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# REVIEW ARTICLE

# Comparison of butyrylcholinesterase and acetylcholinesterase

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## INTRODUCTION

In vertebrates two different enzymes hydrolyse acetylcholine. Acetylcholinesterase (EC 3.1.1.7; AChE) terminates the action of acetylcholine at the post-synaptic membrane in the neuromuscular junction. The other enzyme hydrolyses acetylcholine as well as many other esters, but has no known physiological function. It is called butyrylcholinesterase, pseudocholinesterase, non-specific cholinesterase, and cholinesterase (EC 3.1.1.8). In this review it is called butyrylcholinesterase (BChE), while 'cholinesterases' refers to both AChE and BChE. In vertebrates both enzymes are inhibited by $10^{-5}$ M-eserine, a property which distinguishes them from non-specific esterases. AChE and BChE can be specifically inhibited by BW284C51 and *NN'*-di-isopropylphosphorodiamidic anhydride [1].

Butyrylcholinesterase is studied by pharmacologists because it is responsible for the hydrolysis of succinylcholine, a drug used in surgery as a short-acting blocker of the acetylcholine receptor. Some patients experience prolonged apnea due to slow hydrolysis of succinylcholine which can be related to a genetic variation of the enzyme [2].

The role of AChE in some tissues, as for example the red cell membrane, migrating neurocrest cells [3,4], and early myotendinous junction [5], is not clear. During embryonic development a pattern of organization or succession of BChE and AChE has been reported, leading to the hypothesis that BChE functions as an embryonic acetylcholinesterase [6,7]. Protein sequencing [8], as well as the recently reported cDNA clones and deduced amino acid sequences for these enzymes [9–13], allow a better comparison of AChE and BChE.

A large number of reviews have dealt with cholinesterases [14–16] or more specifically with AChE [17–20] or BChE [21–23]. In this review the two enzymes are compared, the first focus being on the high homologies of the molecular forms, and the homologies in protein sequences. Cholinesterases are the prototype of a new family of related serine hydrolases. Secondly, the distribution and regulation of AChE and BChE is reviewed. In this context the proposed noncholinergic roles for cholinesterases are described. Finally, comparison of the structure and base composition of the genes may give clues to understanding the origin and evolution of AChE and BChE.

## OCCURRENCE OF AChE AND BChE IN ANIMAL PHYLA

All organisms seem to have some kind of enzyme capable of hydrolysing acetylcholine [24], but it is not clear if they can be called cholinesterases by the definitions made for vertebrate AChE and BChE, which are inhibited by $10^{-5}$ M-eserine. *Drosophila* has only one cholinesterase, an AChE [10] with substrate preferences intermediate between AChE and BChE [25]. In addition, *Drosophila* has an unspecific esterase with sequence similarities to the cholinesterases [26]. The presence of a single cholinesterase in insects cannot be generalized to all invertebrates. Three genes coding for AChE seem to exit in the nematode *Caenorhabditis elegans* [27]. In heart of the squid, *Sepia officinalis*, the presence of both BChE and AChE is suggested [28]. The clear demonstration of BChE as well as AChE has been made only in vertebrates. The BChE of *Torpedo* has some of the specificities of AChE. It hydrolyses acetylcholine better than it does butyrylcholine and is responsible for the physiological hydrolysis of acetylcholine in the heart. This suggests that the gene duplication that gave rise to the two enzymes occurred at the emergence of the vertebrates [29].

## COMPARISON OF MOLECULAR POLYMORPHISM

Both BChE and AChE exist as polymers of catalytic subunits (Fig. 1). The globular forms G1, G2 and G4 contain one, two, or four subunits. These forms are either readily extractable in low ionic strength buffers or tightly bound to membranes and require detergent for solubilization. Forms with an elongated shape are called asymmetric, and these do not interact with detergents but are solubilized in buffers with high salt concentration. The asymmetric forms contain one to three tetramers of subunits attached by disulphide bonds to a collagen-like tail. The most complex form, A12, has 12 subunits. The forms can be classified as either hydrophilic, water-soluble or linked to a membrane or extracellular matrix by strong interactions with other molecules.

### Hydrophilic, water-soluble forms

Both AChE and BChE exist as water-soluble forms secreted into body fluids. Human plasma BChE is the soluble form which has been most intensively studied. The G4 tetramer represents 95% of the activity found in plasma. This tetramer is an association of two dimers by strong hydrophobic interactions [30,31]. The two subunits in each dimer are linked by one disulphide bond at Cys-571 [32]. Dimers and monomers found in plasma seem to be degradation products of tetramers [33]. In plasma a size isomer of BChE migrating in electro-

Abbreviations used: AChE, acetylcholinesterase; BChE, butyrylcholinesterase.

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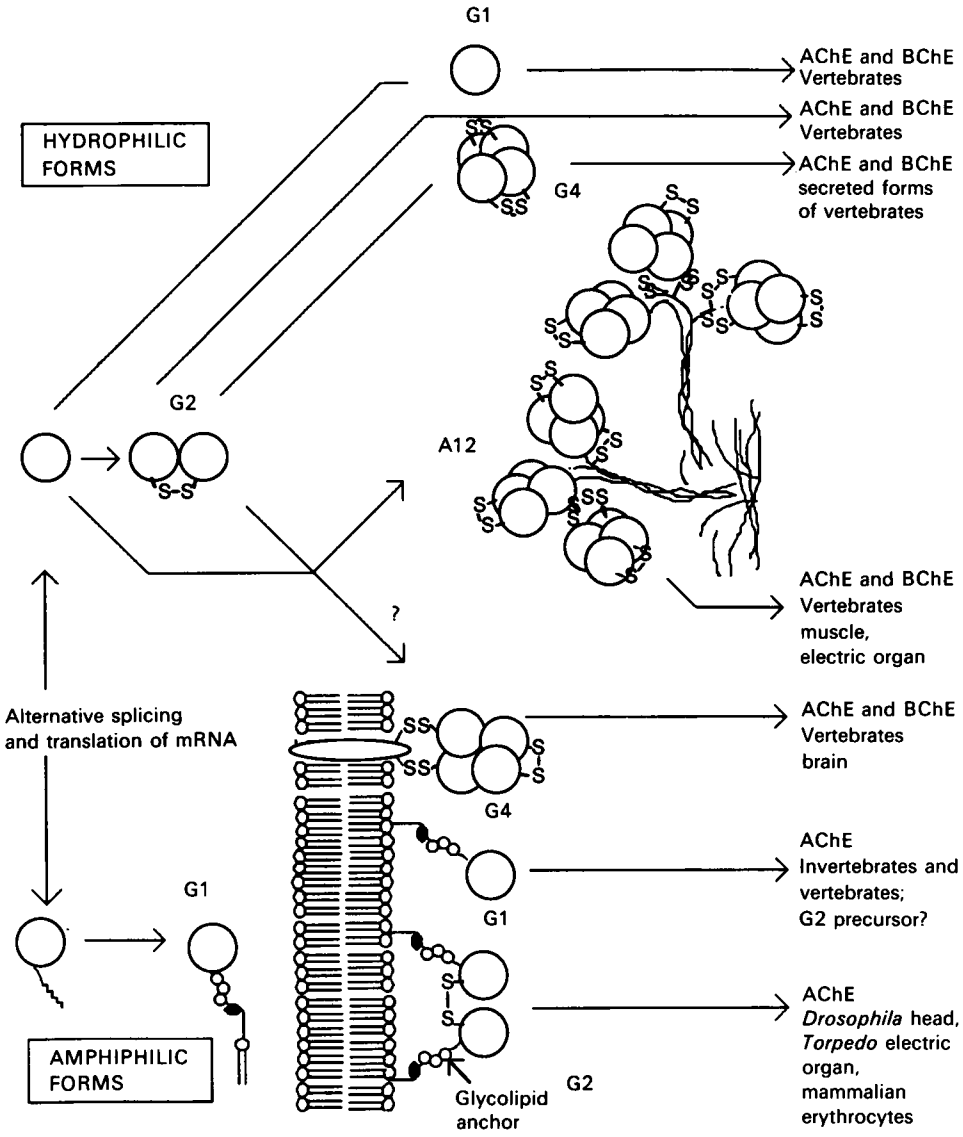


**Fig. 1. Schematic model of the molecular polymorphism of AChE and BChE**

Open circles designate catalytic subunits. Disulphide bonds are indicated by S–S. Hydrophilic forms are G1, G2 and G4 forms. The asymmetric A12 forms have three hydrophilic G4 heads linked to a collagen tail via disulphide bonds. The G4 amphiphilic forms of brain are anchored into a phospholipid membrane through a 20 kDa anchor. The G2 amphiphilic forms of erythrocytes have a glycolipid anchor. In *Torpedo* AChE hydrophilic forms and amphiphilic G2 forms are produced by alternative splicing, so that the proteins are identical at 535 amino acids but are nonidentical at their *C*-termini [116].

phoreses faster than a dimer [31] was shown to be an association by a disulphide bond of a monomer of BChE with albumin (P. Masson, personal communication).

AChE exists as a water-soluble G4 form secreted by adrenal gland [34], by nerve cell cultures [35], muscle cell cultures [36], by peripheral nerve cells *in vivo* or upon stimulation of nerve in hemidiaphragm preparations [37]. AChE is liberated by central nervous cells into the cerebrospinal fluid [38] from which it spills into the amniotic fluid in fetuses with neural tube defect [39]. The 11 S form of eel AChE derived by proteolysis of asymmetric AChE has a hydrophilic G4 structure, similar to human plasma BChE. AChE is found circulating in plasma in adult rabbit and rat and in fetal cow [24]. Fetal bovine serum AChE has the same subunit organization as human BChE [40].

## Immobilized forms

**Asymmetric forms.** The structure of the A12 form of *Torpedo* AChE is a linkage of catalytic subunits with the strands of a triple helical collagen tail (Fig. 1). Three tetramers of catalytic subunits are attached to a collagen tail. Homologous catalytic subunits are used for asymmetric AChE and for hydrophilic BChE (Fig. 1). In addition, a noncatalytic 100 kDa subunit was found in *Torpedo californica* [41].

Asymmetric forms are found in muscle of the primitive vertebrate, the lamprey eel [42], but are not found in invertebrates. AChE and BChE exist in the A12 form in mammals, and birds [15]. In 1-day-old chick muscle three different A12 forms have been described: a major hybrid form containing equal numbers of AChE and BChE

subunits and two minor homogeneous forms with either all BChE or all AChE subunits. The homogeneous BChE form does not react with monoclonal antibodies specific for the collagenous tail of the hybrid form, suggesting that the collagenous part of the molecule is different in the two forms. In chick muscle the AChE and BChE subunits are linked individually to the collagen tail [43]. In contrast, only half of the subunits are covalently linked to the collagen tail in AChE from electric organ.

A small pool of AChE asymmetric forms is found intracellularly. The subunits become attached to a collagen tail in the Golgi [44] and are secreted. The collagen-tailed AChE interacts with heparan sulphate proteoglycans as well as with other components of the basement membrane [45]. Linkage of the collagen tail with the extracellular matrix components is through ionic interactions [46].

**Amphiphilic globular forms.** The membrane-bound globular AChE forms have hydrophobic domains that anchor them in the membrane phospholipid bilayers. Two different amphiphilic forms, G2 and G4, are attached to two different hydrophobic anchors. G4 AChE in mammalian brain has a hydrophobic anchor of 20 kDa that is attached asymmetrically to two catalytic subunits via disulphide bonds. The 20 kDa anchor contains fatty acids but contains no inositol and no ethanolamine or glucosamine with free amino acid groups [47]. Thus the 20 kDa anchor is different from the glycolipid anchor of erythrocyte G2 AChE. The 20 kDa anchor has not yet been characterized with respect to amino acid and carbohydrate content.

Amphiphilic G2 AChE has been found in mammalian erythrocytes [48], platelets, sheep basal ganglia [49], *Drosophila* head [25,50–52], and *Torpedo* electric organ [19,53]. G2 AChE has been characterized in greatest detail in human erythrocytes, where the *C*-terminal amino acid of the catalytic subunit, which is glycine, is covalently bound in amide linkage to phosphatidylinositol. The glycolipid contains 1 molar equivalent each of *myo*-inositol, glucosamine and ethanolamine and 2 equivalents of fatty acids [48]. In contrast to most glycolipid-anchored proteins, the G2 AChE of human erythrocytes is not cleaved by phosphatidylinositol-specific phospholipase C. The reason is the presence of a structural modification in phosphatidylinositol. The hydrophobic domain, which includes the glycolipid, has a size of about 3 kDa. Protease cleavage separates fully active hydrophilic G2 AChE from its hydrophobic domain [54].

Similar membrane-bound BChE forms may exist since detergent-soluble G2 BChE has been reported in heart of *Torpedo marmorata* [29] and superior cervical ganglion of rat [55]. Detergent-soluble G4 BChE has been reported in mammalian brain [55,56].

As cholinesterases have been found in all branches of the animal kingdom [24] and are seen during development in noncholinergic systems, one can wonder whether acetylcholine and cholinesterases first played a role in which the soluble character of cholinesterases was necessary. During evolution AChE may have been recruited for a function for which its fixation to membranes was necessary.

## COMPARISON OF PROTEIN SUBUNITS

### Comparison of amino acid sequences

To date the only cholinesterase sequences known are *Torpedo* AChE, *Drosophila* AChE, 85% of fetal bovine AChE, and human BChE [9–13,57]. Both enzymes have not been sequenced from the same species, so it is not possible to compare BChE and AChE from one source.

There is a high degree of similarity between BChE and AChE despite the fact that the enzymes are from species that are far apart in evolution. Human BChE and *Torpedo* AChE are 54% identical, while human BChE and *Drosophila* AChE are 38% identical. Human BChE and *Torpedo* AChE are more similar to each other than to *Drosophila* AChE. However, bovine AChE is closer to *Torpedo* AChE than to human BChE (about 60% and 50% similar respectively) [57]. The divergence between AChE and BChE probably occurred in the deuterostomian lineage. The disulphide bonds in human BChE [32] and *Torpedo* AChE [58] are in exactly the same positions, and their three disulphide loops contain exactly the same number of amino acids. For both enzymes the cysteine is found four amino acids before the *C*-terminus and is used in a disulphide bond between identical subunits [32,58]. The sequence around the active site serine is conserved so that all four have the sequence GESAG. The lengths of the catalytic subunits are similar: 574 amino acids for human hydrophilic BChE, 575 for hydrophilic *Torpedo* AChE, 537 for mature amphiphilic *Torpedo* AChE, and 577 for amphiphilic *Drosophila* AChE. For hydrophilic fetal bovine AChE the number of residues is estimated to be 577.

The specificity of the cholinesterases for their substrates comes from the presence of an 'anionic site' which binds the choline residue during hydrolysis of choline esters. A sequence GSXF that should be close to the 'anionic site' has been found by photoaffinity labelling with an inhibitor of electric eel AChE [59]. It corresponds to a sequence GSFF in *Torpedo* AChE. Hasan *et al.* [60] argued that the 'anionic site' could be hydrophobic rather than ionic in nature accommodating the trimethylammonium part of the substrate. Evidence for a true anionic component of the 'anionic' subsite comes from the discovery of a point mutation in human BChE of patients carrying the atypical variant of the enzyme. The mutation was known to affect the anionic site [61], and not the sequence around the active serine [62]. The discovery of a single mutation in atypical BChE, replacing Asp-70 by Gly which induces a change of one charge, is in favour of a true anionic site [63]. Asp-70 is also present in *Torpedo* AChE and fetal bovine AChE but not in *Drosophila* AChE. In *Drosophila* AChE the tyrosine which replaces the aspartate could play the role of the aspartate. The importance of the hydrophobic and ionic forces around the 'anionic' site could vary among species. Differences in hydrolysis of charged substrates by AChE were found in fish in response to adaptation to high pressure and low temperature [64]. This could correspond to a smaller hydrophobic binding region around the anionic site.

### Comparison of glycosylation

Both AChE and BChE are glycoproteins. *Torpedo* AChE has four asparagine-linked carbohydrate chains [58] and human BChE has nine [32]. Carbohydrate moieties can differ for AChE from different tissues. This was suggested by Meflah *et al.* [65] who reported differences in lectin binding for AChE from bovine lymphocytes, erythrocytes and brain membrane. Interaction with lectins also distinguishes fetal calf serum AChE

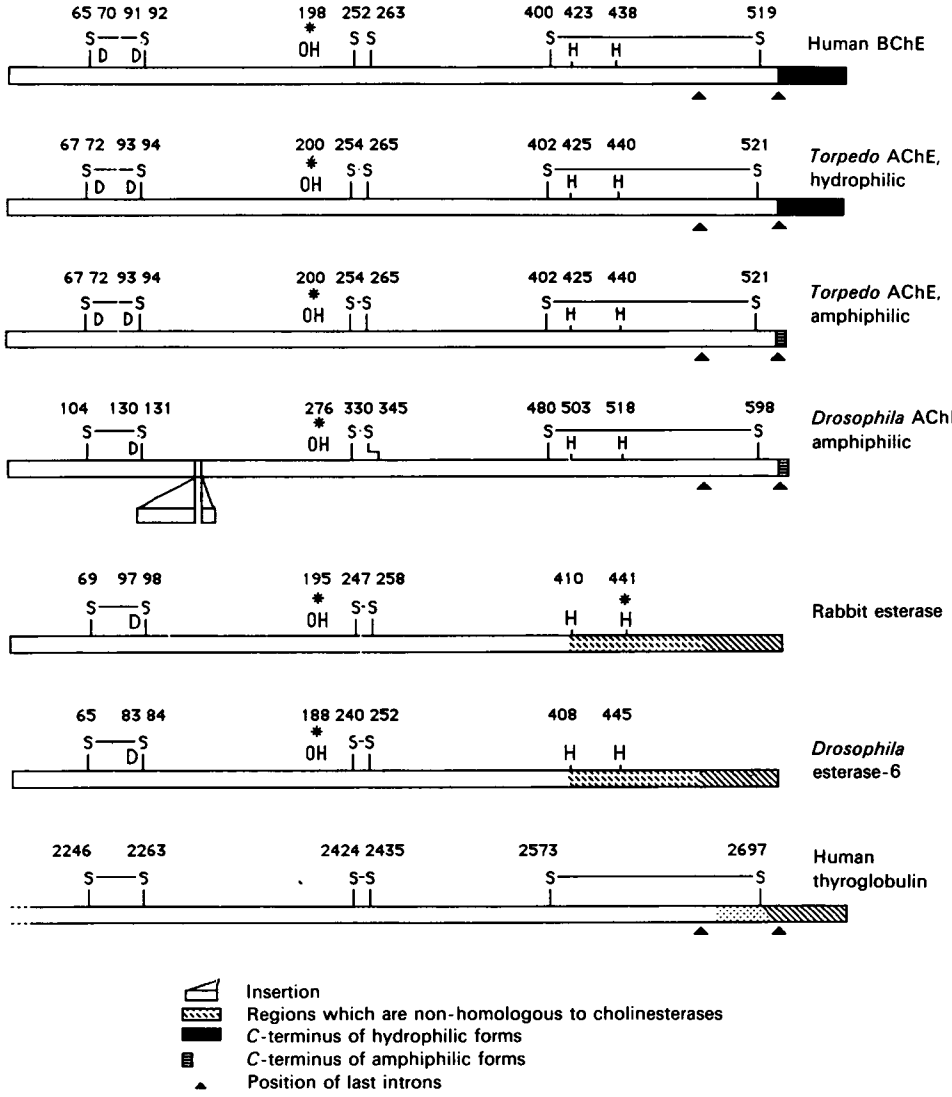


**Fig. 2. Schematic diagram of proteins homologous to cholinesterases**

Conserved residues are shown, including the active site serine (OH*), an aspartic acid that may be part of the catalytic triad (D-91 in BChE), and two histidines (H-423 and -438 in BChE) that may be important for catalysis. Asp-70 of BChE is at the anionic site. Intron locations for cholinesterases are indicated by a triangle. Disulphide bonds (S–S) for human BChE [32] and *Torpedo* AChE [58] were determined. For other proteins the location of disulphide bonds is by homology. Amino acid sequence information is from [8] (human BChE), [9] and [11] (*Torpedo* AChE), [10] (*Drosophila* AChE), [69] (rabbit microsomal liver esterase), [26] (*Drosophila* esterase-6) and [123] (bovine thyroglobulin).

from bovine erythrocyte AChE [40]. A decrease in sialylation of AChE occurs during erythroid differentiation in a human leukaemia cell line [66]. A subset of AChE G2 forms from electric organ of *Torpedo* is recognized by an anti-carbohydrate antibody [67]. Little is known about possible heterogeneity in the glycosylation of BChE.

## Comparison of the active sites of the cholinesterase and serine proteases

Homology of the peptide containing the active site serine in trypsin-like proteases (GDSGG) and cholinesterases (GESAG) could indicate common ancestry. However, the active site serine is closer to the *N*-terminus in the cholinesterases than in serine proteases and the pattern of disulphide bonds is different. The cholinesterases have no histidine in a location similar to the catalytic triad histidine of the serine proteases. This suggests that the similarity of the active site in the trypsin-like proteases and cholinesterases arose from convergent evolution.

## The cholinesterase family of esterases

The cholinesterases seem to belong to a class that is distinct from the serine proteases [32,58,68]. The new class is not limited to cholinesterases, as is shown in Fig. 2. Similarities have been found with other esterases, a rabbit liver microsomal esterase of 60 kDa [69] and esterase-6 of *Drosophila* [26] as well as with thyroglobulin [8,9]. Disulphide bonds on each side of the active serine are conserved. A third sulphide bond at the *C*-terminus is not present in the two unspecific esterases. No sequence homology is found at the *C*-terminus, particularly in the region which can give rise to different forms of cholinesterases. Since the tertiary structure may be partly

conserved it is likely that amino acids involved in the catalytic triad are conserved in all these enzymes. Two histidines (423 and 438 in human BChE) are conserved in the cholinesterases as well as in rabbit liver microsomal esterase and *Drosophila* esterase-6. Both histidines could play a role in catalysis. His-441 of rabbit esterase is phosphorylated by di-isopropylphosphofluoridate at the same time and under the same conditions as the active site serine, suggesting that this histidine is present in the esteratic site [69]. Asp-91 is found in the same position in all the enzymes and could represent the third amino acid of the catalytic triad.

## COMPARISON OF THE DISTRIBUTION OF AChE AND BChE IN TISSUES AND REGULATION DURING DEVELOPMENT

The various cholinesterase forms are tissue-specific. Asymmetric AChE and BChE forms are found only in peripheral nerves and muscles of vertebrates. Membrane-bound G4 AChE and G4 BChE are found in mammalian brain and membrane-bound G2 AChE is found in erythrocytes [15].

Both BChE and AChE have been described in tissues during development. There is evidence that BChE is present transiently in some embryonic cells where it can sometimes be replaced by AChE. This suggests a function for BChE as an embryonic acetylcholinesterase. Embryonic development has been mostly studied in chicken, and in this species the highest BChE activity is in embryonic tissues, thus allowing better comparison of AChE and BChE.

### Appearance and fate of BChE and AChE in muscle

The role of contractile activity in the localization and regulation of the forms of AChE at the neuromuscular junction has been extensively reviewed [44]. In muscle fibres both enzymes are progressively restricted to the neuromuscular junction by aggregating factors [70]. Neurotrophic factors, for example a dipeptide Gly-Gln in chicken and rat muscle cell cultures, seem to be involved in the regulation of AChE [71].

In chicken skeletal muscle the total amounts and forms of AChE change in parallel with those of BChE during development [72]. There is a period of relatively high activity of BChE which lasts until just before hatching. The localization of the two enzymes at this stage is not clear, but the fact that subunits of both participate in the same asymmetric forms implies an identical location [43]. In the rat a similar evolution of activities occurs in the neonate [73] and this can be related to the maturation of neuro-muscular junctions.

### Appearance and fate of BChE and AChE in nervous tissue

AChE associated with the cholinergic system is found in the brain, but its role is unclear in regions where no acetylcholine is present [24]. BChE is present in regions of the brain in positions not related to AChE, namely in capillary endothelial cells, in glial cells, and in neurones [74]. Human BChE is clearly synthesized in the brain, and not derived from plasma, since cDNA clones have been found in brain cell cDNA libraries [12,13]. Human brain and liver BChE and water-soluble G4 BChE from plasma have identical amino acid sequences [23]. Although almost half of the G4 form of brain BChE is membrane-bound [56], no clones coding for an alternative *C*-terminus have been found.

In embryonic chicken brain and eye the expression of BChE and AChE are co-regulated [75,76]. BChE is expressed just before and during mitosis, while AChE is expressed about 11 h after mitosis. The strong correlation between BChE and cell proliferation and between AChE and cellular differentiation suggests cholinesterase involvement in the regulation of these processes [75].

### Appearance and fate of BChE and AChE in blood

The concentration of BChE in human serum is correlated with growth hormone, obesity, pregnancy and parturition [22]. Average adult human plasma contains 3300 ng of BChE/ml and 8 ng of AChE/ml as measured by immunosorbent assay [77]. Some 94% of plasma BChE is in the water-soluble G4 form and its origin is thought to be the liver. Of the AChE in plasma 54% is in the G4 form and 44% is G1 and G2 [78]. While the G1 and G2 AChE forms could possibly be released from red blood cells, the G4 form probably originates from the neuromuscular junction, or autonomic ganglia or the central nervous system. Human fetal serum contains considerably more AChE than is present in adult serum [79]. Fetal bovine serum contains predominantly AChE [40]. The presence in plasma of AChE and BChE and the presence of AChE on red cell membranes suggests a requirement for circulating cholinesterase activity rather than a requirement for one of the cholinesterases. This would explain the dispensable character of BChE as exhibited in humans by homozygote 'silent' individuals with no BChE protein in the plasma.

### Appearance and fate of BChE and AChE in heart

The heart in higher vertebrates is one of the tissues containing a large amount of BChE. A partial switch from BChE to AChE has clearly been shown in the atrioventricular specialized tissue of the rat during early post-natal development [80,81]; the proportion of AChE increased from 6% to 15% [82]. The switch is linked to the development of adrenergic innervation and can be delayed when anti-(nerve growth factors) are used [80]. Although the activity of BChE in the total atria of the adult rat is in excess compared with AChE [83], AChE is responsible for the physiological hydrolysis of acetylcholine liberated by the vagus nerve [82]. In contrast, in a more primitive vertebrate, *Torpedo*, BChE is the only cholinesterase present in the heart and is responsible for the physiological hydrolysis of AChE [29].

### Tissue-specific regulation of AChE and homeostatic regulation of BChE in adults

Edwards & Brimijoin [84] measured AChE and BChE activity in tissues of three strains of adult rats and showed that, whereas there was no correlation of AChE activity between tissues of the rat there was correlation of BChE activity among tissues in individual rats. There was greater variation between BChE activity in the same tissues in different animals or different strains. They also studied the effect of hypophysectomy and found that BChE was more affected than AChE [85]. Their conclusion was that the regulation of AChE is tissue-specific. In contrast, BChE depends on a homeostatic type of regulation throughout the whole body. The discrepancies between studies showing parallel [86] or opposite regu-

lation of AChE and BChE could come from the fact that cholinesterases may have more than one function.

## ROLES OF CHOLINESTERASES NOT RELATED TO THE CHOLINERGIC SYSTEM

### Amidase and peptidase activity

In locations where no acetylcholine is released, the presence of AChE or BChE is puzzling. AChE and BChE were shown to possess other catalytic activities in addition to esteratic activity. Both AChE and BChE have arylacylamidase activity [87,88]. The arylacylamidase activity of BChE is probably responsible for the *C*-terminal deamidation of Substance P. The arylacylamidase activity of BChE is inhibited by the cholinesterase inhibitors eserine and tetraisopropylpyrophosphoramide, suggesting involvement of the active site serine [88]. Though both AChE and BChE have been reported to hydrolyse Substance P [89,90], the suggestion has been made that a contamination by dipeptidylaminopeptidase IV is responsible for Substance P hydrolysis by BChE preparations [91,92]. In fact, two different types of peptidasic activities have been described for human BChE. The first peptidase activity has the substrate specificity of dipeptidylaminopeptidase and has an active site that is distinct from the esteratic site of BChE [93]. The number of peptidasic sites in a highly purified preparation of BChE was very low compared with the number of esteratic sites [94]. The strong association of peptidase and esterase activities on gel electrophoresis [92,94] raises the question of the possible association *in vivo* of cholinesterases with other molecules with different activities.

The second peptidase activity described for BChE is a carboxypeptidase activity towards Substance P [95]. This activity is inhibited by EDTA and was not found in previous experiments due to the presence of chelating agents. This activity is not inhibited by di-isopropylphosphofluoridate or other esterase inhibitors like eserine. The possibility of another contaminant cannot be ruled out, but this activity is immunoprecipitated along with the esteratic activity.

AChE hydrolyses Substance P, enkephalins and their precursors [96,97]. AChE may also hydrolyse chromogranin, a protein secreted by adrenal cells [98]. AChE shows more than one type of peptidase activity, i.e. a trypsin-like activity and a metalloexopeptidase-like activity [99]. The trypsin-like activity is not inhibited by phosphorylation of the serine at the esterasic site but is inhibited by di-isopropylphosphofluoridate at high concentration. This raises the same question as for BChE: are the peptidasic and esteratic sites different or overlapping?

### Other roles not related to neurotransmission

Other noncholinergic roles have been tentatively attributed to AChE and BChE. The injection of purified AChE in the substantia nigra of the brain induced compartmental changes of long effect. BChE did not produce similar effects [100]. Dendritic release of AChE suggested a role in choline re-uptake [101]. The presence of BChE in liver where fatty acid metabolism occurs suggested a role for BChE in this metabolism [102]. Cholinesterases are found in intracellular locations such as the nuclear envelope, in membrane fractions, the transverse tubular system of muscle, and in sarcoplasmic reticulum, and could play an intracellular role as only a fraction of the subunits end up outside the cell [44]. Whatever role is proposed for BChE it has to be compatible with the fact that homozygotes for the silent BChE gene have no BChE in plasma and liver.

## COMPARISON OF THE GENES AND THE TRANSCRIPTIONAL REGULATION OF CHOLINESTERASES

### Alleles and variants

Genetic variants are known for human BChE. Two genetic loci determine BChE activity in plasma. Locus $E_1$ is located on chromosome 3 in the region 3q21-25 [103]. This position has been confirmed by chromosome *in situ* hybridization [104]. The $E_1$ locus controls most of the genetic variants. The best known alleles are the usual gene $E_1^u$, the atypical gene $E_1^a$, the fluoride-resistant gene $E_1^f$, and the silent gene $E_1^s$ [20,21]. Quantitative variants $E_1^j$ and $E_1^k$ have reduced activity [22]. In addition to the homozygotes all combinations of heterozygotes have been found. Both alleles are expressed in heterozygotes and the plasma tetramers show all possible combinations of the two kinds of subunits [105]. The human BChE $E_1$ variants have reduced affinity for substrates and lower activity at standard substrate concentration. The $E_1$ variants are indistinguishable from normal BChE on the basis of size or electrophoretic mobility. The $E_1^a$ variant enzyme has a low affinity for choline esters and positively charged inhibitors [2,61], thus suggesting that the single amino acid mutation is at the anionic site. In atypical BChE a single base substitution at nucleotide 209 converts Asp-70 to Gly [63]. In human populations there seems to be more than one type of silent gene, so that $E_1^s$ homozygotes have either no activity or 2% activity in plasma [21]. Humans with no activity enjoy normal health unless they undergo surgery with injection of the myorelaxant succinylcholine. Two subjects with 'silent' BChE were found to have a frame-shift mutation at nucleotide 351 (GGT→GGAG) which created a stop codon at nucleotide 384 [63]. Their BChE protein should contain 128 amino acids, and should not include the active-site serine. This explains the complete absence of BChE activity in the homozygote 'silent' person.

The $E_2$ locus controls the appearance of a distinct band of BChE on gels in approximately 10% of Caucasians [22]. The band appears to be a slow-moving tetramer called $C_5$. The $C_5$ variant is an autosomal dominant trait nonallelic with the $E_1$ locus [107]. However, the $C_5$ band had the same dibucaine resistance as heterozygote $E_1^uE_1^a$ [107,108], suggesting that $C_5$ was controlled by both genetic loci. To explain how two genetic loci can affect a single protein it was proposed that $C_5$ may be a hybrid of cholinesterase and a second protein [108]. The locus $E_2$ may be located on chromosome 16 [109]. However, there is no clear evidence that the $E_2$ locus is a structural gene for cholinesterase except that probes of BChE cDNA hybridized to chromosome 16 as well as to chromosome 3 [104].

There is only one gene for AChE in *Torpedo* [11] and one gene in *Drosophila* [10]. Genetic variants of AChE with two different subunit molecular masses of 100 and 105 kDa but no differences in activity have been reported for chicken. These are allelic variants and only heterozygotes show the two forms [44,110]. Genetic variants

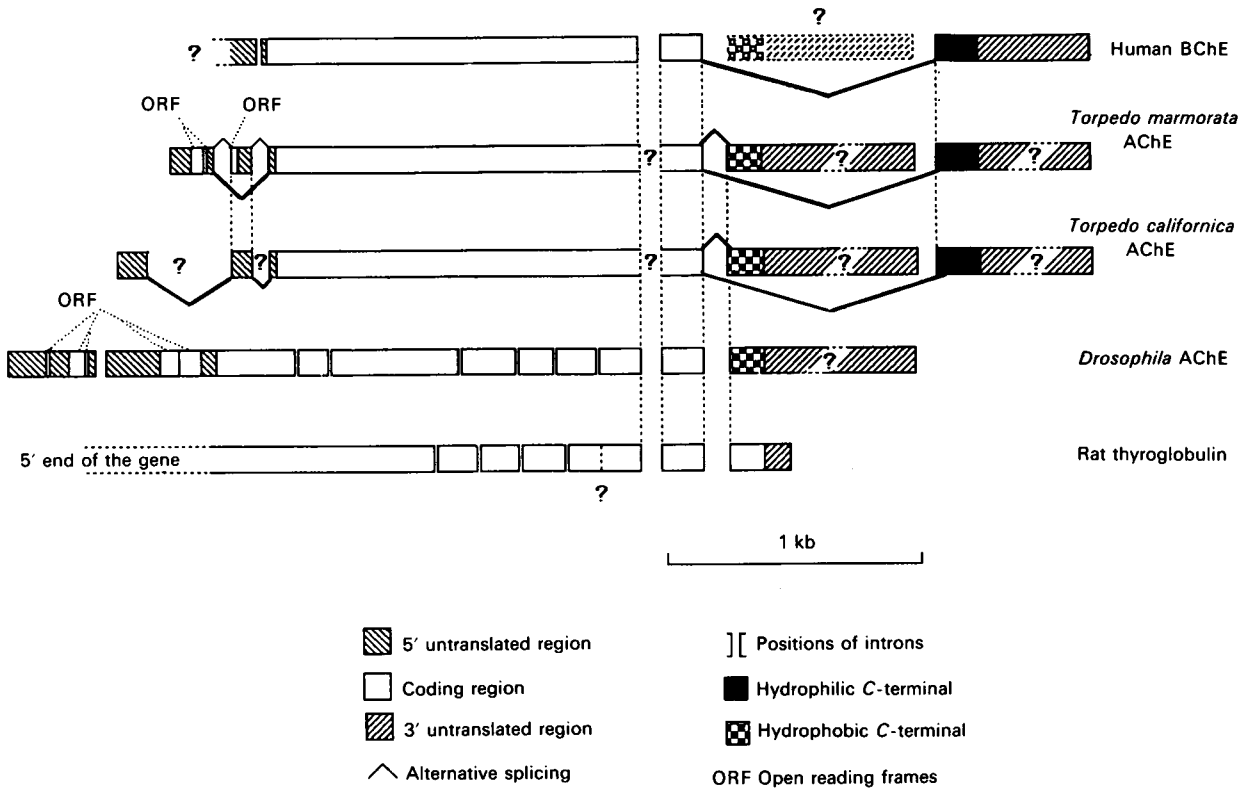


**Fig. 3. Comparison of gene structure of cholinesterases and *C*-terminus of the thyroglobulin gene**

Intron locations but not their lengths are shown. The coding region of human BChE and *Torpedo* AChE is interrupted by two introns. *Drosophila* AChE and rat thyroglobulin have many additional introns in the coding region. The number of introns in the 5′ untranslated regions is still uncertain and the Figure shows the minimum number based on current data. Open reading frames, ORF, containing potential translation initiation sites are present in *Torpedo* and *Drosophila* AChE. At the 3′ end two exons used in alternative splicing are indicated.

containing amino acid alterations have not been reported for human AChE. Insects have genetic variants of AChE that are resistant to organophosphate poisons [111] and *Drosophila* has variants with no AChE activity [112]. Homozygotes with no AChE cannot develop beyond a very early stage of embryogenesis and therefore this mutation can only be maintained in heterozygote populations.

### Structure of the genes for AChE, BChE and thyroglobulin

It is now clear that BChE and AChE are products of different genes. The structure of the gene is similar for *Torpedo* AChE and human BChE (Fig. 3). A common feature is a large exon possessing approx. 80% of the coding sequence starting in the 5′ untranslated region and ending far after the active-site serine. In thyroglobulin, as in *Drosophila* AChE, at least five introns are found in the region where vertebrate AChE and BChE have a unique large exon. The processed character of the coding region (homologous with a mature spliced mRNA) could be due to a retrotranscription event before duplication of the gene that gave rise to AChE and BChE. This may have occurred at the time of emergence of the vertebrates.

The region upstream of the large coding exon is complex in *Torpedo* AChE. Alternative splicing involving at least two exons was deduced from isolation of multiple cDNA and from protection assays of mRNA [11]. *T. marmorata* has multiple translation initiation sites. Use of some of these initiation sites would not result in a protein product because of the presence of in-frame stop codons. Similarly, *Drosophila* AChE has multiple initiation sites, some of which are within small open reading frames. *Drosophila* AChE has an unusually long 5′ untranslated leader sequence of about 1000 bp. Bingham *et al.* [113] have suggested that *Drosophila* AChE is an example of on/off regulation at the level of splicing. In this interpretation the mRNA containing small open reading frames is nonfunctional, and only fully-spliced mRNA is functional.

Human BChE also has multiple ATGs in the 5′ leader sequence. However, it contains no in-frame stop codons and therefore initiation at alternative ATGs would produce functional BChE. The most probable size of the signal peptide in human BChE is 28 amino acids. The signal peptide in *T. marmorata* is 24 amino acids and in *T. californica* is 21 amino acids long.

Downstream of the large coding exon there is a small exon from Gly-478 to Gly-534 in human BChE. The positions of the two introns limiting this small exon are identical in the genes for *Torpedo* AChE, human BChE, *Drosophila* AChE (D. Fournier, personal communication) and rat thyroglobulin [114]. The sequence of this small exon is not very well conserved but the splicing sites are conserved.

The coding sequence terminates with a choice of two possible exons, producing precursors of hydrophilic or amphiphilic forms. When translated, the 'hydrophilic' exon of *Torpedo* AChE gives 40 amino acids. The exon for G2 amphiphilic forms of *Torpedo* AChE is translated in a peptide of 31 amino acids [115]. The terminal 29 amino acids are replaced by a phosphatidylinositol anchor [116]. This 'hydrophobic' exon is found in a minor proportion of mRNA of *Torpedo* AChE and has not yet been found for BChE. The cDNA of BChE so far found by McTiernan *et al.* [13] and Prody *et al.* [12] has a last exon similar to that of the hydrophilic form of *Torpedo* AChE and corresponded to the protein sequence of BChE of plasma [8].

Surprisingly, introns have been found in cDNA clones of BChE and AChE. A cDNA clone of human BChE contained 105 bases at the 5′ side of the last exon [13]. A clone of rabbit BChE contained exon 3 and portions of introns at each extremity (A. Chatonnet, unpublished work). An AChE cDNA clone from *Torpedo marmorata* had an intron at the 5′ end of the cDNA [11]. This would explain why pools of mRNA with very different sizes elicited production of cholinesterase when injected into *Xenopus* oocytes [117]. Slow maturation of pre-mRNA of AChE would explain the high-molecular-mass bands seen in Northern blots hybridizing with AChE probes [9,10,11]. The presence of introns in mRNA may be an indication of regulation at the level of splicing [113].

A cDNA clone with a sequence at the 3′ end that corresponded to neither the exons coding for hydrophilic or amphiphilic AChE was found in *Torpedo*. The cDNA terminated with a poly(A) stretch, suggesting that no other exons followed it. This sequence could be either recognized as an intron and spliced out or used as a continuation of the preceding exon. This sequence was present in a small percentage of the mRNA [115].

### Base composition and codon usage in cholinesterase mRNA

Human BChE mRNA possesses 40% of C+G, which is approximately the proportion of these bases in the total human genome (Table 1). The dinucleotide CG is very under-represented in human BChE mRNA [12,13,118]. We found only 17 CG in the coding sequence of BChE, whereas *Torpedo* AChE has 82 CG and *Drosophila* AChE has 153 CG. This difference in base composition is in marked contrast with the high similarity of amino acid sequences of 54% between *Torpedo* AChE and human BChE. A large number (40) of the CG dinucleotides in *Torpedo* are at the codon–codon boundary (C is the third base of a codon and G is the first base of the next codon). In human BChE the C is often not present and is replaced by a T. This is found in 24 out of the 40 CG present at the codon–codon boundary in *Torpedo* AChE. This reflects a striking difference in the proportion of the four bases at the last base of codons of BChE when compared with AChE.

When compared with the human genes so far sequenced human BChE presents one of the lowest C+G content at the third base of the codons (34%, see Table 1).

Comparison with the cholinergic proteins (nicotinic and muscarinic acetylcholine receptors, choline acetyltransferase and BChE) shows that in contrast with BChE the cholinergic proteins of mammals and birds have a high C+G content.

**Table 1. C+G content in total mRNA and at the third base of codons in cholinergic proteins**

Data were obtained from GENBANK, EMBL, and NBRF. The computer programs were from the Protein Identification Resource, National Biomedical Research Foundation, Washington DC, U.S.A.

| Sequences | C+G | |
|---|---|---|
| | % at third base | % of total |
| Invertebrates | | |
| *Drosophila* AChE | 75.2 | 58.7 |
| Poikilotherm vertebrates | | |
| *Torpedo* AChE | 71.9 | 55.0 |
| *Torpedo* nicotinic ACh receptor α subunit | 37.0 | 39.4 |
| *Torpedo* nicotinic ACh receptor β subunit | 84.2 | 58.9 |
| *Torpedo* nicotinic ACh receptor γ subunit | 43.7 | 44.4 |
| *Torpedo* nicotinic ACh receptor δ subunit | 43.6 | 41.6 |
| Homeotherm vertebrates | | |
| Human BChE | 34 | 40.2 |
| Human nicotinic ACh receptor γ subunit | 79 | 61.1 |
| Human nicotinic ACh receptor α subunit | 67 | 51.4 |
| Mouse nicotinic ACh receptor δ subunit | 74.2 | 56.5 |
| Bovine nicotinic ACh receptor ε subunit | 80.6 | 59.7 |
| Chicken nicotinic ACh receptor δ subunit | 78 | 57.6 |
| Chicken nicotinic ACh receptor γ subunit | 80 | 58.1 |
| Porcine muscarinic ACh receptor | 95 | 66.6 |
| Porcine choline acetyltransferase | 70.9 | 56.4 |

According to this view AChE at the neuromuscular junction should have a high C+G content to be consistent with the high C+G content of muscle genes in higher vertebrates [119]. Plasma BChE is produced by the liver and as expected shows a liver-type C+G content. It should be noted that a comparison cannot be done with *Torpedo* genes where a great variety of C+G contents can be found even between the different subunits of the receptor (C+G 37–86%). Tissue specificity of codon usage does not seem to exist in *Torpedo* and might be restricted to homeotherm vertebrates.

C+G content could be important to regulation at the transcriptional level. The genome of warm-blooded vertebrates can be separated into domains of high and low C+G content called isochores [120]. Studies of this compositional compartmentalization of the genome of vertebrates showed that warm-blooded vertebrates have more genes in isochores of higher C+G content [121]. The C+G content of the genes reflects the proportion of these bases in the domain in which they lie. A low C+G content at the last base of codons is correlated with a low proportion of these bases in untranslated regions and in introns [122]. The position of genes in isochores is conserved during evolution. This is verified for human BChE [12,13] and for rabbit BChE (A. Chatonnet, unpublished work) which have conserved the low C+G content. Other cholinergic proteins are likely to be found in different isochores. The tissue specificity of codon usage could reveal that isochores are not used equally in transcription in different tissues. Thus AChE and BChE, which probably belong to two different transcriptional units of the genome with opposite base content, could be used successively or alternatively in different tissues.

## CONCLUSIONS

Comparison of AChE and BChE shows extensive similarities in protein sequences and in molecular forms. This contrasts with differences in expression during tissue differentiation and development. One cholinesterase replaces another during development, suggesting a complementarity of roles for AChE and BChE. The existence of a family of serine esterases structurally related to cholinesterase, but unrelated to the serine proteases, suggests that serine esterases have evolved to maximize their esterase function.

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