

# A distinct family of acetylcholinesterases is secreted by *Nippostrongylus brasiliensis*<sup>☆</sup>

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## Abstract

A third variant of acetylcholinesterase (AChE A) secreted by the parasitic nematode *Nippostrongylus brasiliensis* has been isolated which shows 63–64% identity to AChE B and AChE C, with a truncated carboxyl terminus and a short internal insertion relative to AChEs from other species. Three of the fourteen aromatic residues which line the active site gorge in *Torpedo* AChE are substituted by non-aromatic residues (Y70T, W279D and F288M). All three enzymes have 8 cysteine residues in conserved positions, including 6 which have been implicated in disulphide bonds in other AChEs. Phylogenetic analysis suggests that these enzymes form a distinct group which evolved after speciation and are most closely related to ACE-2 of *Caenorhabditis elegans*. Recombinant AChE A secreted by *Pichia pastoris* was monomeric and hydrophilic, with a substrate preference for acetylthiocholine and negligible activity against butyrylthiocholine. A model structure of AChE A built from the coordinates of the *Torpedo californica* AChE suggests that W345 (F331 in *Torpedo*) limits the docking of butyrylcholine. This model is consistent with mutational analysis of the nematode enzymes. Expression of AChE A is regulated at the transcriptional level independently of the other 2 secreted variants, with maximal expression by fourth stage larvae and young adult worms. These enzymes thus appear to represent an unusual family of AChEs with conserved structural features which operate outside the normal boundaries of known functions in regulation of endogenous neurotransmitter activity. © 2002 Elsevier Science B.V. All rights reserved.

**Keywords:** Acetylcholinesterase; Nematode; *Nippostrongylus brasiliensis*

## 1. Introduction

Acetylcholinesterases (AChEs) have a well-defined role in cholinergic neurotransmission in both vertebrates and invertebrates [1]. In addition, AChEs are expressed at other sites in the body, where they may have a variety of other roles such as regulation of cell growth and adhesion which appear to be unrelated to their catalytic

properties [2]. Acetylcholine (ACh) is the major excitatory neurotransmitter which regulates motor functions in nematodes, and as expected, AChEs have been localised to neurones and neuromuscular junctions in these organisms [3]. In contrast to the situation in vertebrates, distinct forms of AChE are encoded by separate genes in nematodes. This has been most comprehensively described in *Caenorhabditis elegans*, which possesses four separate genes for AChE, and current studies are aimed at mapping the anatomical location and function of each enzyme [4,5].

Many parasitic nematodes, in particular those which colonise the alimentary tract of their mammalian hosts, also release AChEs to the external environment via specialised secretory glands, although their possible functions have been interpreted in terms of hydrolysis of ACh in intestinal mucosal tissue [6–9]. We are utilising *Nippostrongylus brasiliensis*, which colonises

**Abbreviations:** AChE, acetylcholinesterase; BChE, butyrylcholinesterase; ACh, acetylcholine; BCh, butyrylcholine; ASCh, acetylthiocholine; PSCh, propionylthiocholine; BSCh, butyrylthiocholine.

<sup>☆</sup> **Note:** Nucleotide sequence reported in this paper have been submitted to the GenBank<sup>TM</sup>, EMBL and DDBJ databases with the accession number AY033080.

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the rat intestine, as a model to study this unusual phenomenon. This parasite secretes three monomeric hydrophilic, or non-amphiphilic ( $G_1^{na}$ ) AChEs designated as forms A, B and C which are distinguishable by their electrophoretic properties in non-denaturing gels [6]. These have been purified and defined as independent enzymes with similar molecular masses between 69 and 74 kDa [10]. They are expressed at subtly different time points in the life cycle of the parasite; form A immediately after entry of the fourth stage larvae into the duodenum, and forms B and C shortly after as the adult worms migrate to a more distal position in the jejunum [11,12]. We have previously cloned and characterised AChE B and C, which display 90% identity in amino acid sequence [13,14]. We have now isolated cDNAs for AChE A, and demonstrate that although this form is somewhat divergent from the other enzymes in primary structure, it displays important conserved features which are reflected in similar biochemical properties to the other secreted enzymes.

## 2. Materials and methods

### 2.1. Parasite recovery and culture

*N. brasiliensis* were isolated from the small intestine of Sprague–Dawley rats between days 3 and 10 post-infection as previously described [12]. Secreted proteins were collected from culture supernatants, cleared through 0.2  $\mu$ M filters and concentrated by passage through Centricon 10 microconcentrators (Amicon), washed in 50 mM sodium phosphate (pH 7.4) and the protein concentration determined by the Bradford method.

### 2.2. Cloning, alignment and phylogeny

We used a 5' primer designed to the 6 N-terminal amino acids of AChE B [13] (DDGTPV; 5'-GA(T/C)GA(T/C)GGNACNGT-3') and a 3' primer based on a conserved region of AChEs (WIYGGF) (5'-AGAATCCTCCTCCGTAGATCCA-3') to amplify cDNA fragments, using a standard RT-PCR reaction with 30 ng cDNA of *N. brasiliensis* isolated from rats day 3 post-infection, 500 ng of each primer and 30 cycles of 94 °C for 30 s, 50 °C for 1 min and 72 °C for 2 min. A cDNA fragment of 340 bp was amplified which was found to have homology to AChEs, and in particular 72 and 68% to AChE B and AChE C of *N. brasiliensis*, respectively [13,14]. We then utilised this fragment to screen a cDNA library of *N. brasiliensis* constructed in lambda ZAP. Several positive clones were isolated and sequenced on both strands by dideoxy chain termination. The longest clone (1859 bp) was used for protein expression. AChE sequences from diverse species were

retrieved using ESTHER (<http://www.ensam.inra.fr/cgi-bin/ace/index>), a specialised database on cholinesterases [15]. Alignments of protein sequences were performed via CLUSTAL W 1.8 [16], and modified according to the program 3D-JIGSAW [17]; <http://www.bmm.icnet.uk/servers/3djigsaw/>. For phylogenetic analysis, insertions, deletions and residues which could not be unambiguously assigned were manually removed. Bootstrap support was estimated from 100 resampled data sets (SEQBOOT, PHYLIP Version 3.57c [18]). Phylogenetic trees constructed via PROTPARS with the *C. elegans* esterase as an outgroup were used as input for CONSENSE and the consensus tree displayed using TREEVIEW [19].

### 2.3. Molecular modelling

The model for *N. brasiliensis* AChE A was obtained via 3D-JIGSAW [17], using as input the amino acid sequence of AChE A and the coordinates of the *Torpedo californica* AChE structure (PDB code 1EVE, [20]). As the model contained some short contacts and poor geometry, it was subjected to the model energy minimization program CNS (module model-minimize [21]). Docking of butyrylcholine (BCh) in the active site was generated by overlapping the AChE A model with that of the butyrylcholinesterase (BChE) model [22].

### 2.4. Northern blotting

Total RNA was isolated from adult worms of *N. brasiliensis*, isolated at different times post-infection as described previously [23]. Twenty micrograms of each sample were resolved on 1.2% agarose, 10% formaldehyde gels and transferred to nylon membranes. Hybridisation was carried out at 65 °C,  $5 \times$  SSC and blots washed at high stringency ( $0.1 \times$  SSC, 0.1% SDS at 65 °C) prior to autoradiography.

### 2.5. Expression in *pichia pastoris*

The cDNA clone encoding AChE A was ligated into *Pst*I/*Xba*I-digested pPICZ $\alpha$ B and the plasmid linearised with *Sac*I. The construct, which encoded the mature protein and an N-terminal signal peptide provided by the prepro sequence of the  $\alpha$ -mating factor of *Saccharomyces cerevisiae* and a C-terminal polyhistidine tag, was then used to transform competent *P. pastoris* as described by the manufacturer (Invitrogen). Growth and induction of recombinant yeast were performed as described previously [13]. Yeast culture supernatants were collected, concentrated using an Amicon cell and dialysed against 50 mM sodium phosphate buffer (pH 8.0) containing 0.3 M sodium chloride (buffer A). The dialysed protein solution was then loaded onto a Nickel-agarose column equilibrated previously with buffer A.

The enzyme was eluted with 0.1 M imidazole in buffer A and then used for further experiments.

### 2.6. Denaturing and non-denaturing electrophoresis

The purity of the enzyme was confirmed by 12% SDS-PAGE followed by staining with Coomassie Brilliant Blue. Purified AChE A and secreted products of *N. brasiliensis* recovered from rats day 5 post-infection were resolved on an 8% gel under non-denaturing conditions followed by visualisation of enzyme activity according to Karnovsky and Roots [24].

### 2.7. AChE activity, substrate and inhibitor specificities

AChE activity was determined as previously described [25]. The standard incubation mixture contained 1 mM acetylthiocholine (ASCh) iodide as substrate in the presence of 1 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) in 100 mM sodium phosphate pH 7.0 at 20 °C. The reaction was monitored by measuring the absorbance at 412 nm, and hydrolysis of ASCh calculated from the extinction coefficient of DTNB. One unit of activity is defined as 1  $\mu$ mol of substrate hydrolysed per min at 20 °C. The  $K_m$  values for ASCh, butyrylthiocholine (BSCh), and propionylthiocholine (PSCh) were determined by linear regression from plots of  $1/V$  against  $1/S$ , utilising substrate concentrations at a range of 0.05–1 mM (i.e. below excess substrate inhibition). Substrate inhibition constants ( $K_{ss}$ ) were determined by plotting  $1/V$  against substrate concentrations of 5–30 mM. Inhibition constants ( $K_i$ ) for 1,5-bis(4-allyldimethylammoniumphenyl)pentan-3-one bromide (BW284C51) and propidium iodide were determined using two fixed inhibitor concentrations and six substrate concentrations between 0.05 and 1.0 mM. Kinetic constants were derived using GRAPHPAD PRISM 2.0 Software (Graphpad, San Diego, CA).

## 3. Results

### 3.1. Cloning and sequence of AChE A

A 340 bp cDNA fragment was obtained by RT-PCR as described in Section 2, utilising cDNA from fourth stage larval worms isolated from the duodenum of rats at 3 days post-infection. This fragment was cloned and sequenced, which revealed that it was clearly derived from an AChE gene, but distinct from the 2 cDNAs previously described from *N. brasiliensis* [13,14]. We utilised this fragment to screen a cDNA library constructed in lambda ZAP, and several positive clones were isolated and sequenced.

The longest clone (1859 bp) contained a single open reading frame of 1734 bp and a 3' untranslated region of

125 bp containing a consensus polyadenylation site (AATAAA) and a poly(A) tail (GenBank accession number AY033080). Fig. 1 shows the alignment of AChE A with AChE B and C of *N. brasiliensis* [13,14] and the *Torpedo californica* AChE T subunit [26]. Although the cDNA is just short of an initiator methionine, it has a largely hydrophobic N-terminal stretch of 18 residues similar to that of AChE B and C indicative of a signal peptide. The alignment shows that the three *N. brasiliensis* AChEs have several important conserved residues which are critical molecular features of these enzymes. These include S193, E341 and H463 (S200, E327, H440 in *Torpedo*) that form the catalytic triad, and W79 (W84 in *Torpedo*) which binds the quaternary ammonium group of ACh in the active site. Of the fourteen aromatic residues lining the wall of the active site gorge in the *Torpedo* enzyme [27], eleven of these are conserved or show conservative substitutions. Three residues, T65 (Y70), D280 (W279) and M289 (F288) show non-conservative substitutions, which are essentially conserved in all three parasite enzymes. AChE A has six consensus sequences for N-linked glycosylation, at positions 99, 124, 277, 368, 437 and 477, compared to 3 sites in AChE B and 2 in AChE C.

Like the other 2 *N. brasiliensis* enzymes, the 6 cysteine residues implicated in the formation of three intramolecular disulphide bridges characteristic of *Torpedo* AChE are conserved, as are 2 additional cysteine residues at positions 232 and 263. Determination of total free sulphhydryl groups for all 3 *N. brasiliensis* AChEs by denaturation and reaction with DTNB [28] indicated a value of zero, suggesting that the proteins may have 4 intramolecular disulphide bonds.

Given the divergence in sequence of AChE A from the other 2 secreted AChEs of *N. brasiliensis*, we analysed the relationship of these enzymes with cholinesterases from diverse sources. Phylogenetic analysis using the PHYLIP program shows that all three enzymes are most closely related to ACE-2 of *C. elegans*, yet form an independent cluster, suggesting that they evolved after speciation (Fig. 2). A much more comprehensive analysis of proteins belonging to the superfamily of alpha/beta hydrolases homologous to cholinesterases is provided by ESTHER (<http://www.ensam.inra.fr/cgi-bin/ace/index>).

### 3.2. Expression and biochemical properties

In order to define the characteristics and biochemical properties of AChE A, we expressed the mature enzyme in the methylotrophic yeast *Pichia pastoris*, utilizing the prepro sequence of the  $\alpha$ -mating factor of *Saccharomyces cerevisiae* to direct secretion. The enzyme was released into culture medium and purified to homogeneity, displaying an apparent molecular mass of 74 kDa when resolved under reducing conditions by SDS-

		1	
AChE A	---- <td></td> <td>37</td>		37
AChE B	---MGLPARLLLAICVFTSAADDGPTVVLSSGSKIHGIMDVNGQTVSAYLGVPFATAE		
AChE C	NSCRGLPARLLAICILSTSAADDGPTVVLSSGAKIHGVYMEVDGQTVSAYLGVPFATAE		
Tc	MNLLVTSLSGLVLLHLVLCQADHSELLVNTKSGKVMGTRVPVLSHISAFGLGIPFAEPP		39
		1	
AChE A	----RFAKPTLIETYGEEELSAKELSKTCFQTPDGYFPGFLGSEMWNAPTELLEDCLSLN	▲ O ● ▲	93
AChE B	----RFAMPTLTETY--GGDIEALQLSKTCFQTKDETYPGFDGAEMWNPPTLSEDCLSLN		
AChE C	----RFAKPTLTETY--EKDIEAIQPSKTCFQSKDETYPGFDGAEMWNPPTDLSEDCLSLN		
Tc	VGNMFRFRPEPKPW--SGVWNASTYPNNCQQYVDEQFPFGSGSEMWNPNREMSEDCLYLN		98
AChE A	IWAP--ENPSGNVIVWIYGGGFFSGSPSLALYNGSVLAAKTKAVVNVNINRVSPFGFFYL	■ ● ● ●■	151
AChE B	IWVP--ENPDGNVIVWIYGGGFFSGSPSLALYNGSVLAGKTNVNVNINRVSPFGFFYL		
AChE C	IWVP--QNPDGNVIVWIYGGGFFSGSPSLALYNGSVLAARTGAVFVTNINRVSPFGFFYL		
Tc	IWVPSPRKSTTMVWVIYGGGFYSGSSTLDVYNGKYLAYTEEVVLVLSYRVGAFGLAL		158
AChE A	GDETKAPGNVGLLDQQUALQWIRKHSVFGGNSDKVTLFGESAGGASVTAHLLAPDSRDL	*	211
AChE B	GANSKAPGNVGLLDQQTALKWIHNIEYFKGDPKSVTLFGESAGGTSVTSHELLAPDSHSL		
AChE C	GADSK--PGNVGLLDQQTALKWIHNIEFFKGDPSKVTFLFGESAGGASVTSHELLAPDSHSL		
Tc	HGSQEAPGNVGLLDQRMALQWVHDNIQFFGGDPKTVTIFGESAGGASVGMHILSPGSRDL		218
AChE A	FSKIIVNSGTVRNPWATRSSCRMLYFSMETAKGLGCFDEYEIEQSKTEEDRCTVTKPAVA	● Δ ▲ Δ	271
AChE B	FSKIIVNSGSIHNVWATRSPCTMLHISMKTAKALGCVENYEIKPIREAEGRCTVLGADAD		
AChE C	FSKIIVNSGSIHNVWATRSPCTMLHISMKTAKALGCVENYDFKIIREAEGRCTVLGGNVD		
Tc	FRRAILQSGSPNCPWASVSVAEGRRAVELGRNLNCLNS-----DE		260
AChE A	DIYRCLSGKNTKDIQN--ISDTMYEHMRPMEWPFPGPITGDVNFQGRCTTKKLNLDGDKR	▲ ■ O O ●	329
AChE B	TIYECMKETPEKIQSEGNSDAIYAEMLPMEWPFPGPITYDDNYFKGEVR--RKLFSGEFKT		
AChE C	KIYECMKETPDDIQSAGNSDAIYENMLPMEWPFPGPITYDDNYFKGDVR--RKLFNDEFKK		
Tc	ELIHCLREKKPQELID--VEWNVLPFDSIFRFSFVP--VIDGEFFPTSLESMLNSGNFK-		315
AChE A	DVSAIFGTVKDEGTFWLPHYLYDSGFAFYPE--KSEKENAAIIDEQNYKASLKAFEGYFR	* ● ● ●	387
AChE B	DVSAIFGTVKDEGTFWLPHYLSSESGFSFFPDQSDSEANAAKIDEANYTASMQAFEGYFG		
AChE C	DVSAIFGTVKDEGTFWLPHYLSDSGFSFFPDMGSDSEANAAKIDESNYTASMQAFEGYFG		
Tc	KTQILLGVNKDEGSFLLYGAP-----GFSKDSSEKISREDFMSGVKLSVPH--		362
AChE A	NNSKALKILEDGFEGE--SGKETRYRDGVAQFVGDFYFSCSLYEFVDHIWNTLSTYIYY	■ ▲ ■	445
AChE B	KSSKAIEILKEGFKDL--GDVQTMYRDGVARFVGDFFFTCNLVEFVDHVTQKISEAYMY		
AChE C	KSSKAIEILKDGFKDL--GDEETMYRDGVARFVGDFFFTCNLVEFVDHVAPKIDEAYMY		
Tc	ANDLGLDAVTLQYTDWDDNNGIKNRDGLDDIVGDHVICPLMHFVNKYTKFNGNTYLYF		422
AChE A	FVRSSANPPEWGMVHGHEIEYEFYGFIFINESAYTNLEKDKKVEHFIFGLIGDFIDKG	● * ● ■	505
AChE B	FKARSSANPWPKWMGMVHGHEIEYEFYGFIFINSTAYKEVDKDRITISEEFMQLIKEFVNG		
AChE C	FKARSSANPWPKWMGMVHGHEIEYEFYGFIFINSAAYKEADKQDTISKEFMQLIKEFVENG		
Tc	FNHRASNLVPEWGMVHGHEIEYEFVGLPLVKELNY--TAEELASRRIMHYWATFARTG		481
AChE A	SFD-----DAKWPQYNNEKNTLVIDEDTSKSTIRKNIHKQYCEVINKAKEAAIGADGN	▲	559
AChE B	KFD-----DEWPKY--EGGKVMVIEDDASRKIEDKDIQQKYCKIINDARQALIDEAKGN		
AChE C	KFD-----GKWPKYNEEGKVMVIEDGTSSSIEDKNIQQKYCQIINEARKAVIDEAKGN		
Tc	NPNEPHSQESKWPLFTTKE--QKFIDLNTEPMKVHQLRVRQMCVFNQFLPKLLNATETID		540
Tc	EAERQWKTEFHRWSSYMMHWKNQFDHYSRHESCAEL		575

Fig. 1. Alignment of *N. brasiliensis* and *Torpedo californica* AChEs. The derived amino acid sequence of AChE A is aligned with the other non-neuronal AChEs of *N. brasiliensis* and the T subunit of *T. californica* AChE (Tc). Numbering starts from the amino terminus of the mature proteins (marked 1). Residues in the catalytic triad are marked by (★), and six potential glycosylation sites are marked by (■). The positions of cysteine residues conserved in AChEs from other species is shown by (▲), whereas the two cysteine residues unique to *N. brasiliensis* AChEs are indicated by (Δ). Circles depict the aromatic residues which line the wall of the active site gorge in *Torpedo* AChE. Conserved or conservative substitutions are marked by (●), whereas non-conservative substitutions are marked by (○). A comprehensive alignment of proteins belonging to the superfamily of alpha/beta hydrolases homologous to cholinesterases is provided by ESTHER (<http://www.ensam.inra.fr/cgi-bin/ace/index>).

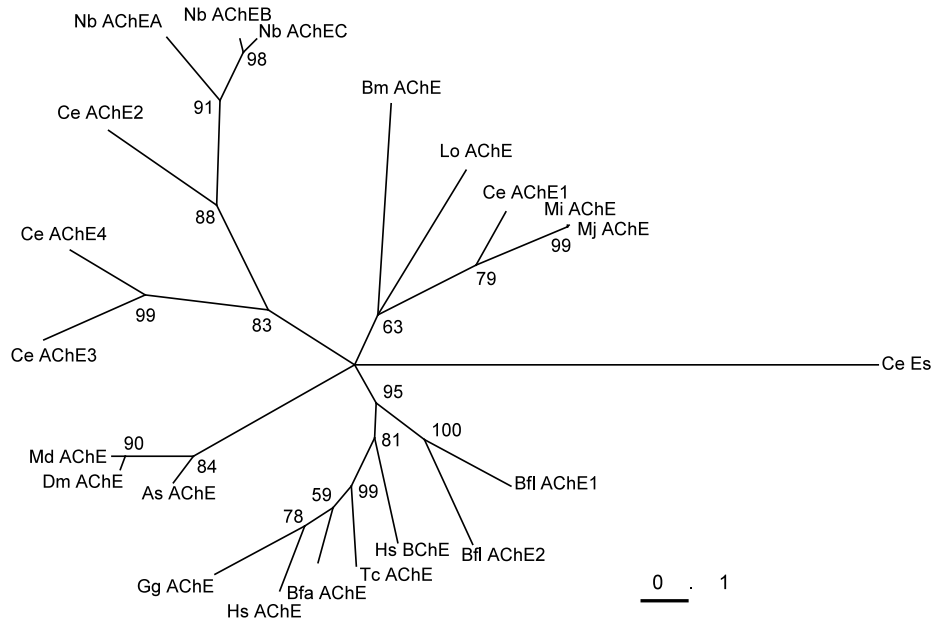


Fig. 2. Nematode secreted AChEs constitute a distinct group of enzymes. Phylogenetic analysis was performed on cholinesterase sequences retrieved from the GenBank™ database and analysed by protein parsimony. Bootstrap support was estimated from 100 resampled datasets (SEQBOOT, PHYLIP Version 3.57c), and values are shown at each branch. The scale bar represents 0.1 nucleotide substitutions per site. Abbreviations and GenBank accession numbers (Swiss-Prot for *Anopheles stephensi*) are as follows: Nb, *Nippostrongylus brasiliensis* AChEA (AY033080), AChEB (AF052508), AChEC (AF194408); Ce, *Caenorhabditis elegans* ACE1 (P38433), ACE2 (AF025378), ACE3 (AF039650), ACE4 (AF025379); Mi, *Meloidogyne incognita* (AF075718); Mj, *Meloidogyne javanica* (AF080184); Bm, *Boophilus microplus* (AJ223965); Lo, *Loligo opalescens* (AF065384); Dm, *Drosophila melanogaster* (X05893); Md, *Musca domestica* (AJ310134); As, *Anopheles stephensi* (P56161); Bfl, *Branchiostoma floridae* ChE1 (U74380), ChE2 (U74379); Tc, *Torpedo californica* (X03439); Bfa, *Bungarus fasciatus* (U54591); Gg, *Gallus gallus* (U03472); Hs, *Homo sapiens* AChE (M55040), BChE (M16541) Ce Es, *Caenorhabditis elegans* esterase (X66104).

PAGE (Fig. 3a). Like AChEs B and C, resolution on sucrose gradients at approximately 4.5 S was unaffected by inclusion of detergents (data not shown), indicative of a monomeric hydrophilic protein.

The specificity of AChE A for choline esters was investigated. Fig. 3b illustrates that the enzyme showed a preference for acetylthiocholine (ASCh), with negligible activity against BSCh. At a substrate concentration of 1 mM ASCh, AChE A had a specific activity of

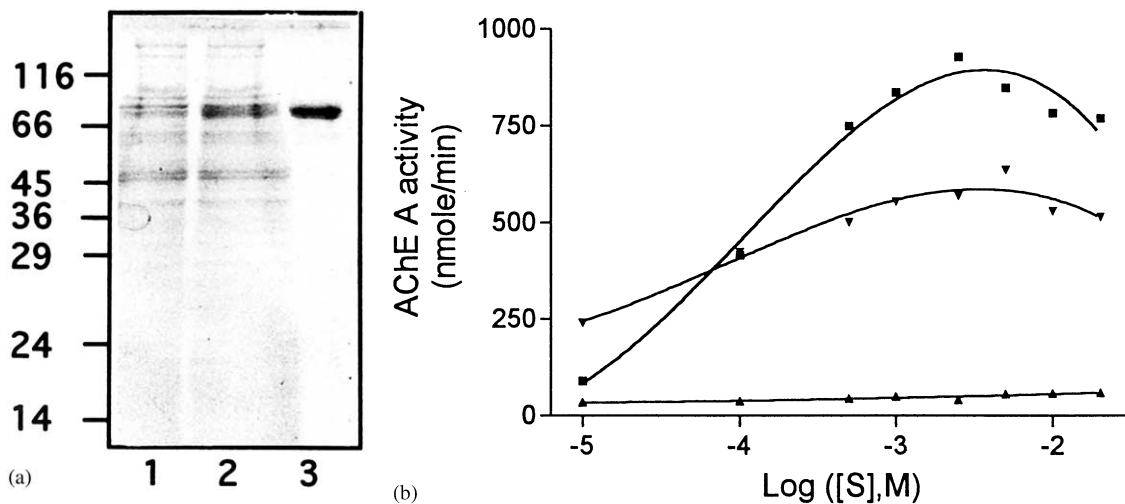


Fig. 3. Properties of recombinant *N. brasiliensis* AChE. (a) Expression in *Pichia pastoris*. Secreted proteins from culture media of wild-type *P. pastoris* X-33 (lane 1), or cells transfected with AChE A cDNA (lane 2) were resolved via 12% SDS-PAGE under reducing conditions and stained with Coomassie Brilliant Blue alongside the purified enzyme (lane 3). The position of molecular mass markers are shown in kDa. (b) Substrate specificity. The rate of hydrolysis of acetylthiocholine (■), propionylthiocholine (▲) and butyrylthiocholine (▼) by AChE A were determined as described in Experimental Procedures, and are shown here as a function of substrate concentration between 0.01 and 20 mM.

2010 U mg<sup>-1</sup>, and the rates of hydrolysis of PSCh and BSCh relative to ASCh were 61.4 and 4.4%, respectively. This figure also shows that the enzyme displayed excess substrate inhibition with ASCh and PSCh at concentrations above 2.5 mM. Kinetic constants of AChE A with ASCh and PSCh as substrates are shown in Table 1. The enzyme was inhibited by BW284C51, a specific inhibitor of AChEs [29], in a competitive manner and by the ‘peripheral’ site inhibitor propidium iodide in a non-competitive manner, with  $K_i$  values determined at  $57.9 \pm 7.9$  nM ( $n = 3$ ) and  $387 \pm 20$  nM ( $n = 3$ ), respectively. Tetraisopropyl pyrophosphoramidate (iso-OMPA), a specific inhibitor of BChE, had no effect at concentrations upto 10 mM (data not shown).

### 3.3. Molecular modelling and implications for substrate specificity

As previously noted [13,14], the strict substrate specificity for ASCh distinguishes the nematode secreted AChEs from other invertebrate AChEs, and appears inconsistent with the linear alignment of residues in the acyl-binding pocket based on comparative analyses of enzymes from different species and mutagenesis studies on vertebrate AChEs [22,30]. We previously demonstrated that a triple mutant (M300G/W302F/W345F) allowed nematode secreted AChE to hydrolyse larger substrates such as PSCh and BSCh [14], and therefore sought to examine the basis for these properties by deriving a model structure for AChE A as described in Section 2. Energy minimization of the preliminary model was achieved after 200 cycles in the CNS module model-minimize program [21], and the total energy of the molecule dropped from 1300 to 26.5 KCal, with a parallel drop of Van der Waals energy from 1200 to 0.5 KCal. The starting model and the energy-minimized one were superimposed by rigid-body transformation, and the root-mean-square deviation of their C-alpha coordinates was found to be 0.85 Å. Docking of BCh in the active site was generated by overlapping the AChE A model with that of the BChE model [22] in which the BCh position was assigned by its known interactions with BChE and by elimination of Van der Waals clashes between the enzyme and its substrate.

Table 1  
Catalytic properties of AChE A

Substrate	ASCh	PSCh
$K_m$	$0.08 \pm 0.01$	$0.38 \pm 0.01$
$K_{ss}$	$33.0 \pm 2.0$	$159.4 \pm 15.2$
$K_{cat}$	$5.22 \pm 0.17 \times 10^5$	$4.9 \pm 0.21 \times 10^5$
$K_{cat}/K_m$	$67.8 \times 10^8$	$12.22 \times 10^8$

$K_m$  (mM),  $K_{ss}$  (mM),  $K_{cat}$  (min<sup>-1</sup>) and  $K_{cat}/K_m$  (M<sup>-1</sup> min<sup>-1</sup>) values for ASCh and PSCh were determined as described in Section 2. Figures represent the mean  $\pm 1$  SD of three independent experiments.

The nematode AChE contains an insertion of 2 residues in its acyl binding pocket, and hence the trajectory of residues 296–302 is different from that of *Torpedo* AChE 285–289 (Fig. 4). In the AChE A model, the active S193 O-gamma is in tetrahedral bonding distance from the butyrate carbon and the carbonyl oxygen in an optimal oxyanion hole position (Fig. 5a). The butyryl moiety of BCh severely clashes with W345 (the shortest distance is 2.9 Å, Fig. 5a). The single mutation W345F is not sufficient to eliminate this short distance as F345 packs in the same site as W345 due to steric tightness. However, the steric tightness can be alleviated by the M299G mutation which eliminates the packing of the Met against either W345 or F345 and allows F345 to adopt a conformation which would not clash with the butyryl moiety (Fig. 5b).

### 3.4. Timing of expression in *N. brasiliensis*

Recombinant AChE A was resolved by non-denaturing polyacrylamide gel electrophoresis (PAGE) and enzymatic activity determined by staining in situ [24]. It resolved as a single band which migrated closely to the native enzyme recovered from secreted products of *N. brasiliensis* (Fig. 6a). Northern blotting, utilising RNA from parasites isolated from the small intestine of rats at different time points post-infection demonstrated that expression of AChE A was regulated at the level of transcription, with the opposite pattern to that seen for forms B and C. Thus, maximal expression was observed in fourth stage larvae and young adult worms immediately following entry into the duodenum, followed by a progressive decline to undetectable levels by day 8 post-infection (Fig. 6b).

## 4. Discussion

The rationale for cholinesterase secretion by parasitic nematodes which colonise the alimentary tract of their vertebrate hosts is still unexplained, despite having been documented for some considerable time [6]. Adult *N. brasiliensis* secretes three variants of AChE designated A, B and C [6,12]. All three forms are monomeric and hydrophilic, with molecular weights and acidic pIs estimated at 74 kDa and 4.0 for form A, 69 kDa and 3.8 for form B, and 71 kDa and 3.6 for form C [10,31]. They all have truncated C-termini in comparison to AChEs from other species, and are similar in this respect to AChEs from *Bungarus fasciatus* venom [32] and from *Boophilus* [33] which possess short polar C-terminal peptides allowing the production of soluble secreted enzymes.

Previous mutagenesis studies on the *Nippostrongylus* secreted AChEs have suggested that M288, W290 and W331 (*Torpedo* numbering) collectively blocked the

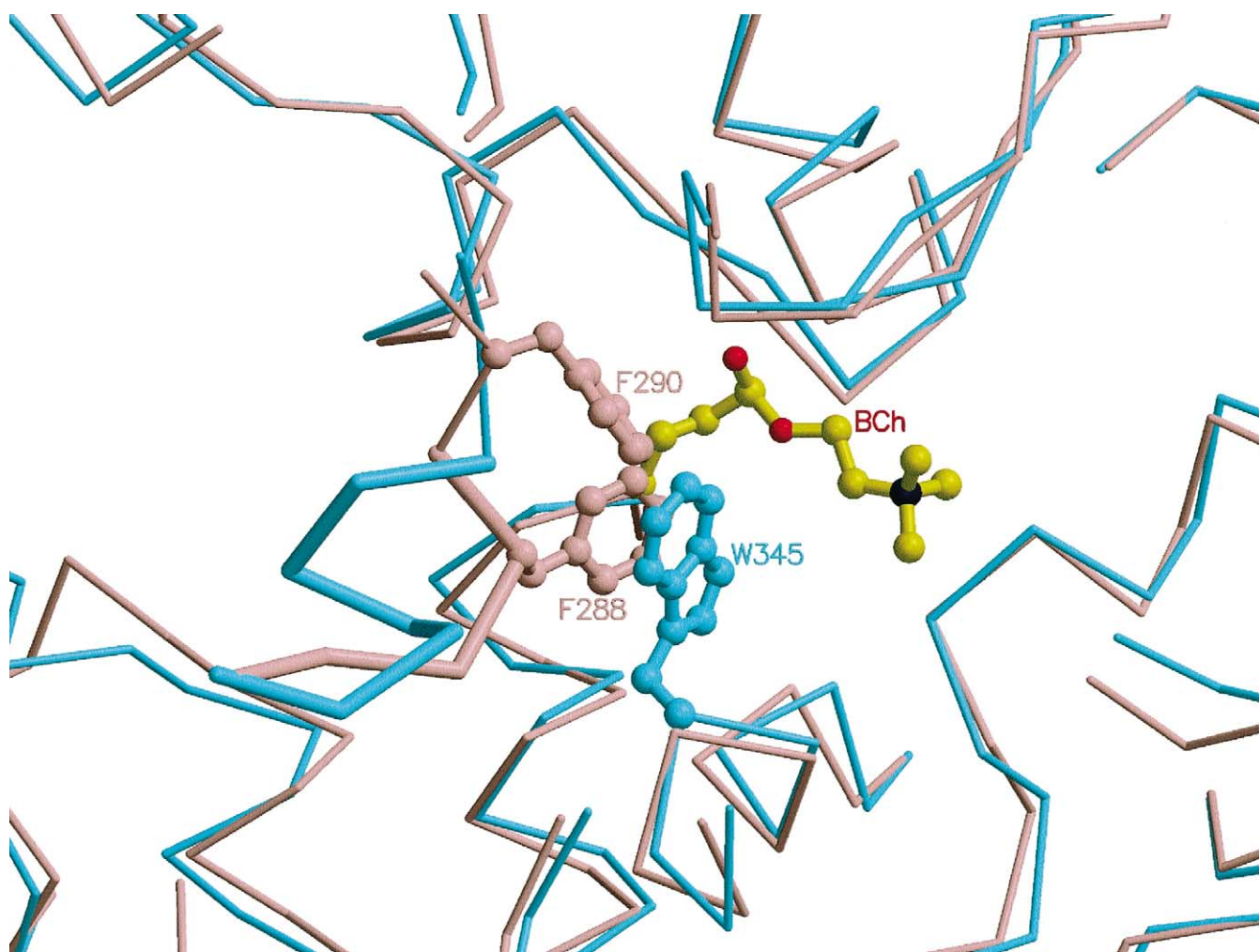


Fig. 4. Superposition of the *T. californica* (Tc) AChE X-ray structure (pink) and the model of BCh-bound *N. brasiliensis* (Nb) AChE A (cyan) at the active site. The sequence 296–302 of Nb AChE A which contains the 2 residue insert and the homologous 285–289 residues of Tc AChE are shown in thick lines. Residues F288 and F290 of the Tc AChE acyl binding pocket and residue W345 of Nb AChE A which occupies some of the same space are shown as ball-and-stick figures. The figure was made with the programmes MolScript [37] and Raster3D [38].

access of substrates larger than ACh [14]. The energy-minimized model of AChE A helps to interpret these data. Docking of BCh indicates a clash of the butyryl moiety of the substrate with W345 (F331 in *Torpedo*), and an alleviation of the steric tightness by mutation of M299 (288 in *Torpedo*) to Gly. It is worth noting that the hydrophobic interaction between the side chains of Met and Trp is well known, and is seen in the complex of *Torpedo* AChE with the toxin fasciculins as well as in other protein structures [34]. The above observations agree with the mutagenesis data which indicate that the single mutants W331F or M288G by themselves do not enhance BCh hydrolysis, while the triple mutant W331F/M288G/W290F does [14].

Despite broadly similar enzymatic properties, native AChE A has been observed to be less sensitive to excess substrate inhibition [10]. This phenomenon is also shown by the recombinant enzyme, with a  $K_{ss}$  of  $33 \pm 2$  mM for ASCh compared to  $1.3 \pm 0.2$  mM for AChE C

[14]. Although we have previously shown that a double mutation of W302F/W345F (F290/F331 in *Torpedo*) rendered AChE C less susceptible to substrate inhibition [14], all 3 parasite enzymes possess tryptophan residues in these positions, and therefore the basis for the relative insensitivity of AChE A to excess substrate inhibition is unclear. Two aromatic residues (Y70 and W279) which have been demonstrated to participate in binding of inhibitory ligands such as gallamine and propidium in *Torpedo* and mammalian AChE are substituted by T/S and D, respectively in the nematode enzymes, which nevertheless all remain sensitive to inhibition by these compounds.

Native AChE A is slightly larger than the other 2 variants, despite having a very similar protein backbone. This is likely due to glycosylation, as the former enzyme has six consensus sequences for N-linked glycosylation at positions 99, 124, 287, 388, 437 and 477, compared to 3 for AChE B (N124, 377 and 480) and 2 for AChE C

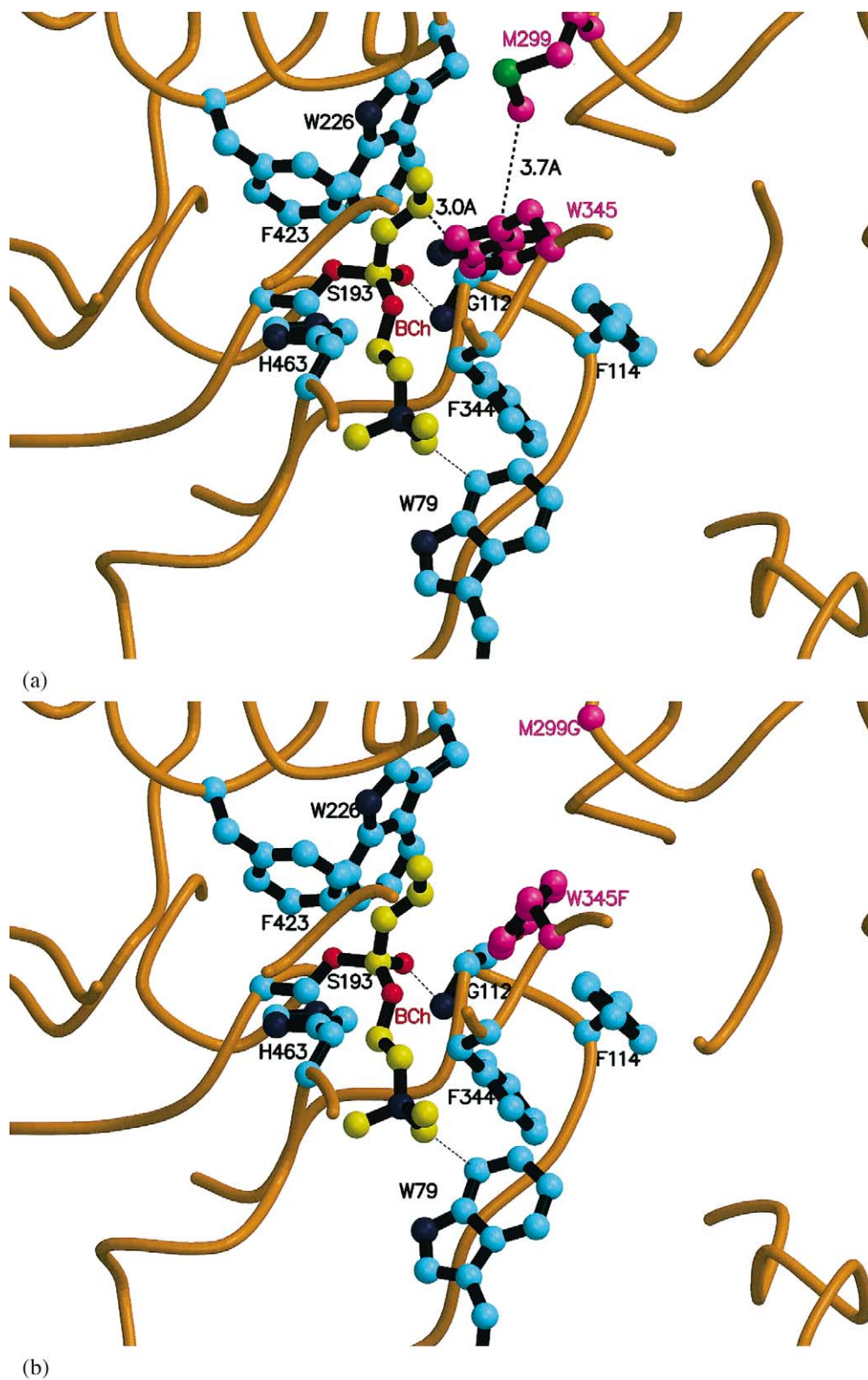


Fig. 5. A close-up view of the model of BCh-bound *N. brasiliensis* AChE A. (a) The BCh is shown in yellow, bound to S193 O-gamma. Interactions of BCh with the anion binding site W79 and the oxyanion hole G112 are shown in thin dashed lines. Aromatic residues surrounding BCh are shown in cyan, and residues involved in the mutagenesis studies are in magenta. Close contacts of the latter to BCh and to each other are shown in thick dashed lines. (b) A similar view with the mutations W345F and M299G. The new conformation of W345F, allowed by the M299G mutation, eliminates the close contact to BCh and allows its binding and subsequent hydrolysis. Figure drawn with programmes MolScript [37] and Raster3D [38].



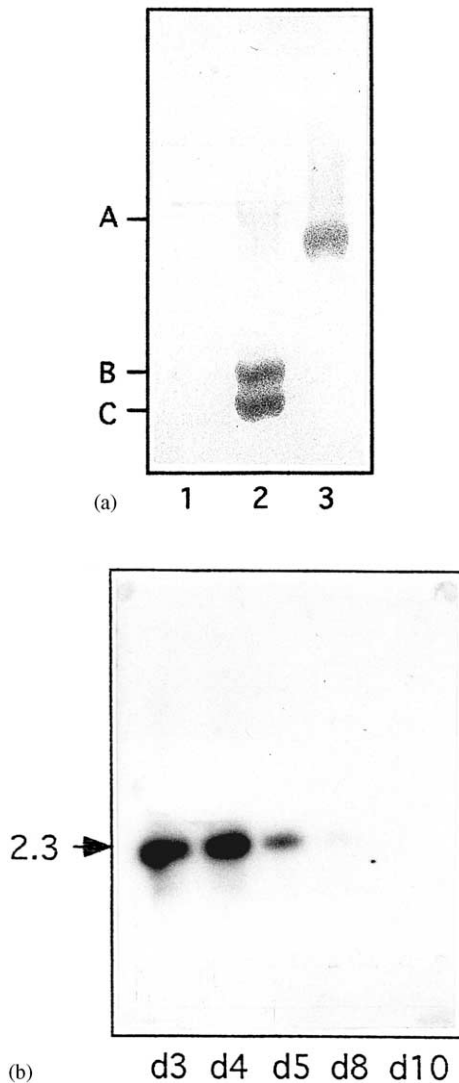


Fig. 6. Resolution and expression of AChE A. (a) Resolution by non-denaturing PAGE. Secreted proteins from culture medium of wild-type *P. pastoris* X-33 (lane 1), recombinant AChE A (lane 3), and native secreted AChEs from parasites collected day 4 post infection (lane 2) were resolved by non-denaturing PAGE and enzyme activity visualised according to Karnovsky and Roots [24]. The migration of native secretory AChEs designated as forms A, B and C is indicated. (b) Developmental regulation of expression. *N. brasiliensis* were recovered from the small intestine of rats at different time points from day 3 (d3) to day 10 (d10) post infection and total RNA isolated. The expression of AChE A was analysed by Northern blotting, utilising the full length cDNA as a probe. A single hybridising mRNA species of 2.3 kb is indicated.

(N124 and 376). The nematode AChEs have an 18 amino acid insertion relative to *Torpedo* which is characteristic of this group of enzymes. This was not included in the phylogenetic analysis, but provides additional evidence that they form a distinct family which has evolved after speciation.

We do not know how many AChE genes are expressed by *Nippostrongylus*. In addition to the three secreted enzymes, we have identified a non-secreted (and

therefore presumably neuromuscular) amphiphilic variant which sediments in sucrose gradients at 10.2 S and displays discrete enzymatic properties, although this preparation may contain more than one enzyme [35]. *C. elegans* possesses four different genes encoding distinct AChE variants [5], and thus it is possible that *Nippostrongylus* has an even greater complement of enzymes. The secreted variants are clearly related most closely to ACE-2 of *C. elegans*, which has a hydrophobic C-terminus and associates into glycolipid-anchored dimers, but comparison with parasite neuromuscular enzyme(s) awaits their isolation and sequencing.

It is unclear why *N. brasiliensis* expresses multiple forms of secreted AChE or why there is a difference in the timing of their expression. Neither infective larvae nor larvae recovered from the lungs 24 h post-infection secrete AChEs. However, by day 3 post-infection, as the fourth stage larvae enter the duodenum they display this behaviour, initially through exclusive expression of AChE A, and subsequently, following differentiation to adult worms, by gradually terminating expression of this variant, which is replaced by forms B and C [11,12]. The rationale for this switch is unclear, particularly given their similar enzymatic properties. All the enzymes show the same substrate specificity, and AChE A is even more catalytically efficient than AChE C [14]. As the parasites mature, they migrate distally down the jejunum. However, the pH optima of the enzymes are not radically different (7.0 for form A compared to 7.4 for forms B and C, data not shown), and thus a switch in expression is unlikely to be made for this reason. It is also unlikely to be made for immune evasion, as altered expression of the enzymes occurs relatively early following invasion of the mammalian host, and antibodies appear to play little role in expulsion of parasites in a primary infection [36]. The presence of multiple forms may be related to other properties of the enzymes such as tissue targeting, but this has yet to be investigated.

Although the role of these proteins is still unknown, the preservation of catalytic activity in all examples documented thus far suggests that this is requisite for their physiological function, and the strong preference for hydrolysis of ACh indicates that this is most likely the *in vivo* substrate. We have suggested that AChE secretion may be related to cholinergic regulation of secretory responses or undefined aspects of leukocyte function in the intestinal mucosa [9], and are working to investigate this central question.

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