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THE IMPORTANCE OF STERIC AND STEREOCHEMICAL FEATURES IN SERUM CHOLINESTERASE SUBSTRATES

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Abstract—Rates of hydrolysis of the optical isomers of the butyryl-α and β-methylcholines of known configuration have been determined by using purified horse serum cholinesterase, horse and human serum†.

Inhibition of serum esterase activity toward butyrylcholine as substrate by using benzoylcholine, *p*-aminobenzoylcholine and the opticali somers of *p*-aminobenzoyl-α and β-methylcholine is described.

The similarity between horse and human serum esterase is discussed and a probable common identity is suggested for the active sites in the mixture of enzymes of serum cholinesterase of a given species.

ROUTINE use of succinylcholine during surgery has led to an interest in the structure of the enzyme pseudocholinesterase (3.1.1.8 acylcholine acylhydrolase) which is responsible for the *in vivo* hydrolysis of this drug. The discovery of genetically determined variants of the enzyme,[1–3] having modified hydrolytic activities, has further increased interest in the nature of this enzyme.

Optically active substrates and inhibitors are increasingly used as sensitive tools with which to map the area in the immediate vicinity of an active site. Simonart[4] early reported the different inhibitory activities of the enantiomorphs of miotine toward pseudocholinesterase, and more recently[5] it has been shown that the optical isomers of acetyl-α and β-methylcholine are hydrolysed by acetylcholinesterase at different rates. It was therefore decided to investigate the rate of hydrolysis of the optical isomers of butyryl-α and β-methylcholine by pseudocholinesterase.

The suitability of both butyrylcholine (BuCh) and benzoylcholine (BzCh) as substrates for pseudocholinesterase was investigated, and BzCh was shown to possess marked inhibitory properties. The *p*-aminobenzoyl-α and β-methylcholines were prepared and the relative inhibitor potency of the optically active forms investigated.

METHODS

Materials

All substrates and inhibitors investigated during the course of this study were prepared in the form of their iodides in these laboratories.

* One of the authors (M.M) is indebted to the Pharmaceutical Society for a Research Scholarship during the tenure of which this work, which forms part of a Ph.D. thesis of the University of London, was carried out.

† Typical human serum.

Acetylcholine (ACh)	m.p. 165–166°
Butyrylcholine (BuCh)	m.p. 92°
DL-Butyryl-α-methylcholine (Bu-α-MeCh)	m.p. 75–76°
DL-Butyryl-β-methylcholine (Bu-β-MeCh)	m.p. 154–155°
D-Butyryl-α-methylcholine	m.p. 76–77°
L-Butyryl-α-methylcholine	m.p. 77°
D-Butyryl-β-methylcholine	m.p. 197–198°
L-Butyryl-β-methylcholine	m.p. 198–199°
Benzoylcholine (BzCh)	m.p. 251° (dec)
p-Aminobenzoylcholine (PABCh)	m.p. 258°
DL-*p*-Aminobenzoyl-α-methylcholine (PAB-α-MeCh)	m.p. 185–186°
DL-*p*-Aminobenzoyl-β-methylcholine (PAB-β-MeCh)	m.p. 187–188°
D-*p*-Aminobenzoyl-α-methylcholine	m.p. 179°
L-*p*-Aminobenzoyl-α-methylcholine	m.p. 179°
D-*p*-Aminobenzoyl-β-methylcholine	m.p. 148–150°
L-*p*-Aminobenzoyl-β-methylcholine	m.p. 148–150°

Horse serum cholinesterase was purified by the method of Strelitz[6] (the last two stages being omitted) and stored as a freeze-dried powder at 0°. The preparation was characterised by its substrate (BuCh, BzCh, acetylcholine (ACh) and tributyrin) and inhibitor Nu 1250† (*N*-*p*-chlorophenyl-*N*-methylcarbamate of m-hydroxyphenyltrimethylammonium bromide) and Nu 683* (dimethylcarbamate of (2-hydroxy-5-phenylbenzyl) -trimethylammonium bromide) specificity.

In both horse and human serum studies, serum was obtained from single individuals and not pooled from different sources. Human serum known to have normal serum esterase activity† was used.

Experimental methods

The hydrolysis and inhibitor studies were carried out using the manometric technique described elsewhere.[7]

A preliminary investigation established that a purified serum esterase (PCE) concentration of 6 μg in 100 ml gave a satisfactory degree of hydrolytic activity, 1·5 ml of enzyme solution in a total volume of 3 ml hydrolysing 6 μmole of BuCh (10^{-2}M) in 30 min. In each case $V_{max.}$ was determined over the concentration range 5×10^{-4}–5×10^{-2}M.

During the inhibitor studies, the inhibitor was preincubated with the enzyme for a period of 30 min at 38° prior to mixing the flask contents. For each substrate (BuCh) concentration (1·25, 2·5 and $4{\cdot}2 \times 10^{-2}$M) investigated, three inhibitor concentrations (1, 2 and 5×10^{-4}M) were used.

RESULTS

Substrate studies

The rates of hydrolysis of BuCh, ACh and BzCh by the purified horse serum esterase were determined for a range of substrate concentrations, and the substrate characteristics of this enzyme preparation are presented in Table 1.

* The human serum was kindly typed by Professor H. Harris, Kings College, London University.

† Nu1250 and Nu683 were supplied by Hofmann La Roche.

TABLE 1. THE SUBSTRATE CHARACTERISTICS OF THE HORSE SERUM CHOLINESTERASE PREPARATION

Substrate	$V_{max.}$ μmole/30 min	K_m M/l.
BuCh	6·7	$1·6 \times 10^{-3}$
ACh	3·0	$2·0 \times 10^{-3}$
BzCh	0·65	$2·7 \times 10^{-4}$

The introduction of an α-methyl substituent into the choline moiety of the butyryl esters reduced the rate of enzymic hydrolysis slightly, but the presence of a β-methyl substituent dramatically reduced the rate as shown in Table 2.

TABLE 2. THE INFLUENCE OF α AND β-METHYL SUBSTITUTION ON THE RATE OF ENZYMIC HYDROLYSIS OF BUTYRYLCHOLINE

Substrate	$V_{max.}$ μmole/30 min	% (BuCh 100%)	K_m M × 10^3
DL-butyryl-α-methylcholine	4·6	69	1·4
D-butyryl-α-methylcholine	4·4	66	1·55
L-butyryl-α-methylcholine	5·3	81	1·3
DL-butyryl-β-methylcholine	0·24	3·5	2·8
D-butyryl-β-methylcholine	No detectable enzymic hydrolysis		
L-butyryl-β-methylcholine	0·29	4·5	1·4

The above determinations were repeated using freshly prepared (a) horse serum (1:50 dilution) and (b) human serum 1:50 dilution) as the enzyme source and the results are shown in Table 3.

TABLE 3. THE SUBSTRATE SPECIFICITY PATTERN OBTAINED USING FRESH HORSE SERUM AND HUMAN SERUM WITH SUBSTITUTED CHOLINE ESTERS

Substrate (3×10^{-2}M)	Horse serum μmole/30 min	%	Human serum μmole/30 min	%
BuCh	5·26	100	7·07	100
ACh	2·43	46	3·32	47
BzCh	0·69	13	1·11	15·5
DL-butyryl-α-methylcholine	4·24	81	5·82	82
D-butyryl-α-methylcholine	4·08	78	5·62	79
L-butyryl-α-methylcholine	4·86	93	6·58	93
DL-butyryl-β-methylcholine	0·20	4	0·82	5
D-butyryl-β-methylcholine	0·0	0	0·0	0
L-butyryl-β-methylcholine	0·29	5·5	0·55	5

Inhibitor studies

BzCh inhibited the enzymic hydrolysis of BuCh by the purified serum esterase preparation and the results are presented in Table 4. The inhibition characteristics of the *p*-aminobenzoylcholine analogues are presented in Table 5.

TABLE 4. THE RELATIVE INHIBITOR PROPERTIES OF BzCh AND NU683 ON THE HYDROLYSIS OF BuCh BY PCE

Substrate (3×10^{-2})	Inhibitor	Molar Ratio Substrate: Inhibitor	μ mole hydrolysed in 30 min	% inhibition
BuCh	—	—	8·93	—
BzCh	—	—	1·36	—
BzCh	Nu683	10^5:1	0·13	90
BuCh	BzCh	3 :1	1·34	85
BuCh	Nu683	3×10^5:1	0·98	85

TABLE 5. THE INHIBITOR CHARACTERISTICS OF *p*-AMINOBENZOYLCHOLINE AND ITS OPTICALLY ACTIVE METHYL CHOLINE ANALOGUES ON THE HYDROLYSIS OF BuCh BY PCE

Inhibitor	(S) M $\times 10^2$	I_{50} M $\times 10^4$	K_i M/l.
p-aminobenzoylcholine	1·25	1·0	$3{\cdot}5 \times 10^{-5}$
	2·5	1·3	
	4·2	17·5	
L-*p*-aminobenzoyl-α-methylcholine	1·25	0·4	2×10^{-5}
	2·5	0·6	
	4·2	0·9	
D-*p*-aminobenzoyl-α-methylcholine	1·25	1·25	6×10^{-5}
	2·5	1·5	
	4·2	2·1	
L-*p*-aminobenzoyl-β-methylcholine	1·25	3·75	$9{\cdot}5 \times 10^{-5}$
	2·5	5·0	
	4·2	13·0	
D-*p*-aminobenzoyl-β-methylcholine	1·25	36·0	1×10^{-3}
	2·5	62·5	
	4·2	80·0	

The rates of enzymic hydrolysis of all of the *p*-aminobenzoylcholines studied were extremely low at the concentration of enzyme used during these investigations; they could not be measured accurately by the manometric technique.

DISCUSSION

Horse and human serum esterase substrate specificity

The use of the β-isomer of acetylmethylcholine was early proposed as a specific substrate for acetylcholinesterase (AChE).[8] Since then, the specificity of AChE toward the optical isomers of both acetyl-α- and β-methylcholine has been investigated.[5]

The substrate specificity pattern obtained with the purified enzyme preparation was very similar to that obtained with horse serum (Tables 2 and 3); the α isomers were hydrolysed more rapidly by the serum preparation when compared to BuCh, but the relative pattern remains unaltered. Other esterases in serum probably account for this apparent increase. The other serum constituents do not appear to influence significantly the relative rates of hydrolysis of these substrates.

As reported by Myers[9] a similar substrate specificity pattern was obtained using horse serum and human serum, the relative rates of hydrolysis of the isomeric substrates being almost identical as shown in Table 6. Therefore the areas in the region of the active sites of human and horse serum cholinesterase are very similar.

TABLE 6. THE SUBSTRATE SPECIFICITY OF HORSE AND HUMAN SERUM CHOLINESTERASE

Substrate (6×10^{2}M)	% Rate		
	Purified Horse Serum cholinesterase	Horse Serum	Human Serum
BuCh	100	100	100
ACh	44	46	47
BzCh	15	13	15·5
L-butyryl-α-methylcholine	81	93	93
D-butyryl-α-methylcholine	66	78	79
L-butyryl-β-methylcholine	4·5	5·5	5·0
D-butyryl-β-methylcholine	0	0	0

The similarity between the relative rates obtained with a purified preparation and with horse serum establishes that the specificity pattern obtained with serum is due primarily to serum cholinesterase activity.

The influence of methyl substitution on the enzymic hydrolysis of BuCh

The present investigation demonstrated that C-methyl substitution in BuCh decreased its rate of hydrolysis by PCE in a manner similar to that of the ACh/AChE system. There are however, significant differences apparent from a comparison of Tables 6 and 7.

TABLE 7. THE RELATIVE RATES OF HYDROLYSIS OF ACh AND ITS METHYL ANALOGUES BY BOVINE ERYTHROCYTE ACETYLCHOLINESTERASE[5, 11]

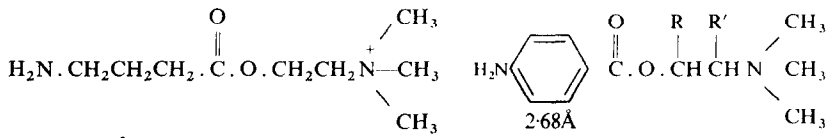
Substrate	Rate of hydrolysis (expressed as % of ACh)
ACh	100
L-acetyl-α-methylcholine	97
D-acetyl-α-methylcholine	97
L-acetyl-β-methylcholine	54
D-acetyl-β-methylcholine	0

Substitution of an α-methyl group in BuCh leads to a slight loss in the ability to be hydrolysed by PCE and this loss is greater for the D- than the L-isomer. A similar result was obtained with the corresponding acetylcholine analogues using acetylcholinesterase; substitution of the β-methyl group in BuCh to give the L-isomer produces a much greater reduction in hydrolysis by PCE than occurred with the corresponding L-isomer of acetylcholine using acetylcholinesterase. With both enzymes the appropriate -β-isomer was not hydrolysed.

It would appear that the spatial limitations for the serum cholinesterase site are more critical than for acetylcholinesterase. An α-methyl substituent prevents an "ideal fit" between enzyme and substrate, and a β-methyl substituent seriously impedes substrate enzyme interaction (Beckett and Mitchard, in press).

The relative inhibitor potencies of the p-*aminobenzoylcholines*

BzCh was hydrolysed at only 15 per cent of the rate of BuCh by PCE (Table 6) but the affinity of BzCh for the enzyme appears to be greater than that of BuCh (Table 1) for BzCh was shown to inhibit competitively the hydrolysis of BuCh (Table 4). The addition of an amino substituent to BuCh as in γ-aminobutyrylcholine prevents its hydrolysis by serum esterase,[10] and since the amino ($-NH_2$) to carbonyl ($C = O$) distances in both γ-aminobutyric and *p*-aminobenzoic acids are similar, it was considered that two features, viz. the high affinity of benzoylcholine for PCE and the absence of any enzymic hydrolysis of γ-aminobutyrylcholine might be combined in the *p*-aminobenzoylcholine analogues, to give compounds which would strongly inhibit the hydrolysis of BuCh and ACh by PCE. It was decided to investigate the value of the optically active, methyl substituted *p*-aminobenzoylcholine esters (IV) as inhibitors of this enzyme.



2·6Å

(III)

2·68Å

(IV)

It was established that the *p*-aminobenzoylcholine esters are competitive inhibitors of serum cholinesterase (Table 5). Significantly, β-methyl substitution reduces the inhibitor potency more than α-methyl and in each case the L-isomer was the more effective inhibitor. It is therefore probable that the "active conformation" of each of the *p*-aminobenzoylcholine inhibitors is similar to that of the BuCh substrate analogues.

The fact that serum cholinesterase is not a single enzyme but a mixture of enzymes[12–15] would at first appear to minimise the value of the present investigation. However, in the first place it has been shown that many esterases have a similar amino acid sequence (B group)[16–17] associated with the active site, and that these esterases hydrolyse substrates by a similar mechanism, and secondly, the pattern of results obtained in the present inhibitor and substrate studies indicate that fundamentally only one active site was being studied. The similarity in the stereo specificity of PCE and AChE indicates that a similar conformation exists in the region of the active sites of these closely related enzymes. It is therefore reasonable to assume that within the "family" of serum cholinesterases occurring in a given species, a common active site region exists having the same steric requirements and limitations for substrate fit. The technique required to separate the mixture of enzymes, "two dimensional electrophoresis",[14, 15] exploits very small differences in the properties of the constituent proteins, a difference which could be produced by the change of a single amino acid residue in a position remote from the active site. The atypical serum esterases appear to result from changes in the individual amino acid residues of the protein, or their sequence, so that the active site becomes distorted (Mitchard: to be published). The possibility exists that the apparent mixture of serum cholinesterases is due to partial degradation products of the protein produced by the isolation procedures; utilisation

of the isomers currently described as substrates or inhibitors for each isolated component would help to substantiate these conclusions.

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