolysed at all. It is striking that the stereospecificity of both enzymes is not shown in the hydrolysis of acetyl-β-methylcholine: both isomers are hydrolysed by BuCE more slowly by a factor of 1.5 than AC[34], while the rate of hydrolysis by ACE is the same as that of AC[33].

Thus the stereospecificity of cholinesterases is not manifested in all hydrolytic reactions of optically active substrates: it depends significantly on the position of the asymmetric carbon atom in the substrate molecule. The stereospecificity depends also on the nature of the enzyme. As stated above, ACE can distinguish between the D- and L-isomers of lactoylcholine, while BuCE cannot.

The replacement of two hydrogen atoms in the ethylene group by a methyl group in the α- and β-positions lowers the rate of hydrolysis of this ester by ACE[35]. Since there are two asymmetric carbon atoms in the acetyl-α,β-dimethylcholine molecule, this ester should exist in the form of four isomers, forming two pairs: the *erythro*-isomers and the *threo*-isomers, each pair constituting optical enantiomers. It was found that ACE hydrolyses only the *threo*-α(R), β(S)-dimethyl-AC racemate, while the rate of hydrolysis of *erythro*-α(R), β(S)-dimethyl-AC is zero[55].

The esters having the general formula

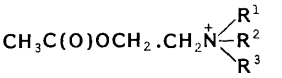


which are synthesised from ephedrine and pseudoephedrine and are the α-methyl and β-phenyl derivatives of AC, are not hydrolysed by cholinesterases and behave as reversible inhibitors[36].

When the ethylene group in the AC molecule was replaced by the "rigid" cyclopropyl group (A = ), the optically active *trans*- and *cis*-isomers were obtained[37]. The rates of hydrolysis of the (+)- and (-)-*trans*-isomers by ACE were 96% and 59% of that of AC respectively. They were hydrolysed at a lower rate by BuCE: 61 and 33% respectively. The *cis*-isomers were not hydrolysed by these enzymes.

The data described here lead to the conclusion that BuCE is more sensitive to changes in the structure of the ethylene group in the AC molecule than ACE. This conclusion is also confirmed by the results of experiments with AC analogues in which the ethylene group has been replaced by a longer alkyl chain: A = $-(CH_2)_n-$. The ester with the trimethylene chain is hydrolysed by the rat brain homogenate ACE at 70% of the rate of hydrolysis of AC, while the ester with the tetramethylene chain is hydrolysed at 10% of the AC rate. On the other hand, under the influence of BuCE, the rates of their hydrolysis are 7.2 and 0.6% respectively[18,38,39]. Analogous data indicating a decrease of the catalytic activity of cholinesterases with increase of *n* have been obtained in experiments with ammonium and sulphonium compounds[40,41]. The high sensitivity of BuCE to an increase in the length of the alkyl chain has also been demonstrated in experiments with analogous BuC derivatives. The esters with the trimethylene and tetramethylene chains are hydrolysed by this enzyme respectively 5 and 70 times more slowly than BuC[42].

4. Substrates Having the General Formula



The susceptibility of various analogues of AC to hydrolysis by cholinesterases depends also on the structure of the ammonium group. Thus the replacement of three methyl groups at the onium nitrogen in AC by ethyl groups resulted in a sharp decrease of the rate of their hydrolysis by BuCE but hardly affected the rate of hydrolysis by ACE[43].

It follows from the data in Table 3[44] that, among the compounds investigated, only the dibutylmethyl derivative (XXIX) proved to be an inhibitor and not a substrate of cholinesterases. Evidently such a large increase in the bulk of the ammonium group should result in a loss by AC derivatives of susceptibility to cholinesterase hydrolysis. Other AC derivatives, except compound (XXI), were hydrolysed by cholinesterases at high concentrations in the reaction mixture but at a lower rate than AC: this is indicated by the values of a_C listed in Table 3. According to the literature[43], a change in the composition of the ammonium group entails a more marked alteration of a_C in experiments with BuCE. Apparently the presence of a charge is most important for ACE, while a change in the structure of the ammonium group is less significant than for BuCE.

When the methyl groups in the AC molecule are replaced by ethyl groups, as in compound (XXVII), the parameter a_C decreases by a factor of 3 for BuCE and only by 1/3 for ACE. Analogous ratios were obtained also for other derivatives, except the pentyl derivative (XXIV), for which a_C in experiments with ACE fell by a factor of two compared with a_C for AC, while in experiments with BuCE the decrease was only by 1/4. It is of interest that the isopropyl derivative (XXV) is very similar to its unbranched analogue (XXII) as a substrate of BuCE and ACE.

The values of K_M for many compounds, with the exception of (XXI), (XXIV), (XXV), and (XXVII) in experiments with ACE and (XXVIII) in experiments with BuCE, proved to be lower than for ACE.

The observed differences between the values of a_C for cholinesterases in the enzyme hydrolysis of AC analogues as a function of the composition of the ammonium group conflict with Scheme 2 and Eqn.(1) which follows from it. Since all these compounds are acetate esters, the deacetylation rate constants k_3 for their ES′ complexes should be the same. The acetylation rate constants k_2 should also be virtually the same, since, according to quantum-chemical calculations, all the compounds are characterised by the same electron density at the carbonyl carbon atom and the strengths of the ester linkages should not therefore differ either. Thus, in conformity with this finding, when Scheme 2 is valid for all the compounds quoted, the values of a_C should also be identical in experiments with both ACE and BuCE. In fact these quantities vary within wide limits.

Table 3. Kinetic parameters of the hydrolysis of AC analogues having the general formula $CH_3C(O).OC_2H_4X$ by BuCE and ACE[44].

Compound	X	BuCE						ACE					
		a_c, min^{-1}	K_M, M mol $litre^{-1}$	a_c/K_M, litre mol^{-1} min^{-1}	p	K_M^*, M mol $litre^{-1}$	K_b, M mol $litre^{-1}$	a_c, min^{-1}	K_M, M mol $litre^{-1}$	a_c/K_M, litre mol^{-1} min^{-1}	p	K_M^*, M mol $litre^{-1}$	K_b, M mol $litre^{-1}$
(XX)	$\overset{+}{N}(CH_3)_3$ (AX)	$6.0\cdot10^4$	$5.0\cdot10^{-4}$	$1.2\cdot10^8$	1	$5\cdot10^{-4}$	—	$3.0\cdot10^5$	$2\cdot10^{-4}$	$15\cdot10^8$	1	$2.0\cdot10^{-4}$	—
(XXI)	$\overset{+}{N}(CH_3)_2C_2H_5$	$6.0\cdot10^4$	$3.3\cdot10^{-4}$	$1.8\cdot10^8$	1	$3.3\cdot10^{-4}$	—	$3;1\cdot10^5$	$2\cdot10^{-4}$	$15\cdot10^8$	1	$2\cdot10^{-4}$	—
(XXII)	$\overset{+}{N}(CH_3)_2C_3H_7$	$1.9\cdot10^4$	$3.7\cdot10^{-4}$	$0.5\cdot10^8$	0.12	$12\cdot10^{-4}$	$5.4\cdot10^{-4}$	$2.2\cdot10^5$	$1.8\cdot11^{-4}$	$12\cdot10^8$	0.73	$2.5\cdot10^{-4}$	$6.7\cdot10^{-4}$
(XXIII)	$\overset{+}{N}(CH_3)_2C_4H_9$	$2.2\cdot10^4$	$2.3\cdot10^{-4}$	$1.0\cdot19^8$	0.37	$6.2\cdot10^{-4}$	$3.7\cdot10^{-4}$	$1.6\cdot10^5$	$3.8\cdot10^{-4}$	$4\cdot10^8$	0.54	$6.9\cdot10^{-4}$	$8\cdot10^{-4}$
(XXIV)	$\overset{+}{N}(CH_3)_2C_5H_{11}$	$4.8\cdot10^4$	$2.8\cdot10^{-4}$	$1.7\cdot10^8$	0.8	$3.5\cdot10^{-4}$	$14\cdot10^{-4}$	$1.6\cdot10^5$	$1.4\cdot10^{-4}$	$11\cdot10^8$	0.54	$2.6\cdot10^{-4}$	$3.1\cdot10^{-4}$
(XXV)	$\overset{+}{N}(CH_3)_2CH(CH_3)_2$	$1.9\cdot10^4$	$4.1\cdot10^{-4}$	$0.5\cdot10^8$	0.32	$13\cdot10^{-4}$	$6\cdot10^{-4}$	$2.5\cdot10^5$	$1.6\cdot10^{-4}$	$16\cdot10^8$	0 85	$1.9\cdot10^{-4}$	$10\cdot10^{-4}$
(XXVI)	$\overset{+}{N}(C_2H_5)_2CH_3$	$4.5\cdot10^4$	$4.4\cdot10^{-4}$	$1.0\cdot10^8$	0.75	$5.9\cdot10^{-4}$	$18\cdot10^{-4}$	$2.6\cdot10^5$	$1.7\cdot10^{-4}$	$15\cdot10^8$	0.87	$2.0\cdot10^{-4}$	$13\cdot1^{-4}$
(XXVII)	$\overset{+}{N}(C_2H_5)_3$	$2.1\cdot10^4$	$2.9\cdot10^{-4}$	$0.7\cdot10^8$	0.35	$8.3\cdot10^{-4}$	$4,5\cdot10^{-4}$	$1,9\cdot10^5$	$2.6\cdot10^{-4}$	$7\cdot10^8$	0.64	$4.1\cdot10^{-4}$	$7.2\cdot10^{-4}$
(XXVIII)	$\overset{+}{N}(C_3H_7)_2CH_3$	$0.3\cdot10^4$	$12\cdot10^{-4}$	$0.02\cdot10^8$	0.05	$240\cdot10^{-4}$	$13\cdot10^{-4}$	$0.32\cdot10^5$	$9\cdot10^{-4}$	$0.4\cdot10^8$	0.11	$82\cdot10^{-4}$	$10\cdot10^{-4}$
(XXIX)	$\overset{+}{N}(C_4H_9)_2CH_3$	0	$1\cdot10^{-3}$ [a]	—	0	—	—	—	$1\cdot10^{-4}$ [a]	—	0	—	—

Note. K_M^* is the true Michaelis constant, K_b the equilibrium dissociation constant of the inactivated complex, and p a coefficient defining the fraction of productive binding of the substrate in enzyme hydrolysis.

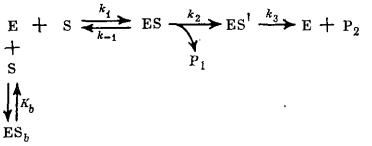
[a] The reversible inhibition constant K_i is quoted.

The resulting contradiction can be readily resolved on the hypothesis of productive and non-productive binding[45-47]. According to this hypothesis, enzyme hydrolysis can occur only for the correct orientation of the substrate molecule on the active surface of the enzyme, i.e. under the conditions of productive binding where the carbonyl carbon atom of the substrate occupies a rigorously defined position relative to the serine hydroxyl, which attacks this carbon atom.

Evidently the probability of the correct orientation is not sufficiently high for all the AC analogues. If, following the binding of the substrate, its carbonyl carbon atom is not in the immediate vicinity of the serine hydroxyl, hydrolysis cannot occur. Such binding with an incorrect orientation is referred to as non-productive binding.

Taking into account non-productive binding, the scheme for cholinesterase hydrolysis becomes:

Scheme 3



where K_b is the equilibrium dissociation constant of the inactive complex ES_b. Scheme 3 leads to a modification of the Michaelis–Menten equation:

$$v=\frac{a_c[E]_0[S]}{K_M+[S]}=\frac{(k_2+k_3)/(k_2\cdot k_3)\cdot K_b/(K_b+K_M^*)[E]_0[S]}{(K_M^*K_b/(K_b+K_M^*))+[S]}=\frac{a_c^*p[E]_0[S]}{K_M^*p+[S]}\quad\ldots,\quad(5)$$

where K_M and a_c are the Michaelis constant and the activity of the catalytic centre, which are determined experimentally, K_M^* and a_c^* are the true values of these parameters expressed by Eqns.(1c) and (1b), and p is a coefficient which defines the fraction of the productive binding of the substrate in the enzyme hydrolysis:

$$p=\frac{[ES]+[ES']}{[ES]+[ES']+[ES_b]}=\frac{K_b}{K_b+K_M^*}\quad.\qquad(6)$$

If $K_b > K_M^*$, then $[ES_b] \to 0$ and $p = 1$, so that $a_c = a_c^*$ and $K_M = K_M^*$. In this case there is no non-productive binding and each substrate molecule sorbed on the active centre hydrolyses. On the other hand, if $K_b < K_M$, non-productive binding mainly takes place: $p = K_b/K_M^* \to 0$, $a_c = a_c p \to 0$, and under these conditions the ester is a reversible inhibitor and not a substrate. Probably at equal enzyme concentrations $[E]_0$ the differences between the experimental values of $a_c = a_c p$ [see Eqn.(5)] for the AC analogues are related mainly to the different values of the coefficient p; on the other hand, the true catalytic activities a_c for these substances should be the same for the reasons indicated above. In this case the ratio of the values of a_c for two substrates should be equal to the ratio of the coefficients p:

$$a_{c;1}/a_{c;2}=a_{c;1}^*p_1/a_{c;2}^*\cdot p_2=p_1/p_2\quad.\qquad(7)$$

The value of p for each AC analogue with a modified composition of the ammonium group can be estimated assuming that $p = 1$ in the enzyme hydrolysis of AC neglecting thereby the non-productive binding of AC on both ACE and BuCE:

$$p=a_c/a_c(AX)\quad.\qquad(8)$$

The values of p found for the acetates investigated make it possible to calculate for them the K_b and the true Michaelis constants K_M^*:

$$K_M^*=K/p\quad,\qquad(9)$$

$$K_b=K_M^*p/(1-p)\quad.\qquad(10)$$

The ratio a_c/K is of fundamental importance for the kinetics of enzymic reactions. Firstly, it does not depend on the non-productive sorption and, when account is taken of Eqns.(8), (9), and (10), we have

$$a_c/K_M=a_c^*\cdot p/K_M^*p=a_c^*/K_M^*=\frac{k_2k_3k_1(k_2+k_3)}{(k_2+k_3)(k_{-1}+k_2)k_3}=k_1\frac{k_2}{k_{-1}+k_2}\quad.\qquad(11)$$

Secondly, this ratio makes it possible to estimate the rate constant for the formation of the Michaelis complex—since $k_2/(k_{-1} + k_2) < 1$, it follows from Eqn.(11) that $k_1 > a_c/K$.

Thirdly, the ratio a_c/K_M characterises the rate of reaction for $[S] \ll K_M$, whereupon Eqn.(1) becomes

$$v = \frac{a_c}{K_M} \cdot [E]_0 [S] .$$

Calculations have shown that the markedly lower a_c for the butyrylcholinesterase hydrolysis of the AC analogues investigated compared with the acetylcholinesterase hydrolysis is associated with a more significant alteration of p. If compound (XXII) is excluded, then a_c is reduced by a factor less than two in experiments with ACE for all other AC analogues, while in experiments with BuCE the reduction is by a factor of three. When account was taken of non-productive binding, the true Michaelis constants K_M^* increased appreciably. In most cases they are inversely proportional to the ratios a_c/K_M, which, as stated above, characterise to a first approximation the rate constant for the productive binding of the substrate to the enzyme (k_1). Indeed substrates with the highest K_M^* are characterised by the lowest ratios a_c/K: (XII) and (XXV) in experiments with BuCE and (XXIII) and (XXVII) in experiments with ACE. The observed correlation between K_M^* and a_c/K_M shows that the changes in the true Michaelis constants K_M^* for the AC analogues investigated are associated mainly with the changes in k_1, while the rate constants for other steps in enzyme hydrolysis, namely k_2 and k_3, and possibly also k_{-1}, do not vary[45-47].

Compound (XXVIII), which can be logically regarded as an inhibitor, is not an exception. It is characterised by the highest K_M^* and the lowest ratio a_c/K_M in view of the extremely small contribution of productive sorption. On the basis of the idea of productive and non-productive binding, the calculated K_b should be smallest for substrates with the smallest p. Such a relation has been traced in relation to many AC analogues in experiment with BuCE: compounds (XXIV) and (XXVI) have the highest values of p and the highest K_b, while compounds (XXII), (XXIII), and (XXVII) have the lowest p and K_b. In experiments with ACE this relation is less clear-cut. The observed scatter of the calculated data is associated, firstly, with the assumption that $p = 1$ for AC and, secondly, with the inevitable errors in the experimental determination of V and K_M, particularly for ACE, whose catalytic activity is suppressed by high substrate concentrations. The question arises of the cause responsible for the alteration of the contribution of productive binding for the AC analogues investigated. In the cholinesterase hydrolysis of choline esters, the sorption of the latter is ensured in the first place by the ion–ion interaction of the ammonium group of the substrate with the anionic site of the CE. Furthermore, the hydrophobic interaction of the hydrocarbon groups of the "cationic head" of the substrate with the hydrophobic region surrounding the anionic centre plays a significant role.

The complementarity of the substrate molecule in relation to the active surface of the enzyme, which is ultimately responsible for productive sorption, depends in general on the extent to which the ion–ion, ion–dipole, and hydrophobic interactions are concerted. When the interactions are concerted, the substrate molecule occupies a position on the active centre such that the necessary conditions are created for the formation of a reactive Michaelis complex (see Scheme 1). In such productive binding of the substrate, the activation of the serine hydroxyl, which executes a nucleophilic attack on the carbonyl carbon atom, is ensured.

When the above interactions of the substrate molecule with the active surface of the enzyme are not concerted, conditions, for the formation of the Michaelis complex are not created. An enzyme–substrate complex ES_b, inert with respect to the reaction, is formed instead.

In the light of this hypothesis, the enhancement of the hydrophobic interaction as a result of the introduction of more complex hydrocarbon groups into the substrate molecule should not always result in an increased contribution of the productive binding of the substrate to the enzyme. For example, in experiments with BuCE the parameter a_c decreases respectively by a factor greater than two, 2.7, and only 1.3 when the methyl group in the AC molecule is replaced by a propyl group [compound (XXIV)], by a butyl group [compound (XXV)], and by a pentyl group [compound (XXVI)].

Theoretical conformational analysis showed that such an unusual alteration of a_c on gradual substitution of one methyl group at the onium nitrogen atom by more complex groups is associated with the alteration of the steric configuration of the substrate molecules occurring under these conditions. It has been shown that even a slight change in the ammonium group alters the number of stable conformations, among which only some are compatible with the active surface of the enzyme. Hence the change in the contribution of the non-productive sorption of the substrate is associated with the change in the quantitative ratio of the conformers, both complementary and non-complementary, in relation to the active surface of the enzyme[48].

III. ESTERS WITH A CYCLIC AMMONIUM GROUP IN THE ALCOHOL COMPONENT OF THE MOLECULE

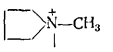
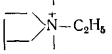
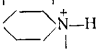
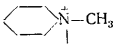
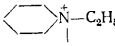
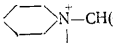
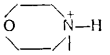
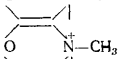
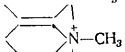
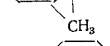
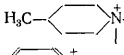
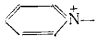
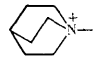
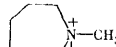
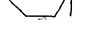
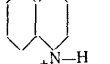
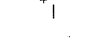
Since 1939[18] numerous esters of this kind have been investigated. Among them, extremely valuable cholinesterase substrates have been discovered. This applies in the first place to the chomogen and fluorogen substrates, which differ significantly in their optical properties from their hydrolysis products[49]. The use of these substrates makes it possible to determine the activities of cholinesterases with a high accuracy even at very low enzyme and substrate concentrations.

This section deals with two types of acetates of cyclic aminoalcohols: esters in which the acetoxyethyl group is linked to an onium nitrogen [$CH_3C(O)OCH_2CH_2\overset{+}{N}$] and esters in which the acetoxy-group is linked to rings containing an onium nitrogen atom. In addition, certain thio-derivatives are also considered.

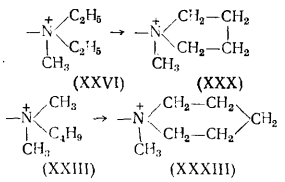
1. Esters Having the General Formula $CH_3C(O)OCH_2CH_2\overset{+}{N}$

These esters can be regarded as AC analogues. Their study was begun comparatively recently[47]. The compounds listed in Table 4 have been synthesised and the kinetics of their cholinesterase hydrolysis have been investigated by large teams of chemists and biochemists at the Institute of Bio-organic Chemistry of the Academy of Sciences of the Uzbek SSR, the Institute of Experimental Medicine of the Academy of Medical Sciences of the USSR, and the Sechenov Institute of Evolutionary Physiology and Biochemistry of the USSR Academy of Sciences[4,36,44,47,50-64]. Compounds (XXX)–(XXXIII) are of special interest, because the effect of the cyclisation of the ammonium group can be demonstrated in

Table 4. Kinetic parameters of the reactions of the cyclic aminoalcohol acetates $CH_3C(O)OC_2H_4X$ with BuCE and ACE.

Compound	X	BuCE			ACE		
		a_c, min^{-1}	K_M, mol $litre^{-1}$	a_c/K_M, litre $mol^{-1}min^{-1}$	a_c, min^{-1}	K_M, mol $litre^{-1}$	a_c/K_M, litre $mol^{-1}min^{-1}$
(XXX)		$1.3\cdot10^4$	$1.7\cdot10^{-4}$	$7.7\cdot10^7$	$2.5\cdot10^5$	$2.6\cdot10^{-4}$	$1.0\cdot10^9$
(XXXI)		$3.2\cdot10^4$	$2.5\cdot10^{-4}$	$1.3\cdot10^8$	$2.5\cdot10^5$	$1.8\cdot10^{-4}$	$1.4\cdot10^9$
(XXXII)		0	—	—	0	—	—
(XXXIII)		$3.0\cdot10^3$	$4.0\cdot10^{-5}$	$7.5\cdot10^7$	$1.9\cdot10^5$	$5.0\cdot10^{-4}$	$3.8\cdot10^8$
(XXXIV)		$2.0\cdot10^4$	$1.8\cdot10^{-4}$	$1.1\cdot10^8$	$1.6\cdot10^5$	$5.0\cdot10^{-4}$	$8.0\cdot10^8$
(XXXV)		$3.9\cdot10^4$	$3.3\cdot10^{-4}$	$1.2\cdot10^8$	$9.0\cdot10^4$	$3.3\cdot10^{-4}$	$2.7\cdot10^8$
(XXXVI)		0	—	—	0	—	—
(XXXVII)		$4.1\cdot10^3$	$8.0\cdot10^{-4}$	$5.1\cdot10^6$	$1.4\cdot10^5$	$6.7\cdot10^{-4}$	$2.1\cdot10^8$
(XXXVIII)		$3.1\cdot10^3$	$3.4\cdot10^{-4}$	$9.0\cdot10^6$	$1.1\cdot10^5$	$7.0\cdot10^{-4}$	$1.6\cdot10^8$
(XXXIX)		$1.2\cdot10^4$	$1.8\cdot10^{-4}$	$6.7\cdot10^7$	$1.9\cdot10^5$	$1.5\cdot10^{-3}$	$1.3\cdot10^8$
(XL)		0	—	—	0	—	—
(XLI)		0	—	—	0	—	—
(XLII)		$4.0\cdot10^3$	$8.0\cdot10^{-4}$	$5.0\cdot10^6$	$1.4\cdot10^5$	$6.7\cdot10^{-4}$	$2.1\cdot10^8$
(XLIII)		0	—	—	0	—	—
(XLIV)		$1.6\cdot10^3$	$2.1\cdot10^{-4}$	$7.5\cdot10^6$	$3.2\cdot10^4$	$1.2\cdot10^{-4}$	$2.6\cdot10^8$
(XLV)		$1.8\cdot10^3$	$2.8\cdot10^{-5}$	$6.5\cdot10^7$	$1.7\cdot10^4$	$3.0\cdot10^{-5}$	$5.6\cdot10^8$
(XLVI)		$1.7\cdot10^3$	$2.2\cdot10^{-5}$	$7.2\cdot10^7$	$1.5\cdot10^4$	$3.1\cdot10^{-5}$	$4.9\cdot10^8$
(XLVII)		$6.0\cdot10^2$	$1.2\cdot10^{-4}$	$5.0\cdot10^6$	$7.4\cdot10^3$	$1.2\cdot10^{-4}$	$6.0\cdot10^7$
(XLVIII)		0	—	—	0	—	—

relation to them:



(XXVI) (XXX)

(XXIII) (XXXIII)

These processes are accompanied by a sharp decrease of a_C for BuCE but hardly affect the reactions with ACE. The expansion from the five-membered ring in compound (XXX) to the seven-membered ring in compound (XLII) is accompanied by a gradual decrease of a_C by a factor of 3–4 for BuCE and less than two for ACE. The data presented demonstrate as a whole a greater sensitivity of BuCE to changes in the structure of the ammonium group. This conclusion is consistent with that based on the study of the substrates (XX)–(XXIX) in Table 3.

The introduction of a methyl group in the α-position of the piperidine ring [compound (XXXVIII)] reduces a_C for ACE, while the introduction of a methyl group in the γ-position [compound (XXXIX)] increases a_C for BuCE. On the other hand, the derivative with a pyridinium group in the α-position [the anabasine derivative (XLVIII)] is not hydrolysed by these enzymes. The replacement of the pyridinium group by the piperidinium group [compound (XL)] or by the rigid quinuclidinium group (XLI) results in a complete loss of susceptibility to hydrolysis by cholinesterases.

The increase in the bulk of the cyclic ammonium group [compounds (XLIV), (XLV), (XLVI), and (XLVII)] is accompanied by an appreciable decrease of a_C for ACE and BuCE. There is little difference between the substrate properties of the piperidinium compound and the more hydrophilic morpholinium compound (XXXVII). The *N*-ethyl derivatives (XXXI) and (XXXIV) are hydrolysed by BuCE much more effectively than their *N*-methyl analogues.

The constants K_M for the substrates investigated vary: by a factor of 35 for BuCE [compounds (XLII) and (XLVI)] and by a factor of 50 for ACE [compounds (XXXIX) and (XLV)]. As pointed out above, the constants K_M do not by themselves characterise the susceptibility of substrates to hydrolysis by cholinesterases. They are important in estimates of this susceptibility at low substrate concentrations, where the rate of hydrolysis is determined by the quantity a_C/K_M. It is striking that compound (XLVII), with the smallest values of a_C for these enzymes, has also the smallest value of a_C/K_M and that the maximum value of a_C/K_M corresponds to the maximum value of a_C: compound (XXXI) for ACE and compound (XXXV) for BuCE.

The fact that the tertiary analogues of the cyclic esters (XXXII), (XXXVI), and (XLIII) are not hydrolysed by ACE and BuCE is of special interest. Here it is appropriate to recall that dimethylaminoethyl acetate, a tertiary analogue of AC, is hydrolysed 1.5–3 times more slowly by BuCE and ACE than AC. According to the results of theoretical conformational analysis[65], the cyclic conformation of dimethylaminoethyl acetate with the C=O...H–N hydrogen bond is the most favourable. As stated above, the formation of a hydrogen bond between the carbonyl oxygen of AC and the imidazole ring of histidine in the active centre of the enzyme plays an important role in cholinesterase catalysis and such cyclic conformers are not therefore hydrolysed although their sorption on the catalytic surface is not ruled out.

As regards the lack of susceptibility of the cyclic aminoalcohol tertiary esters (XXXII), (XXXVI), and (XLII) to cholinesterase hydrolysis, conformational calculations have shown that the structure of the acetoxyethyl component in the equatorial position relative to the nitrogen-containing ring is energetically favourable. This less compact conformation, compared with the axial conformation, should suffer from steric hindrance by the hydrophobic groups surrounding the anionic centre of the enzyme, which is an additional cause whereby one can explain their lack of susceptibility to hydrolysis by cholinesterases.

2. Esters Having the General Formula $RC(O)S(CH_2)_2R$

It follows from Table 2 that the replacement of the ester oxygen by sulphur in the choline substrate molecules does not entail a significant change in their susceptibility to cholinesterase hydrolysis. Large changes in such susceptibility are observed when O is replaced by S in acetyl-β-methylcholine (AMC). Whereas AMC itself is hydrolysed by BuCE at a very low rate ($a_C = 3 \times 10^3$ min^{-1}), its thiol analogue (AMTC) is hydrolysed by this enzyme 20 times faster ($a_C = 6.5 \times 10^4$ min^{-1}).[28] The parameter a_C for ACE increases only by a factor of two (1.0×10^5 and 2.1×10^5 min^{-1} respectively)[28]. The replacement of O by S in the AMC molecule therefore results in a sharp decrease of specificity: the ratios a_C(ACE)/a_C(BuCE) for AMC and AMTC are respectively 30 and 3.

It was therefore of great interest to compare the substrate properties of the oxygen and thiol analogues of esters with a cyclic ammonium group[63,66]. Table 5 lists the kinetic parameters of the cholinesterase hydrolysis of carboxylic acid thioesters. Two groups of compounds attract attention in the first place: the morpholine derivatives (XLIX)–(LII) and the α-pipecoline derivatives (LIII)–(LVI). Like the choline esters (Table 2), the acetates (XLIX) and (LIII) are hydrolysed at the highest rate by ACE. However, whereas BuTC is not hydrolysed by ACE, among the morpholinium derivatives the butyrate is hydrolysed, albeit 20 times more slowly than the acetate. Furthermore, the valerate is also hydrolysed at an appreciable rate. On the other hand, the change in the catalytic activity of BuCE following an increase in the bulk of the acyl component is different from that in the choline esters. Among the morpholinium derivatives, the propionate (L) is hydrolysed at the highest rate, while among the α-pipecolinium derivatives the rate of hydrolysis of the acetate (LII) is highest. Hence it follows that the dependence of the susceptibility of esters to cholinesterase hydrolysis as a function of the increase in the bulk of the acyl component of the molecule depends on the nature of the onium group.

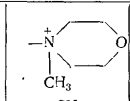
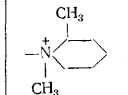
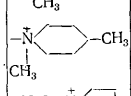
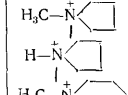
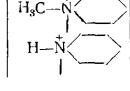
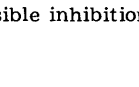
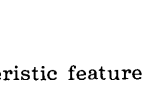
The hydrolysis by both enzymes of the protonated forms of the bases of the cyclic aminoalcohol esters (LX) and (LXII), in contrast to their oxygen analogues (see above), is of interest. Comparison of the values of a_C listed in Table 5 for the thioesters and those for their oxygen analogues (Table 4) reveals a definite regularity: when O is replaced by S, the rate of hydrolysis by BuCE either does not change or increases. The thio-substrates investigated therefore exhibit a lower specificity in relation to ACE. As shown below (Table 8), the replacement of O by S, as in the substrates (XXXII) and (XXXVII) resulted in a sharp decrease of specificity.

3. Esters of Cation-Containing Heterocycles

Table 6 lists the available comparative literature for the acetoxy-derivatives of ammonium-containing heterocycles as BuCE and ACE substrates. In contrast to all the compounds described above, the acyl residue in these esters is linked to

the onium nitrogen via a ring. The pyridinium derivatives (XXX)–(XXXV) are of great interest[67]. Despite the significant structural differences indicated, the rule that BuCE is more sensitive to changes in the alcohol component of the ester compared with ACE is valid for these compounds too. Thus a change in the position of the acetoxymethyl group in the methylated derivatives (XXX)–(XXXII) reduces sharply the rate of enzyme hydrolysis by BuCE but does not affect the hydrolysis by ACE. This was also observed for the ethylated derivatives (XXXIII) and (XXXIX). The high specificity of compound (XXXII) in relation to ACE is noteworthy.

Table 5. The interaction of the thioesters $RC(O)S(CH_2)_2R'$ with BuCE and ACE[63,66].

Compound	R	R′	BuCE		ACE	
			a_c, min^{-1}	K_M, mol litre^{-1}	a_c, min^{-1}	K_M, mol litre^{-1}
(XLIX)	CH_3		$6.6\cdot10^3$	$1.8\cdot10^{-4}$	$2.3\cdot10^4$	$6.0\cdot10^{-5}$
(L)	C_2H_5		$2.3\cdot10^4$	$8.9\cdot10^{-5}$	$1.6\cdot10^4$	$2.5\cdot10^{-4}$
(LI)	C_3H_7		$3.7\cdot10^3$	$7.8\cdot10^{-5}$	$1.2\cdot10^3$	$2.1\cdot10^{-4}$
(LII)	C_4H_9		$1.9\cdot10^3$	$1.9\cdot10^{-4}$	$8.8\cdot10^2$	$6.7\cdot10^{-4}$
(LIII)	CH_3		$3.6\cdot10^3$	$4.8\cdot10^{-4}$	$2.9\cdot10^4$	$2.4\cdot10^{-5}$
(LIV)	C_2H_5		$1.8\cdot10^3$	$4.8\cdot10^{-4}$	$4.4\cdot10^2$	$2.5\cdot10^{-4}$
(LV)	C_3H_7		$2.2\cdot10^2$	$8.3\cdot10^{-5}$	0	$1.4\cdot10^{-5}$ *
(LVI)	C_4H_9		0	$1.6\cdot10^{-3}$ *	0	$1.8\cdot10^{-5}$ *
(LVII)	CH_3		$1.1\cdot10^4$	$7.1\cdot10^{-5}$	$1.3\cdot10^4$	$7.4\cdot10^{-5}$
(LVIII)	C_2H_5		$2.5\cdot10^4$	$6.3\cdot10^{-5}$	$8.5\cdot10^3$	$4.0\cdot10^{-4}$
(LIX)	CH_3		$1.8\cdot10^4$	$5.3\cdot10^{-5}$	$1.4\cdot10^5$	$3.3\cdot10^{-5}$
(LX)	CH_3		$2.8\cdot10^3$	$1.2\cdot10^{-4}$	$7.0\cdot10^4$	$4.3\cdot10^{-4}$
(LXI)	CH_3		$4.4\cdot10^3$	$2.8\cdot10^{-5}$	$4.2\cdot10^4$	$2.4\cdot10^{-4}$
(LXII)	CH_3		$4.8\cdot10^2$	$2.4\cdot10^{-3}$	$1.4\cdot10^4$	$3.0\cdot10^{-4}$

* The reversible inhibition constant K_i is quoted.

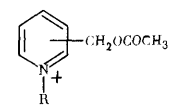
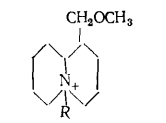
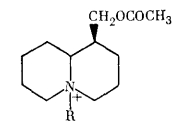
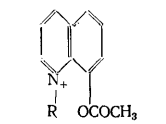
A characteristic feature of the lupinine and epilupinine derivatives (XXXVI) and (XXXVII) is that both cholinesterases are insensitive to epimeric transformations, but ACE hydrolyses these esters faster than BuCE[52,68].

Quinolinium derivatives have found extensive applications in laboratory practice. Table 6 presents data solely for 8-acetoxyquinolinium (XXXVIII)[69]. Other derivatives have been investigated only with ACE from the electric organ of *Torpedo mormorato* [? *Electrophorus electricus*][70,71]. The rate of hydrolysis of compound (XXXVIII) can be determined with a high accuracy by colorimetric and fluorimetric methods. It is hydrolysed by ACE at an even higher rate than AC.

IV. ESTERS OF ALIPHATIC AND AROMATIC ALCOHOLS

As early as 1949, it was established that cholinesterases are capable of hydrolysing not only the esters of cationoid alcohols but also the carboxylic acid esters of aliphatic alcohols[72–74]. Although the rates of hydrolysis were low, this fact was of fundamental importance. As for choline esters, on increase of the length of the acyl component of the molecule from the acetate to the butyrate, their rate of hydrolysis by ACE decreases while the rate of hydrolysis by BuCE increases. As regards the alcohol component, the carbon chain made up of four atoms is optimal for both enzymes. The branching of the alkyl chain has a major influence. The most complementary alkyl group for both cholinesterases is the 3,3-dimethylbutyl group, which is regarded as a carbon analogue of choline.

Table 6. The relative rates of hydrolysis v of the acetoxy-derivatives of certain ammonium-containing heterocycles by BuCE and ACE[72–74].

Compound	R	Position of CH_3OCOCH_3	Heterocycle	v*	
				BuCE	ACE
(LXIII)	CH_3	2		55	50
(LXIV)	CH_3	3		20	60
(LXV)	CH_3	4		3	50
(LXVI)	C_2H_5	2		125	35
(LXVII)	C_2H_5	4		10	35
(LXVIII)	C_3H_7	2		50	35
(LXIX) **	CH_3	—		7	21
(LXX)	CH_3	—		8	22
(LXXI)	CH_3			55	140

* The rate of hydrolysis of AC was adopted as 100.
** (LXIX) is lupinine, (LXX) is epilupinine, and (LXXI) is 8-hydroxyquinoline.

Carboxylic acid esters of aromatic hydroxy-compounds are frequently used in laboratory practice, namely those of phenol[75], nitrophenols, naphthols, and indole[76,77]. The esters of these aromatic alcohols are hydrolysed at a higher rate by cholinesterases. A whole series of resorufin and anthracene derivatives have been proposed for a highly sensitive fluorimetric method for the determination of cholinesterase activity[77].

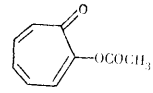
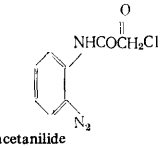
Carboxylic acid esters of indophenol and its halogeno-derivatives occupy a special place among non-cationoid substrates[4,78]. They are widely used in colorimetric methods for the determination of the cholinesterase activity not only with the aid of instruments but also visually. This is because the esters have a pale-red colour and their hydrolysis products (indophenols) are intensely blue. It is striking that ACE hydrolyses only the unsubstituted indophenyl ester, while BuCE hydrolyses also the halogeno-derivatives.

All the esters enumerated in this Section suffer from an important disadvantage: in contrast to the choline esters, they are readily hydrolysed by other esterases. In order to determine the cholinesterase activity of biological tissues with the aid of such chromogenic substrates, one finds initially the overall esterase activity and then, after treating

the tissue with a specific CE inhibitor, the esterase activity is remeasured. The difference between these two measurements characterises the activity of cholinesterases.

It should be noted particularly that certain types of cholinesterases do not hydrolyse all chromogenic substrates: the CE from the nerve ganglia of the cockroach do not hydrolyse *p*-nitrophenyl acetate, which is used to determine the activities of the carboxy-esterases of this insect[79].

Table 7. The hydrolysis of new non-cationogenic substrates by the ACE from the electric organ of *Torpedo mormorato* [?*E. electricus*].

Compound	Substrate	a_c, min^{-1}	K_M, mol $litre^{-1}$
(LXXII)	AC	$9.8 \cdot 10^5$	0.4
(LXXIII)	vinyl acetate $CH_2 = CHOC(O)CH_3$	$10.5 \cdot 10^5$	67.0
(LXXIV)	but-1-enyl acetate $CH_3CH_2CH = CHOC(O)CH_3$	$5.7 \cdot 10^5$	1.1
(LXXV)	propylidene diacetate $CH_3CH_2CH(OCOCH_3)_2$	$6.0 \cdot 10^5$	1.7
(LXXVI)	benzylidene diacetate $C_6H_5CH(OCOCH_3)_2$	$6.6 \cdot 10^5$	1.5
(LXXVII)	 tropolone acetate	$10.2 \cdot 10^5$	0.55
(LXXVIII)	 *o*-nitrochloroacetanilide	$1.6 \cdot 10^4$	2.5
(LXXIX)	*o*-nitrodichloroacetanilide	$1.2 \cdot 10^4$	0.6

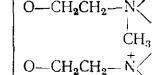
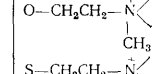
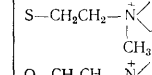
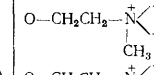
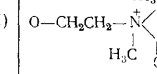
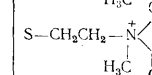
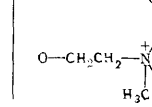
New unusual substrates of the ACE from the electric organ of *Torpedo mormorato* [? *Electrophorus electricus*] have been investigated recently[80]. Table 7 shows that these non-cationoid esters are hydrolysed by ACE a little less effectively than AC, while tropolone acetate (LXXVII) is actually more effectively hydrolysed. The alkylidene diacetates (LXXV) and (LXXVI), where the 2-acetoxy-groups are attached to a single carbon atom, are of interest. The usual dicarboxylic acid esters of dihydric alcohols are slowly hydrolysed by cholinesterases. For example, the hydrolysis of 9,10-diacetoxyanthracene by BuCE is much slower than that of 9-acetoxyanthracene, i.e. the second acetoxy-group interferes with the enzymic reaction[77]. In contrast to such dicarboxylic acid esters, alkylidene diacetates are hydrolysed by the ACE from the electric organ of *Torpedo mormorato* [? *Electrophorus electricus*] only 1.5 times more slowly than AC. One cannot rule out the possibility that the enzyme hydrolysis of the first acetoxy-group is followed by the spontaneous hydrolysis of the second. The possibility of the cholinesterase hydrolysis of the amide bond was demonstrated in the same study in relation to the non-cationoid acetanilides (LXXVI) and (LXXIX).

V. CONCLUSION

The data presented in this review indicate a limited specificity of the catalytic activity of ACE and BuCE. Both enzymes are capable of hydrolysing an extremely large number of choline and non-choline carboxylic acid esters. The difference between the specificities of these enzymes consists in the fact that ACE is more sensitive to the structure of the acid component of the esters, while BuCE is more sensitive to the structure of the alcohol component. It has been shown that ACE and BuCE are stereospecific in relation to optical isomers. Both enzymes are sensitive to the optical isomers of substrates in which the asymmetric carbon atom is in the β-position with respect to the choline component of the molecule. Furthermore, ACE is capable of distinguishing between the optical isomers of substrates in which the asymmetric carbon atom is in the acid component.

Apart from the specificity of the catalytic activity of cholinesterases, there exists also the concept of specific substrates. A specific substrate for a given enzyme is believed to be one whose rate of reaction under the influence of the given enzyme is greater by a factor of tens than the rate of its reaction under the influence of another enzyme. The specificity of the substrate can vary appreciably as a function of the enzymic process conditions: pH, ionic strength, and particularly substrate concentrations. As shown above, at high concentrations of the substrate its specificity with respect to the two cholinesterases is determined by the ratio $a_{c,1}/a_{c,2}$, while at low concentrations it is determined by the ratio $(a_c/K_M)_1/(a_c/K_M)_2$. Until recently only BuC and BeC for BuCE and AMC for ACE were believed to be specific substrates for these enzymes. The study of the acetates of cyclic aminoalcohols led to the discovery of several specific ACE substrates with a more pronounced selectivity (Table 8).

Table 8. The specificity of the acetates $CH_3C(O)X$ to ACE[48,81].

Compound	X	$\frac{a_c\ (ACE)}{a_c\ (BuCE)}$	$\frac{(a_c/K_M)\ (ACE)}{(a_c/K_M)\ (BuCE)}$
(XX)	$O-CH_2CH_2\overset{+}{N}(CH_3)_3$	5	12
ATC	$S-CH_2CH_2\overset{+}{N}(CH_3)_3$	2	11
(LXIII)	$O-CH(CH_3)CH_2\overset{+}{N}(CH_3)_3$	30	32
(LXIV)	$S-CH(CH_3)CH_2\overset{+}{N}(CH_3)_3$	3	10
(XXX)		20	15
(XXXIII)		65	5
(LXI)		10	1
(XXXVII)		50	30
(XXXVIII)		35	18
(LIII)		8	160
(XLII)		35	40

The most specific ACE substrates at high ester concentrations (in terms of the ratio of the parameters a_c) are compounds (XXXIII) and (XXXVII), for which the ratios are 65

and 50 and which are greatly superior to AMC as regards this parameter (the ratio of the a_C is 30).

The esters (XXXVII) and (XLII) proved to be highly specific ACE substrates at both high and low concentrations. Among the newly discovered specific substrates, the derivatives of piperidine (XXXIII) and morpholine (XXXVII) are widely used in laboratory practice.

As stated above, the thio-analogues of AC and AMTC exhibit a lower specificity in relation to ACE. This has been confirmed also for the piperidine derivatives (LII) and (LIII). As regards the α-pipecoline analogues (XXXVIII) and (LIII), the specificity fell only when their concentrations were high, while at low concentrations the specificity (the a_C/K_M ratio) increased sharply. The thioester (LXI) is so far the only thio-substrate which can be regarded as specific to ACE[63,66].

The data presented in this review, indicating an extremely high sensitivity of BuCE to changes in the structure of the ammonium group of the substrate, suggest that the dimensions of the anionic centre in BuCE are limited[48,81]. Presumably in the region of the anionic centre there is a pit whose shape and dimensions determine the limiting structures of the cationic group of such a substrate for which hydrolysis is still possible. Theoretical conformational analysis of AC derivatives with different structures of the ammonium group has shown that the substrate molecule exists in the form of a mixture of conformations separated as a rule by high potential barriers. If the substrate interacting with BuCE has a conformation whose dimensions exceed the limiting size of the pit in the anionic centre, non-productive sorption of the substrate takes place. The conformations of the ester in which the cationic group fits within the limits of the anionic centre of BuCE are susceptible to hydrolysis. Accordingly, the conformations of the same substrate can be divided into productive and non-productive. The analysis carried out here has demonstrated a very satisfactory correlation between the relative populations of the productive conformations of substrates and the rate of hydrolysis by BuCE.

At first sight it would seem that an increase in the size of the *N*-alkyl group should hinder sorption in the region of the anionic centre and hence reduce the rate of hydrolysis by BuCE. However, according to the results of theoretical conformational analysis, conformers with a more compact packing of the alkyl groups than in the propyl derivative (XXII) are characteristic of the pentyl derivative (XXIV).

Thus it is possible to explain the "anomalous" variation of the rate of hydrolysis in the sequence (XX), (XXII), and (XXIV); compound (XXII) is hydrolysed less effectively by factors of 3 and 2.5 than compounds (XX) and (XXIV) respectively. This can also be said of the *N*-alkylpiperidinium derivatives (XXXII), (XXXIV), and (XL), among which the isopropyl analogue (XL) proved to be more compact than the methyl derivative (XXXIII).

This new approach leads to the possibility of investigating the characteristics of the structure of the catalytic surface of cholinesterases by substrate analysis and permits direct recommendations for the synthesis of highly specific substrates.

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