Comparative Inhibitory Effects of Various Physostigmine Analogs against Acetyl- and Butyrylcholinesterases

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ABSTRACT

A number of carbamoyl- and N(1)-substituted analogs of physostigmine were synthesized and their in vitro potencies (IC_{50} values) vs. human erythrocyte and brain (cerebral cortex and caudate nucleus) acetylcholinesterase (AChE) and electric eel AChE and against human brain and plasma butyrylcholinesterase (BChE) were compared to the potencies of physostigmine and other traditional anticholinesterases. In general, increasingly hydrophobic, simple nonbranching carbamoyl groups (as in octyl-, butyl- and benzylcarbamoyl eseroline) did not greatly alter potency vs. AChE whereas increasingly hydrophobic N(1)-substitutions [i.e., N(1)-allyl-, -phenethyl and -benzylphysostigmine] decreased potency vs. AChE. In contrast, increasing the hydrophobicity of both the carbamoyl and N(1) groups increased the potency of the compound against BChE. Furthermore, quaternarization at the N(1) position (physostigmine methosulfate) increased potency vs. AChE but reduced potency vs. BChE. Bulky, branched carbamoyl groups (e.g., N-benzyl-N-benzyl-allophanyl eseroline) were all poor anticholinesterases. N-phenylcarbamoyl eseroline was as potent as benzylcarbamoyl eseroline against AChE yet was 50 to 100 times less potent than the benzyl analog vs. BChE. Therefore, the phenyl substitution appears to increase greatly the selectivity of the compound for AChE. Although it is not possible to determine whether physostigmine analogs that are potent in vitro might be of interest in vivo, these results do show that the structure of physostigmine can be changed significantly while retaining biological activity.

The role of organophosphate inhibition of AChE in the development of both nerve gases and insecticides has resulted in extensive studies of the mechanism of action of such compounds. Consequently, the structure of the active site of AChE and the mechanisms of action of not only organophosphates (such as DFP, Sarin, Soman and Tabun) but also carbamates (physostigmine and neostigmine), are relatively well understood (for reviews see Long, 1963; Usdin, 1970; Silver, 1974; Main, 1976). The active site of AChE (and BChE) contains two subsites, the anionic subsite, which interacts with the quaternary nitrogen of ACh, and the esteratic subsite, which contains a serine hydroxyl group that attacks the carbonyl group of ACh. With respect to physostigmine (eserine), a tertiary pyrollidine nitrogen is protonated at physiological pH values, ($pK_a = 8.1$; Main, 1976), and physostigmine is attracted to the anionic subsite. The carbonyl group of the physostigmine carbamoyl side chain is oriented such that it is attacked by the esteratic subsite serine. Consequently, the enzyme becomes carbamylated (or phosphorylated in the case of organophosphates) in a reaction analogous to the formation of the acylated-enzyme

intermediate in the hydrolysis of ACh. However, whereas the acylated-enzyme intermediate formed during ACh hydrolysis is hydrolyzed rapidly, the carbamylated (or phosphorylated) enzyme intermediate is very stable. The inhibition of cholinesterases (E) by carbamate inhibitors (I) can be summarized as:

$$E + I = E - I \rightarrow E' + \text{eseroline} \rightarrow E + \text{carbamate}$$

where E' is the stable carbamylated enzyme intermediate.

Physostigmine is a carbamate ester alkaloid originally isolated from the seed (Calabar bean) of the West African climbing vine physostigmina venenosum. It has proved to be an invaluable tool for elucidating the mechanisms of cholinergic neurotransmission (Karczmar, 1970) and has also been used clinically in the treatment of glaucoma (Laqueur, 1877), myasthenia gravis (Walker, 1934) and, more recently, AD (reviewed by Becker and Giacobini, 1988). In subjects with AD, the major cholinergic projection to the cerebral cortex degenerates (Perry, 1986) putatively resulting in the memory dysfunction in these patients (Smith and Swash, 1978; Bartus et al., 1982). Despite evidence that physostigmine can enhance memory performance in normal subjects (Bartus et al., 1982), clinical trials of physostigmine in AD patients aimed at prolonging the action of

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ABBREVIATIONS: AChE, acetylcholinesterase; DFP, diisopropylphosphofluoridate; BChE, butyrylcholinesterase; ACh, acetylcholine; AD, Alzheimer's disease; THA, tetrahydroaminoacridine; iso-OMPA, tetramonoisopropylpyrophosphortetramide; BW284c51, 1:5-bis (4-allyldimethylammoniumphenyl) pentan-3-one dibromibe.

ACh released from remaining cortical cholinergic nerve terminals have been largely disappointing (Becker and Giacobini, 1988). However, after attaining peak plasma concentrations in humans at approximately 30 to 60 min, physostigmine is cleared from plasma with a half-life of about 30 min (Gibson et al., 1985; Sharpless and Thal, 1985; Whelpton and Hurst, 1985; Hartvig et al., 1986; Sherman et al., 1988). Therefore, the limited efficacy of physostigmine in the treatment of AD might be due to its relatively short half-life. Indeed, remarkable improvements have been reported recently in AD patients given THA (Summers et al., 1986), an anticholinesterase with a longer half-life than physostigmine (Summers et al., 1980).

Because the clinical usefulness of physostigmine might be limited by its relatively short half-life, we assessed the in vitro anti-AChE and anti-BChE properties (IC$_{50}$ values) of a number of physostigmine derivatives as a first step in the identification of physostigmine analogs that might be clinically useful. A comparison was made between the inhibition of AChE and BChE because an inhibitor that is more potent against AChE than BChE would interact less with BChE, particularly in the BChE-rich plasma, and might therefore have more specific, cholinergic effects.

Methods

The syntheses of both the N(1)-substituted and carbamate physostigmine analogs have been described in detail elsewhere (Yu et al., 1988a, b). Except where specific references are made to (+)- or (±)-physostigmine, the term physostigmine will refer to the (−)-isomer. In brief, the N(1)-substituted analogs were synthesized (fig. 1) using O-methyl,N(1)-nor-eseroline as starting material. After N-benzylation and ether cleavage, the resulting compound, N(1)-benzyl eseroline, was reacted with methylisocyanate to yield N(1)-benzylphysostigmine. Debenzylation of this latter compound yielded N(1)-nor-physostigmine, which was further reacted with either allylbromide, methylisocyanate or phenethylbromide to yield, respectively, N(1)-allylphysostigmine, eseramine or N(1)-phenethylphysostigmine.

The majority of the carbamate analogs (fig. 2) were synthesized by reacting eseroline with the appropriate isocyanate (e.g., octylisocyanate + eseroline → octylcarbamoyl eseroline). For the synthesis of N-benzyl-N-methyl-allophanyl eseroline, the isocyanate (N-benzylisocyanate) was reacted with physostigmine rather than with eseroline. Instead of reacting eseroline with an isocyanate, eseroline was reacted with N-dimethylcarbamoyl chloride or N-diethylcarbamoyl chloride to yield, respectively, N-dimethylcarbamoyl eseroline and N-diethylcarbamoyl eseroline. Physostigmine methosulfate was synthesized by reacting physostigmine with dimethylsulfate. The (+)-isomer of physostigmine was synthesized by reacting (+)-eseroline with methylisocyanate as described elsewhere (Yu and Brossi, 1988).

For comparative purposes, several commercially available (Sigma Chemical Co., St. Louis, MO.) anticholinesterases: BW284c51; neostigmine (Prostigmine); DFP; THA (Tacrine); edrophonium (Tensilon); iso-OMPA; and ethopropazine (Lysivane) were also evaluated. These compounds were chosen to represent agents that were either structurally related to physostigmine (neostigmine), selective inhibitors of AChE (BW284c51 and edrophonium) or BChE (ethopropazine and iso-OMPA), a well characterized, classical organophosphate inhibitor (DFP) or of clinical interest in the treatment of AD (THA).

Preparation of cholinesterase-containing tissues. Samples of postmortem human brain (frontal cortex and caudate nucleus) were obtained from a 57-year-old man who died of a myocardial infarction. The meninges and subcortical white matter were removed from the sample of frontal cortex and the remaining gray matter (approximately 10 g) was homogenized in 10 volumes of buffer (0.1 M sodium phosphate buffer, pH 7.4, containing 1 M NaCl and 0.5% Triton X-100). The cortical homogenate was then diluted 1:2 or 1:1 for AChE and BChE

assays, respectively. The caudate nucleus sample (0.05 g) was homogenized in 50 ml of buffer and the homogenate was then diluted 10-fold.

To prepare erythrocyte AChE and plasma BChE, 10 ml of blood were collected in a heparinized tube and centrifuged at 4000 × g for 10 min. The plasma was removed and diluted 1:200 with 0.1 M sodium phosphate buffer, pH 7.4. The erythrocytes were washed 3 times in isotonic saline followed by lysis in 9 volumes of 0.1 M sodium phosphate buffer containing 0.5% Triton X-100. The lysed cells were then diluted an additional 40-fold in 0.1 M phosphate buffer, pH 7.4 (final dilution = 1:400).

Electric eel AChE (type III, 1000 U/mg of protein) was obtained from Sigma Chemical Co. and was diluted 1:40,000 in 0.1 M phosphate buffer, pH 7.4 All final preparations were frozen and stored at −70°C.

Assay procedures. AChE and BChE activities were determined in 0.1 M sodium phosphate buffer, pH 8.0, using the spectrophotometric method of Ellman et al. (1961). Acetyl-β-methylthiocholine (0.5 mM) and butyrylthiocholine (0.5 mM) were used as specific substrates for the assay of AChE and BChE, respectively. For each cholinesterase preparation, 25 μl were added to a final incubation volume of 1.0 ml.

Compounds were dissolved in dimethylsulfoxide and diluted at one-third log intervals to give a range of final incubation concentrations of 10^{-3} to 10^{-10} M (i.e., 10^{-3}, $10^{-3.33}$, $10^{-3.67}$ M etc.) and preincubated with the enzyme preparations for 30 min before the initiation of the reaction (addition of substrate). The amount of dimethylsulfoxide (25 μl) used in the incubation medium (1.0 ml) did not affect either AChE or BChE activity. The production of the yellow thionitrobenzoate anion was measured at a wavelength of 412 nm for a period of 30 min after initiation of the reaction (during which time the reaction rate was found to be a linear function of time: data not shown). To correct for nonspecific substrate hydrolysis, aliquots of each cholinesterase preparation were incubated under conditions of complete inhibition of either AChE ($+10^{-4}$ M BW254c51) or BChE ($+10^{-4}$ M iso-OMPA) and the change in absorbance under these conditions was subtracted from that observed with varying drug concentrations.

The enzyme activity at each concentration, expressed as a percentage of the activity observed in the absence of inhibitor, was transformed to a logit format (logit = ln (% activity/[100 − % activity]) and plotted as a function of log concentration of compound. The IC$_{50}$ was defined as the concentration, in nanomoles, at which logit = 0 (i.e., logit = ln (50/100−50). For each compound for which the IC$_{50}$ was determined, 50% or greater inhibition was achieved. Values shown are mean ± S.D. of three to five separate assays.

Results

Table 1 shows the IC$_{50}$ values of the compounds studied against human AChE (cerebral cortex, caudate nucleus and erythrocyte), electric eel AChE and human BChE (cerebral cortex and plasma). The potencies of several physostigmine analogs relative to physostigmine are illustrated in figure 3 and the relative potencies of some traditional anticholinesterases are shown in figure 4.

Comparison of inhibitor sensitivity of different AChEs and BChEs. The potencies of different physostigmine derivatives were very similar against human brain (both cerebral cortex and caudate nucleus) and erythrocyte AChE. Thus, for each compound studied, the IC$_{50}$ values of cerebral cortex, caudate nucleus and erythrocyte AChE were very similar as were the inhibition profiles of each of the human AChEs (figs. 3 and 4). These results suggest that, at least with respect to their sensitivity to anticholinesterases, human brain and erythrocyte AChE cannot be distinguished. Therefore, the term human AChE will be used to identify the enzyme irrespective of its source (i.e., brain or erythrocyte).

The electric eel AChE inhibition profiles with both physostigmine analogs and classical anticholinesterases (figs. 3 and

Synthesis of N(1)-substituted physostigmine analogues
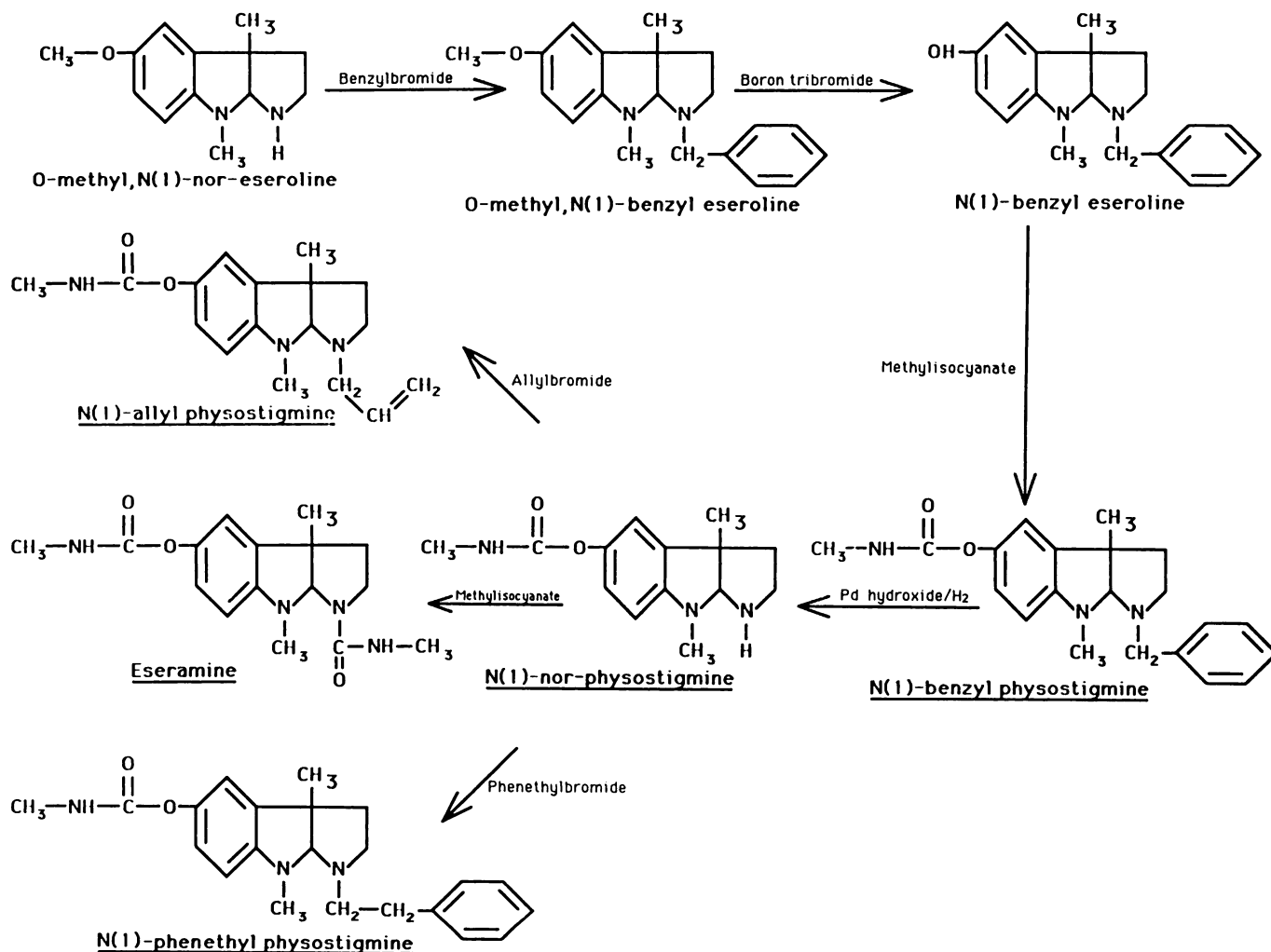

Fig. 1. Synthesis of N(1)-substituted analogs of physostigmine. The compounds whose names are underlined were tested for potency vs. AChE and BChE. A more detailed description of the synthesis of these compounds can be found elsewhere (Yu et al., 1988b).

4) differed from those for human AChE. For example, benzyl-carbamoyl eseroline is as potent as physostigmine against human AChE but has only approximately 10% of the potency of physostigmine against electric eel AChE. These results suggest, therefore, that although there seems to be no intraspecies variability in the anticholinesterase sensitivity of AChE, there is considerable interspecies variability (Silver, 1974; Andersen et al., 1977).

In general, human BChE from cerebral cortex and plasma had the same qualitative sensitivity to different anticholines-terases. Thus, compounds that inhibited cerebral cortex BChE also inhibited plasma BChE, although the inhibition profiles of cerebral cortex and plasma BChE (figs. 3 and 4) were not as similar as the profiles for different human AChEs. However, there was a marked difference in the quantitative sensitivity of these two enzymes. For example, the IC_{50} of cerebral cortex BChE and plasma BChE were, respectively, 129 ± 55 and 15 ± 5 nM for physostigmine and 5 ± 3 and 0.2 ± 0.03 nM vs. DFP. Overall, plasma BChE was approximately 10 times more sensitive to anticholinesterases than was cerebral cortex BChE.

Inhibitory properties of different physostigmine analogs. The inhibition by physostigmine of the different enzyme

types studied was stereospecific because the (+)-isomer of physostigmine was a poor anticholinesterase with an IC_{50} approximately 1000 times higher than that of the (−)-isomer. As expected, racemic (±)-physostigmine had an IC_{50} approximately twice that of the (−)-form. Eseroline, which is presumably the major metabolite of physostigmine, was a very poor anticholinesterase having an IC_{50} of greater than 10,000 nM against all enzyme types studied.

We identified seven physostigmine analogs with potencies against human AChE similar to that of physostigmine; octyl-, butyl-, benzyl- and N-phenyl-carbamoyl eseroline, N(1)-nor- and N(1)-allylphysostigmine and physostigmine methosulfate. Of these compounds, butyl-, benzyl- and N-phenyl-carbamoyl eseroline were less potent against electric eel AChE than against human AChE, emphasizing the interspecies variability in AChE anticholinesterase sensitivity. Two of these seven compounds, N-phenylcarbamoyl eseroline and physostigmine methosulfate, were relatively selective inhibitors of human AChE rather than human BChE, being 5 to 100 times more potent against AChE compared to BChE.

An additional three compounds, N(1)-phenethyl- and N(1)-benzylphysostigmine and methoxyphenylcarbamoyl eseroline,

A. Structures of physostigmine and physostigmine analogues
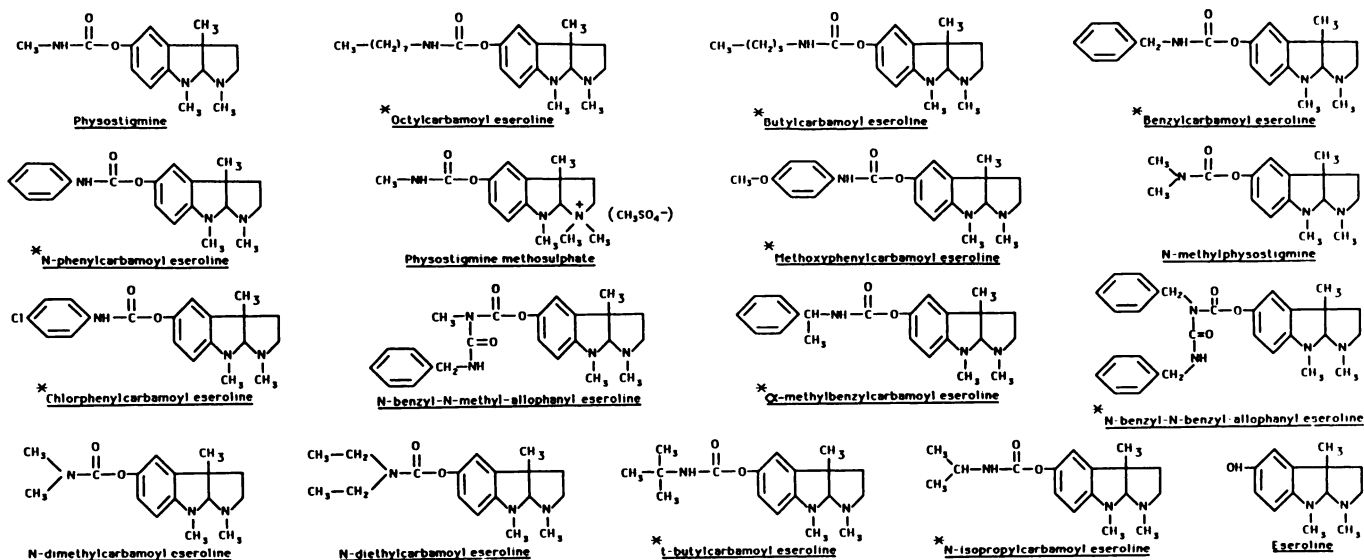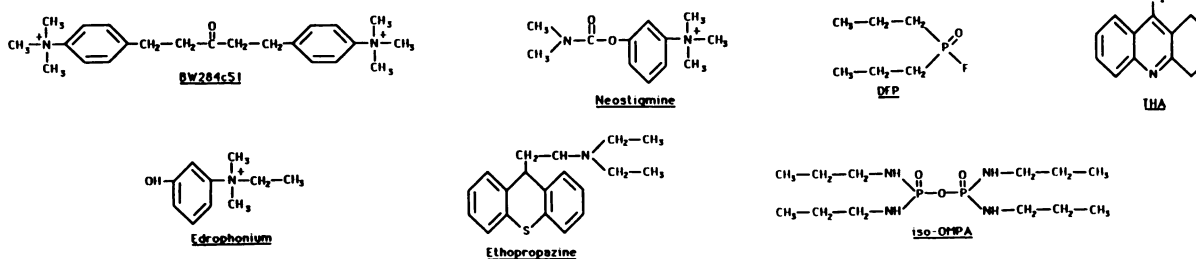

B. Structures of other anticholinesterases

Fig. 2. A, structures of physostigmine and carbamoyl analogs. Most compounds (shown with a *) were synthesized by reacting eseroline with the appropriate isocyanate (*e.g.*, eseroline + octylisocyanate → octylcarbamoyl eseroline). The synthesis of other compounds is described under "Materials and Methods." B, structures of classical anticholinesterases used in the present study for comparative purposes.

had potencies relative to physostigmine of 5 to 20% against AChE but equivalent or higher potencies against cerebral cortex and plasma BChE. Most notable was N(1)-phenethylphysostigmine which, although about one-fifth as potent as physostigmine *vs.* human AChE, was approximately 10 and 20 times more potent than physostigmine against human plasma and cerebral cortex BChE, respectively. Moreover, as N(1)-phenethylphysostigmine had an IC₅₀ against BChE that was 20 to 100 times lower than against AChE, it may be considered as being a relatively selective inhibitor of BChE.

Both N-methylphysostigmine and eseramine (which is also found in extracts of Calabar beans; Robinson and Spiteller, 1964) had relative potencies of approximately 10% against both AChE and BChE. All the other physostigmine derivatives studied had relative potencies of less than 10%. Interestingly, a chlorphenyl substitution of neostigmine (compound NU 1250) does not greatly alter the potency of the compound (Main *et al.*, 1976) whereas an analogous substitution of physostigmine reduced the potency of the compound (chlorphenylcarbamoyl eseroline) about 15-fold.

Inhibitory properties of different classical anticholinesterases. These results confirm that BW284c51 is a selective inhibitor of AChE whereas iso-OMPA and ethopropazine are selective inhibitors of BChE. Neostigmine was 6 to 50 times

and edrophonium more than 3 times more potent against human AChE than against BChE. On the other hand, compared to human AChE, DFP was about 10 times more potent against human brain BChE and approximately 250 times more potent against plasma BChE.

THA was about 3 times more potent against human BChE than human AChE. Similar, but more pronounced selective inhibition of BChE by THA has been reported previously. Heilbronn (1961) reported IC₅₀ values of THA *vs.* human erythrocyte AChE and plasma BChE of 630 nM and 25 nM, respectively, whereas Ho and Freeman (1965) observed IC₅₀ values for THA of 72 and 7500 nM against human plasma BChE and rat brain AChE, respectively.

The ratios of IC₅₀ values against human erythrocyte AChE and plasma BChE for iso-OMPA (51) and DFP (380) are comparable to previously published values (56 and 270, respectively; Usdin, 1970). In the present study, neostigmine was more potent against erythrocyte AChE than against plasma BChE whereas neostigmine has been reported previously to be less potent against erythrocyte AChE than against plasma BChE (see Usdin, 1970).

Discussion

Comparison of inhibitor sensitivity of different AChEs and BChEs. The present results show clearly that despite

TABLE 1

IC_{50} values (nanomolar) of various physostigmine analogs vs. human and electric eel AChE and human BChE

Compound	AChE				BChE	
	Cortex	Caudate	Erythrocyte	Electric eel	Cortex	Plasma
	nM				*nM*	
(−)-Physostigmine	31 ± 8[a]	35 ± 12	28 ± 7	57 ± 11	129 ± 55	15 ± 5
(±)-Physostigmine	72 ± 28	72 ± 14	70 ± 22	92 ± 40	265 ± 150	35 ± 7
(+)-Physostigmine	22,000	26,000	25,000	53,000	26,000	4,000
Octylcarbamoyl eseroline	15 ± 1	12 ± 10	16 ± 4	110 ± 110	9 ± 4	4 ± 1
Butylcarbamoyl eseroline	21 ± 4	21 ± 6	18 ± 9	152 ± 9	16 ± 4	4 ± 1
N(1)-nor-physostigmine	23 ± 1	19 ± 1	21 ± 1	57 ± 2	35 ± 7	2 ± 1
Benzylcarbamoyl eseroline	23 ± 5	32 ± 12	26 ± 8	460 ± 80	19 ± 9	3 ± 2
N(1)-allylphysostigmine	32 ± 10	41 ± 18	45 ± 26	69 ± 25	16 ± 6	3 ± 2
N-phenylcarbamoyl eseroline	36 ± 3	21 ± 1	24 ± 6	350 ± 90	2,500 ± 1,100	1,300 ± 400
Physostigmine methosulfate	37 ± 7	29 ± 4	30 ± 5	34 ± 1	1,100 ± 200	190 ± 60
N(1)-phenethylphysostigmine	150 ± 30	190 ± 60	220 ± 40	1,000 ± 400	7 ± 1	2 ± 1
N(1)-benzylphysostigmine	190 ± 90	220 ± 110	330 ± 100	1,000 ± 450	55 ± 20	10 ± 4
Methoxyphenylcarbamoyl eser-oline	230 ± 40	280 ± 7	350 ± 14	1,700 ± 390	230 ± 50	28 ± 2
N-methylphysostigmine	310 ± 110	220 ± 50	210 ± 40	970 ± 260	3,000 ± 480	420 ± 120
Eseramine	320 ± 60	400 ± 90	450 ± 190	9,300	1,400 ± 650	200 ± 35
Chlorphenylcarbamoyl eseroline	420 ± 90	450 ± 30	660 ± 40	1,800 ± 110	4,500 ± 1300	4,000 ± 540
(−)-α-methylbenzylcarbamoyl eseroline	440	660	620	11,000	N/D[b]	1,600
(+)-α-methylbenzylcarbamoyl eseroline	730	840	860	9,900	1,400	160
N-benzyl-N-methyl-allophanyl eseroline	1,500	1,500	2,000	6,300	4,800	500
N-benzyl-N-benzyl-allophanyl eseroline	15,000	16,000	>10,000	>10,000	17,000	1,600
N-dimethylcarbamoyl eseroline	16,000	8,300	9,700	10,000	170,000	130,000
N-diethylcarbamoyl eseroline	21,000	31,000	39,000	40,000	72,000	40,000
t-Butylcarbamoyl eseroline	32,000	33,000	23,000	>10,000	11,000	2,000
N-isopropylcarbamoyl eseroline	44,000	55,000	51,000	35,000	>100,000	>100,000
Eseroline	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000
	Other Anticholinesterases					
BW284c51	28 ± 12	22 ± 8	18 ± 8	8 ± 1	300,000	48,000
Neostigmine (Prostigmine)	35 ± 3	29 ± 6	27 ± 1	91 ± 6	1700 ± 530	180 ± 10
DFP	44 ± 18	67 ± 38	76 ± 48	480 ± 400	5 ± 3	0.2 ± 0.03
THA (Tacrine)	240 ± 10	180 ± 60	190 ± 40	76 ± 25	45 ± 10	47 ± 10
Edrophonium (Tensilon)	3,700 ± 300	2,900 ± 240	2,500 ± 40	780 ± 190	>10,000	>10,000
iso-OMPA	180,000	250,000	340,000	>1,000,000	6,700 ± 4,000	980 ± 550
Ethopropazine (Lysivane)	210,000	180,000	260,000	120,000	210	300

[a] Values shown are mean ± S.D. of three to five separate assays except in the case of certain compounds where single determinations were made.
[b] N/D, not determined.

their different quaternary structures, human brain AChE (primarily globular tetramers; Atack *et al.*, 1986) and erythrocyte AChE (globular dimers; Ott *et al.*, 1982) are similar with respect to their inhibition sensitivity to various anticholinesterases. Similarly, human brain, erythrocyte and muscle AChEs have been reported to show very similar sensitivities toward a number of anticholinesterases, including neostigmine and DFP (Grob and Harvey, 1958). Although human brain and erythrocyte AChE have globular quaternary associations whereas electric eel AChE molecular forms are asymmetric (Massoulié and Bon, 1982; Atack *et al.*, 1986), it is unlikely that these differences in quaternary structure account for the differences in inhibitor sensitivities between these AChEs. Thus, within a given species, there appears to be no difference in the inhibitor sensitivity of different AChE molecular forms (Vigny *et al.*, 1978; Friboulet *et al.*, 1987). However, there are well characterized species differences in substrate specificity of AChE (and BChE) (Silver, 1974; Main, 1976) suggesting that there are interspecies differences in active site configurations which would be expected to result in interspecies differences in inhibitor sensitivity (see, for example, Andersen *et al.*, 1977).

Although human brain and plasma BChE had qualitatively

similar anticholinesterase sensitivities, plasma BChE was approximately 10 times more sensitive than cerebral cortex BChE. Although the brain and plasma BChE used in the present study were not from the same subject, it is unlikely that interindividual variability would account for the different susceptibilities of brain and plasma BChE as we observed very similar IC_{50} values for the same compounds tested against BChE from different individuals (data not shown). There are differences in the distribution of BChE molecular forms in human brain and plasma (Atack *et al.*, 1986, 1987a,b), but differences in inhibitor sensitivity probably cannot be ascribed to differences in quaternary structures (see above). Moreover, although human brain BChE (50% membrane-bound) and plasma BChE (soluble) differ in their solubility characteristics, it has been shown that soluble and membrane-bound electric eel AChE and rat brain AChE do not have differential inhibition properties (Vigny *et al.*, 1978; Lenzy *et al.*, 1984). These data, however, emphasize the importance of stating the origin of the cholinesterase used in studying anticholinesterase potencies.

Carbamoyl-substituted analogs. Because it is the carbamoyl group that interacts with the esteratic subsite of AChE and BChE, changes in potency after carbamoyl substitutions
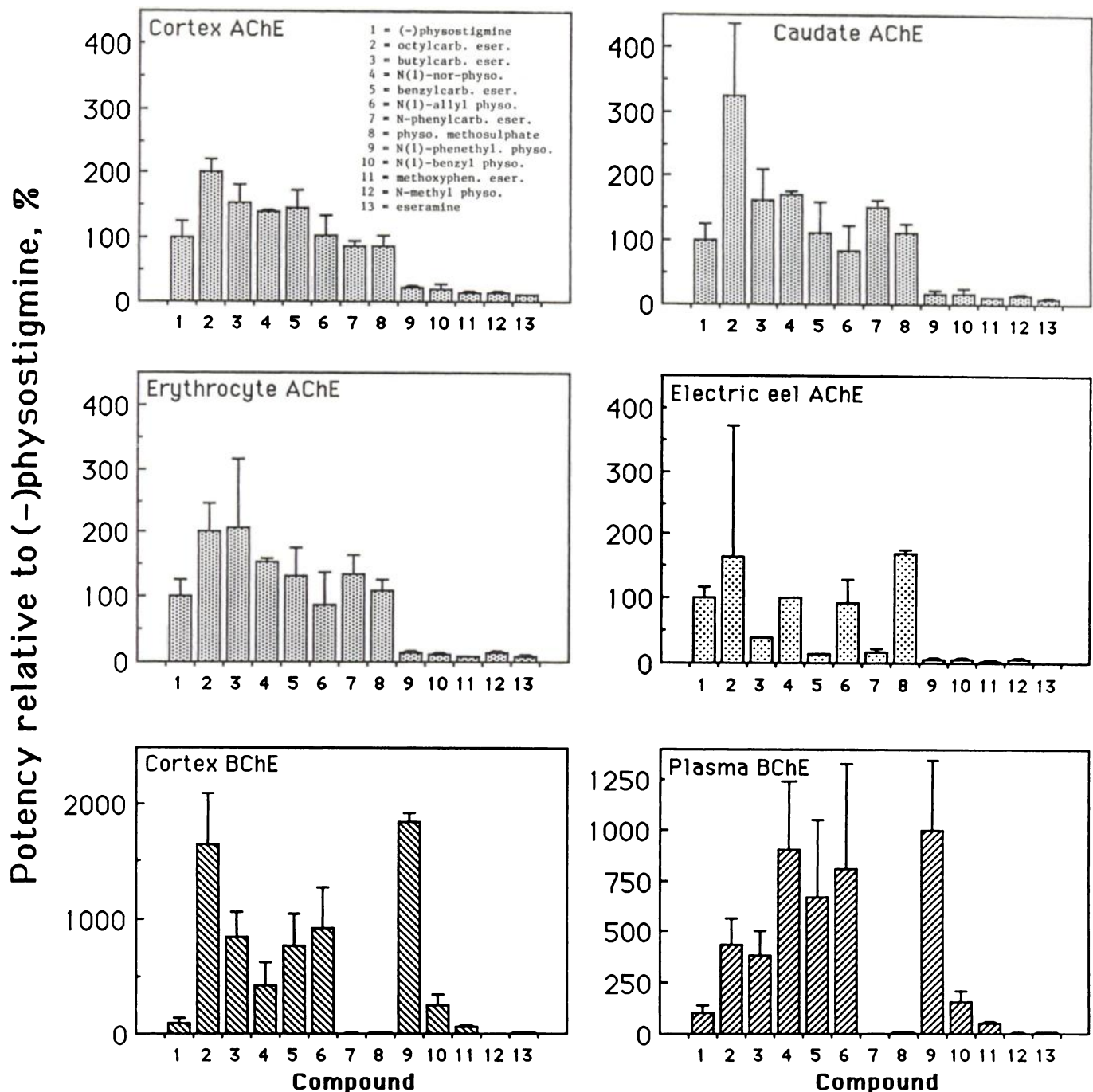
Fig. 3. Potency of various physostigmine analogs against different AChEs and BChEs expressed relative to the potency of physostigmine. Error bars show S.D.s of values obtained in three to five separate assays.

presumably reflect changes in the interaction between the physostigmine analog and the esteratic subsite of the enzyme. In general, increasing the hydrophobicity of the carbamoyl side group (*i.e.*, addition of octyl, butyl and benzyl groups) increased the potency of the compound against BChE, whereas potency increased only slightly *vs.* human AChE and decreased against electric eel AChE. This suggests that binding of the inhibitor to human BChE, and to a lesser extent to human AChE, is enhanced by increasing hydrophobic interactions at the esteratic (or an adjacent) subsite. This hydrophobic binding site presumably is not found in electric eel AChE. Although the hydrophobic binding site seems to be of more consequence in human BChE than in human AChE (*i.e.*, the potency of hydro-

phobic carbamoyl analogs increased more against BChE than AChE), it also seems to be more restricted in BChE than in AChE. Thus, whereas the phenyl and benzyl analogs have comparable potencies *vs.* human AChE, the phenyl derivative is about 50- to 100-fold less potent against human BChE than the benzyl derivative. The presence of a hydrophobic binding site in BChE might also explain why BChE hydrolyzed higher (more hydrophobic) choline esters, such as butyrylcholine, in preference to ACh (Silver, 1974).

Interestingly, the dimethylcarbamic analog (N-methylphysostigmine) was less potent than physostigmine, which is a monomethylcarbamic compound. We were unable to replicate observations that N-methylphysostigmine is about 20 times
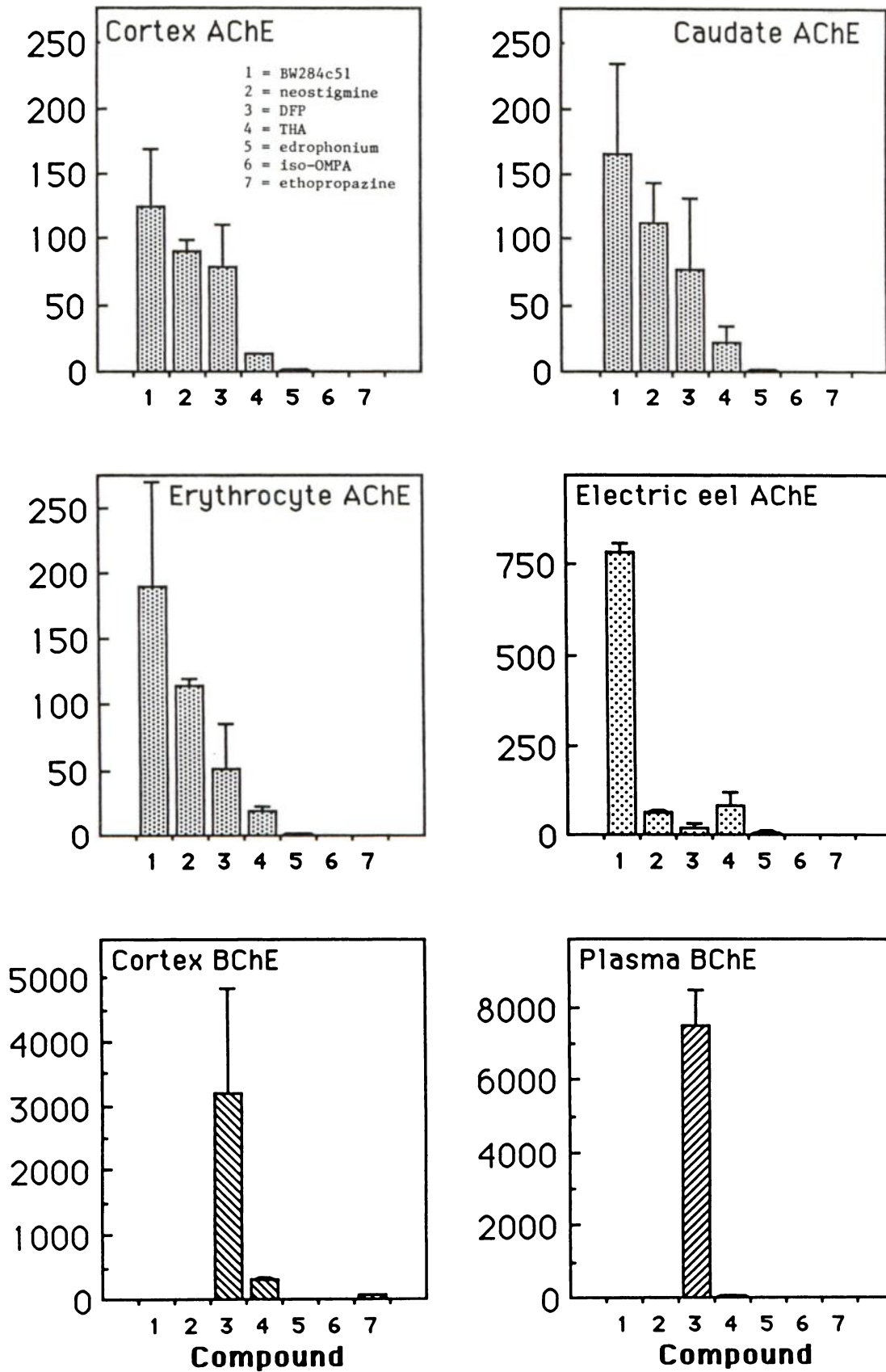
Fig. 4. Potency of various classical anticholinesterases against different AChEs and BChEs expressed relative to the potency of physostigmine. Error bars show S.D.s of values obtained in three to five separate assays. [Because of the scales used, the relative selectivities of edrophonium for AChE and iso-OMPA and ethopropazine for BChE (table 1) cannot be appreciated.]

more potent than physostigmine against electric eel AChE (Brossi et al., 1986). Furthermore, although dimethylcarbamic phenol esters have been reported to be more potent in vivo than monomethylcarbamic esters (Aeschlimann and Reinert, 1931), Wilson et al. (1961) reported that a number of mono-methylcarbamates were more potent anticholinesterases than the corresponding dimethylcarbamates.

N(1)-substituted analogs. The ring structure of physostigmine is important for binding to the anionic subsite of AChE and BChE and consequently changes in potency after substitutions at position N(1) of physostigmine would be expected to reflect changes in the interaction between the physostigmine analog and the anionic subsite of the enzyme.

Hydrophobic (allyl, phenethyl and benzyl) N(1) substitutions did not increase potency vs. AChE. However, as with substitutions of the carbamoyl group, hydrophobic substitutions in position N(1) increased potency vs. BChE, whereas introduction of a quaternary nitrogen (physostigmine methosulfate) reduced potency. The results suggest that electrostatic interactions are more important than hydrophobic binding at the anionic subsite of AChE. On the contrary, at the anionic site of BChE, hydrophobic bonding seems to be of more significance than electrostatic forces (c.f. the selectivity of charged inhibitors for AChE rather than BChE, such as BW284c51 and edrophonium).

Metabolism of physostigmine. The major route of physostigmine metabolism is presumably the hydrolysis of the ester bond by AChE and/or BChE to yield carbamic acid and eseroline, both of which are poor anticholinesterases (table 1) (Long, 1963; Hemsworth and West, 1970; Main, 1976; c.f. Galli et al., 1982 who reported that eseroline had significant anticholinesterase activity). Furthermore, although eseroline can undergo oxidation to yield rubreserine, this compound is also a poor anticholinesterase (Long, 1963; Hemsworth and West, 1970).

An additional route of physostigmine metabolism might be via demethylation at position N(1), to yield N(1)-nor-physostigmine, particularly inasmuch as N-demethylation of tertiary amines in drug metabolism is well documented (Brossi et al., 1965). Although demethylation of physostigmine to N(1)-nor-physostigmine did not greatly affect the potency vs. AChE, it did increase the potency vs. BChE 4- to 8-fold. Consequently, if demethylation is a route of physostigmine metabolism, it would have the effect of making the compound a preferential inhibitor of BChE (c.f. IC$_{50}$ values of erythrocyte AChE and plasma BChE against N(1)-nor-physostigmine of 21 ± 1 and 2 ± 1 nM, respectively).

In vitro vs. in vivo potency. Although in the present study we have identified compounds that inhibit AChE in vitro, pharmacokinetic factors will ultimately determine which of these compounds, if any, is useful in inhibiting brain AChE in vivo. The major pharmacokinetic factors involved in determining whether a potent in vitro AChE inhibitor is of use in vivo include: 1) plasma protein binding; 2) interaction with BChE; 3) the permeability of the compound across the blood-brain barrier; and 4) half-life of the compound. For example, alterations in hydrophobicity may greatly alter the plasma protein binding of physostigmine derivatives (Unni and Somani, 1985). Furthermore, although physostigmine methosulfate is a preferential inhibitor of AChE rather than BChE, its quaternary N(1) nitrogen (which is analogous to the quaternary nitrogen in neostigmine) presumably would prevent this compound

crossing the blood-brain barrier into the central nervous system. Consequently, this compound might, like neostigmine, be of more use as an inhibitor of peripheral rather than central AChE.

With respect to the effects of interaction with BChE, N-phenylcarbamoyl eseroline is of potential interest for in vivo studies as it is 50 to 100 times more potent against AChE than against BChE and might therefore be expected to have more selective cholinergic effects. In addition, although the octyl, butyl and benzyl derivatives have potencies against AChE similar to physostigmine, they are more potent than physostigmine against BChE. Consequently, in vivo these compounds may preferentially bind to BChE, thereby reducing their anticholinergic potency. Nevertheless, heptylcarbamoyl eseroline reportedly has a longer duration of serum and brain AChE inhibition and is less toxic than physostigmine (Brufani et al., 1986, 1987). Compounds with both selectivity for brain AChE and sustained action in the brain will presumably be of most use for in vivo studies. In vivo testing will be necessary to determine which of the compounds identified in the present study as potent in vitro may have clinical utility.

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