Anatomy of Cholinesterase Inhibition in Alzheimer’s Disease: Effect of Physostigmine and Tetrahydroaminoacridine on Plaques and Tangles

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The histochemical distribution of cholinesterases in the cerebral cortex and their response to cholinesterase inhibitors such as physostigmine and tetrahydroaminoacridine (THA) were investigated in brains from patients with Alzheimer's disease and control subjects. In the temporal neocortex of the control subjects, most of the cholinesterase activity was located within axons and cell bodies belonging to cholinergic pathways. In keeping with their well-known cholinomimetic effects, physostigmine and THA effectively inhibited this cholinesterase activity. Cholinesterase-containing normal axons (and in some cases cells) were severely depleted in the cerebral cortex of patients with Alzheimer's disease. Although the cerebral cortex of these patients continued to display abundant cholinesterase activity, the location of this enzyme was largely shifted to the neuritic plaques and neurofibrillary tangles. In fact, the majority of these pathological structures demonstrated intense acetylcholinesterase and butyrylcholinesterase activities. Physostigmine and THA were potent inhibitors of these plaque- and tangle-bound cholinesterases as well. In patients with Alzheimer's disease, cholinesterase inhibitors would therefore appear to have a major and widespread effect directly upon the enzymatic activity of plaques and tangles. Consequently, the clinical effects of anticholinesterases in Alzheimer's disease may be based on mechanisms that are different from those that apply to the normal brain.


Cholinesterase inhibitors such as physostigmine (eserine) yield small but consistent enhancements of memory function in normal subjects [5]. Similar improvements are also obtained when physostigmine is administered to patients with amnestic disorders caused by head trauma [8], herpes simplex encephalitis [18], and even Alzheimer's disease (AD). In fact, of all the therapeutic interventions attempted in AD, physostigmine has been associated with the most consistent (albeit modest and unsustained) improvements of memory [17, 24]. A more recent and as yet unrepli-cated study on patients with presumed AD reported the marked therapeutic effects of tetrahydroaminoacridine (THA), another cholinesterase inhibitor [21].

The cholinergic hypothesis of memory function and the cholinomimetic effect expected from acetylcholinesterase (AChE) inhibitors provide the rationale for using physostigmine in the treatment of amnestic conditions [3]. The expectation that physostigmine is likely to act as a cholinomimetic in the central nervous system is based on several lines of evidence. It is a potent cholinergic agonist in the peripheral nervous system [13], crosses the blood–brain barrier [24], influences the cortical electroencephalogram [19], and rapidly reverses the memory deficits caused by central nervous system cholinergic antagonists such as scopolamine [6]. Furthermore, anatomical observations in experimental animals have shown that the vast majority of AChE activity in the cerebral cortex is located along cholinergic axons and cholinceptive neurons [16]. Inhibiting the hydrolysis of the acetylcholine released by these cholinergic axons should increase the impact of this transmitter upon cholinceptive receptor sites and therefore yield a net cholinomimetic effect.

Most of the information on cortical AChE has been derived from laboratory animals. Considerably less detail is available on the cholinesterases of the human cerebral cortex. In this report, we describe the anatomical distribution of these enzymes in the human brain.

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and their response to cholinesterase inhibitors. Our observations in nondemented individuals are in keeping with the predominantly cholinomimetic effect expected from cholinesterase inhibitors. However, our observations also indicate that the distribution of cholinesterases is markedly altered in patients with AD. As a consequence of this altered anatomical distribution, cholinesterase inhibitors appear to have a major effect directly upon the neuritic plaques and neurofibrillary tangles of patients with AD. This unanticipated site of action suggests that the potential role of cholinesterase inhibition in the treatment of AD need not be confined to cholinomimetic effects. The altered anatomical distribution and enzymatic properties of cortical cholinesterases also raise new questions concerning pathophysiological mechanisms associated with AD.

**Methods**

The observations reported here are based on 7 brains. Extensive clinical information was available for each specimen. Four of the brains came from individuals (43-, 63-, 69-, and 91-year-old men) without dementia; the other 3 were obtained from patients (78-year-old man, two 88-year-old women) with a clinical history typical of severe AD [12].

The interval from death to autopsy varied from 2 to 24 hours. Coronal brain slabs 1 to 2 cm thick were fixed by immersion for 24 to 30 hours in a 4% paraformaldehyde solution buffered with phosphate (0.1 M at pH 7.4). After fixation, the tissue was taken through gradually increasing concentrations (10 to 40% w/v) of sucrose dissolved in the same phosphate buffer. The brain slabs were then frozen on dry ice and cut into 40-μm sections with a microtome. The temporal lobe was available in all brains. In 4 of the brains, additional tissue from the frontal and cingulate regions was also examined.

Sections stained with Bielschowsky's silver method, thioflavin-S, hematoxylin-eosin, and thionin provided the information for diagnostic evaluation. This material did not show senile plaques, neurofibrillary tangles, or other pathological findings in the brains of the 43-, 63-, and 69-year-old nondemented individuals. The brain of the 91-year-old man (normal mental state until a terminal cerebellar hemorrhage) contained neurofibrillary tangles mostly confined to limbic areas of the temporal lobe and neuritic plaques in many cortical areas. The number and distribution of plaques and tangles in this brain were consistent with age-related changes seen in nondemented very old individuals [12]. In addition to the terminal cerebellar hemorrhage, rare cortical microinfarctions were noted. The brains of the 3 individuals with progressive dementia contained dense accumulations of plaques and tangles in all limbic and neocortical areas that were examined. These observations led to a definitive diagnosis of advanced AD in these 3 brains.

The observations reported here are based on the visualization of cholinesterase activity with the help of a new and highly sensitive method. The principles of this method (incubation in a dilute Karnovsky-Roots medium followed by metal ion–diaminobenzidine intensification) were described by Hanker and co-workers [9] and Tago and associates [22]. We introduced a number of changes, outlined below. We had used this method to demonstrate intense cholinesterase activity in the plaques and tangles of patients with AD [15]. Traditional cholinesterase methods (e.g., the Koelle or Karnovsky-Roots procedures [14]) do not yield comparable results.

The cholinesterase solution was prepared by adding 4 liters of 1,000 ml distilled water: 17.186 gm maleic acid (disodium salt), 147 mg sodium citrate, 75 mg copper sulfate, and 16.4 mg potassium ferricyanide. The pH was adjusted to the desired value by titrating with drops of glacial acetic acid or 10 N sodium hydroxide. To this solution, 106 mg acetylthiocholine iodide (molecular weight [MW] 289.2) or the equimolar amount of 116 mg butyrylthiocholine iodide (MW 317.2) was added to demonstrate AChE or butyrylcholinesterase (BChE) activities, respectively. Cholinesterase inhibitors such as ethopropazine hydrochloride (MW 348.9), physostigmine salicylate (MW 413.5), or 1,2,3,4-tetrahydrol-9-aminoacridine hydrochloride (MW 234.73) were added in the desired amounts. The brain sections used in these experiments were first mounted on slides, air dried overnight, and rinsed in 0.1 M maleate buffer (at the same pH as the subsequent cholinesterase incubation) for 5 to 10 minutes. The sections were then incubated in the cholinesterase solution for 3 to 5 hours. At the end of the incubation, the sections were rinsed in an 0.1 M tris buffer at pH 7.6 for 5 to 10 minutes. This was followed by an intensification step for 10 minutes in a solution of 0.5% (w/v) cobalt chloride in the same tris buffer [1]. The sections were then rinsed for 5 to 10 minutes in the tris buffer and incubated for 3 to 5 minutes in a solution containing 50 mg of dianisobenzidine tetrahydrochloride and 3.3 ml of 0.3% (w/v) hydrogen peroxide per 100 ml of the tris buffer. This was followed by a final rinse in the tris buffer and the sections were then air dried, dehydrated in graded alcohols, cleared in xylene, and coverslipped under Permount.

Pilot studies showed that ethopropazine hydrochloride at a concentration of 7.2 mg/liter in the cholinesterase incubation solution led to a near total inhibition of staining with butyrylthiocholine as the substrate but had almost no effect on the staining of neurons, axons, plaques, and tangles with acetylthiocholine as the substrate. This concentration of ethopropazine was therefore used in the incubation medium to obtain specific AChE staining with acetylthiocholine as the substrate.

The following histochemical procedures were used: (1) For the selective demonstration of AChE, 7.2 mg of ethopropazine hydrochloride per liter was added to the incubation medium with acetylthiocholine as a substrate. (2) For the demonstration of BChE, butyrylthiocholine was used as the substrate without the addition of an inhibitor. (3) For some sections, both acetylthiocholine and butyrylthiocholine were added to the incubation medium (without any inhibitors) to reveal the combined cholinesterase staining (AChE + BChE) in the tissue. (4) In additional experiments with each of the two substrates, physostigmine salicylate or THA was added to the incubation medium in concentrations ranging from 10⁻⁷ M to 10⁻⁴ M to determine the characteristics of cholinesterase inhibition. No ethopropazine was used in these experiments.
To assess the time course of the physostigmine and THA inhibition, some experiments included a brief initial exposure to these inhibitors followed by variable intervals (up to 15 hours) of soaking in maleate buffer. The sections were then incubated in the cholinesterase solution to determine the reversibility of the initial exposure to the inhibitor.

For the purpose of counting, no distinction was made between strongly and lightly stained plaques and tangles. All comparisons within a given brain were based on matching tissue sections, and all comparisons within and across specimens were done in the same cytoarchitectonic region.

Results
Effects of pH
In a limited number of sections, the pH of the incubation solution was varied in 0.2 increments from 6.0 to 8.4. The cholinesterase staining in cortical axons and probably also in cell bodies was strongest at pH 8.0. However, the best cholinesterase staining of plaques and tangles was obtained at pH 6.8 to 7.0. On the basis of these observations, the incubations were conducted at pH 6.8 (or 7.0) and 8.0. At pH 6.8 and 7.0 cholinesterase activity was present in mature as well as immature plaques, in the amyloid core as well as in the neuritic halo, and in intracellular as well as in extracellular tangles.

Normal Brain Versus AD
Limbic and cortical areas in the brains of all nondemented individuals contained a dense plexus of AChE-rich axons and probably also in cell bodies was strongest at pH 8.0. In most cases, these sections also contained many AChE-rich cell bodies, especially in Layers 3 and 5 of neocortex (Fig 1A). When the incubation was carried out at pH 7.0, the AChE fiber staining was of lesser intensity. At this pH, the sections from the 91-year-old nondemented man also showed some AChE-rich neuritic plaques (Fig 1B). The BChE reaction (at pH 7 and 8) was essentially negative (except for some staining in extracellular tangles) in the limbic and cortical areas of the 43-, 63-, and 69-year-old nondemented individuals. A few neurons in the deep cortical layers gave a positive BChE reaction, but this was inconsistent. At pH 7.0, the brain of the 91-year-old nondemented man also contained BChE-rich plaques (Fig 1C).

The cholinesterase distribution in the brains from individuals with AD was drastically different from that of nondemented individuals (Table). In the neocortical areas of the temporal lobe, there was an almost total absence of AChE-rich fibers even when the incubation was carried out at pH 8.0 (Fig 1D). Some AChE-rich fibers were seen in the hippocampal, entorhinal, amygdaloid, cingulate, and frontal regions but were much reduced when compared to the staining of the 91-year-old nondemented man. In some cases, but not in all, the AChE-rich cortical cell bodies were also diminished. At pH 7.0, the brains of patients with AD showed dense accumulations of AChE-rich plaques and tangles (Fig 1E). The BChE reaction was essentially negative at pH 8.0, but showed extensive plaque and tangle formations at pH 7.0 (Fig 1F). We previously reported that deeper cortical layers (e.g., Layer 5) contained both BChE- and AChE-rich tangles, whereas the more superficial layers (e.g., Layer 3) contained mostly AChE-rich tangles [15]. The same pattern was observed in these specimens. Neuritic plaques containing AChE or BChE were seen in almost all cortical layers and in all areas available for examination.

Quantitative Importance of Cholinesterase-Containing Plaques and Tangles
Friede [71 reported that neuritic plaques are positive for AChE and BChE activities but did not indicate what proportion of plaques contained these enzymes. We examined cholinesterase positivity in neurofibrillary tangles and reported that at least 50% of thioflavin-S positive neurofibrillary tangles were likely to be rich in AChE [15]. To gain a semiquantitative impression of how many plaques and tangles were likely to contain cholinesterase activity, we incubated a limited number of sections in the presence of both butryrythiocholine and acetylthiocholine. The total AChE plus BChE staining was then compared to thioflavin-S staining in immediately adjacent sections from the same brain (Fig 2). In many brain regions this combined cholinesterase stain revealed at least as many plaques as the thioflavin-S stain and approximately 50 to 75% as many tangles. Given that thioflavin-S is one of the most sensitive stains for visualizing plaques and tangles [27], it would appear that cholinesterase staining in plaques and tangles is widespread if not universal.

Inhibition with THA and Physostigmine
The cholinesterase activity of plaques and tangles was exquisitely sensitive to both THA and physostigmine. The two substances had comparable effects and therefore will be described jointly. In the AChE incubation at pH 6.8, a slight decrease in the intensity of AChE activity within plaques and tangles was already visible at an inhibitor concentration of $10^{-7}$ M. Only ghosts of plaques and tangles remained at a concentration of $4 \times 10^{-7}$ M (Fig 3A, B). By the time a concentration of $3 \times 10^{-5}$ M was reached, nearly all AChE activity within cortical plaques and tangles was eliminated (Fig 3C).

Both THA and physostigmine were more potent in inhibiting BChE than in inhibiting AChE. At a concentration of $10^{-7}$ M, there was a severe depression of BChE staining intensity, and at $4 \times 10^{-7}$ M all plaque and tangle staining was inhibited (Fig 3D–F).

In normal controls as well as in patients with AD,
the AChE in normal axons and cell bodies (visualized by incubation at pH 8.0) was severely depressed at a concentration of $4 \times 10^{-7}$ M and almost completely abolished at $1.9 \times 10^{-6}$ M. On occasion, THA appeared to be slightly more potent (by a factor of 2) than physostigmine for inhibiting cholinesterases in plaques, tangles, axons, and cell bodies, but this was not a consistent finding.

**Time Course of Cholinesterase Inhibition**
Sections from the cingulate cortex of an 88-year-old woman with AD were first incubated for 1 to 5 minutes in a 1.6 $\times 10^{-5}$ M solution of THA or physostigmine. These sections were placed in a 0.1 M maleate buffer at pH 6.8 for up to 15 hours and were then processed for the demonstration of AChE and BChE at the same pH. The resultant staining was qualitatively compared with matched sections that had not been exposed to the inhibitor and also with matched sections that were incubated in a solution containing the same concentration of the inhibitor. This material showed that approximately 75% of the inhibition obtained by having the THA or physostigmine directly in the incubation medium could be obtained with a 1- to 5-minute exposure to the inhibitor followed by a 15-hour interval in an inhibitor-free medium. At least in this in vitro system, therefore, the cholinesterase inhibition can last for many hours after an initial exposure.

**Discussion**
In the normal brain, the great majority of cortical cholinesterase activity is expressed in the form of axonal and perikaryal AChE. These AChE-positive intracortical fibers have their cell bodies within the nucleus basalis and convey cholinergic impulses to the AChE-containing cholinceptive neurons of the cerebral cortex [16]. Our histochemical observations and many additional lines of evidence show that physostigmine and THA are potent inhibitors of the AChE along these cholinergic pathways. The resultant cholinomimetic effect is thought to underlie the memory improvement that emerges upon the administration of physostigmine to normal subjects and to patients who have amnestic conditions caused by head injury and encephalitis.

The evidence in this report shows that the distribution of AChE is markedly altered in the brains of patients with AD. Relatively little AChE remains within normal axons. Instead, intense AChE activity appears in the neuritic plaques and neurofibrillary tangles. In some regions of the brain, these pathological structures appear to contain virtually all of the cortical AChE. Abundant BChE activity is also seen in the plaques and tangles, whereas this enzyme is not conspicuous at all in the neuronal structures of the normal cerebral cortex.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>pH</th>
<th>Normal Young Adult</th>
<th>Nondemented Aged</th>
<th>Alzheimer's Senile Dementia</th>
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<tr>
<td></td>
<td></td>
<td>Perikaryon</td>
<td>Axon</td>
<td>Plaque</td>
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<tr>
<td>AChE</td>
<td>6.8-7</td>
<td>3</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>3</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>BChE</td>
<td>6.8-7</td>
<td>1/0</td>
<td>0</td>
<td>0</td>
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<tr>
<td></td>
<td>8</td>
<td>1/0</td>
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*0 = absent; 1 = occasional; 2 = moderate; 3 = frequent; 4 = dense.

Two numbers indicate range within that group.

AChE = acetylcholinesterase; BChE = butyrylcholinesterase.
Fig 2. (A) Brain tissue from Layers 3 to 5 of midtemporal cortex (Area 21) in one of the 88-year-old women with Alzheimer's disease. The tissue was incubated at pH 6.8 to show combined acetylcholinesterase and butyrylcholinesterase activity in plaques (single arrow) and tangles (double arrow). (B) Same region from an immediately adjacent section stained with thioflavin-S for the immunofluorescent dark-field demonstration of plaques (single arrow) and tangles (double arrow). A comparison of these two panels shows that each method demonstrates a comparable number of plaques and tangles. Therefore, the cholinesterase positivity of these structures appears to be a widespread phenomenon. (× 100 before 15% reduction.)
tex. Therefore, assays of BChE activity (at the appropriate pH) in biopsy samples and in the cerebrospinal fluid may provide particularly useful diagnostic information for identifying patients with AD.

The cholinesterase positivity of cortical plaques and tangles is not an occasional event but a widespread and perhaps universal phenomenon. AChE-rich tangles are distributed somewhat differently than BChE-rich tangles [15]. However, we do not know yet whether these enzymes identify independent populations of plaques and tangles or whether there is also some coexistence. Physostigmine and THA effectively inhibit not only the AChE of normal axons and perikarya but also the cholinesterases of plaques and tangles, especially the BChE.

These observations suggest that the effects of physostigmine and THA are likely to be different in patients with AD and in normal individuals. In the cerebral cortex of patients with AD, THA and physostigmine should have a substantial impact directly upon plaques and tangles since most of these structures are intensely cholinesterase positive and since they frequently contain most of the cortical cholinesterase activity. Furthermore, the cholinomimetic effect of cholinesterase inhibitors is likely to be attenuated because there is such a marked depletion of AChE-containing cholinergic axons and cholinergic perikarya. The encouraging clinical results reported after administration of cholinesterase inhibitors to patients with AD could thus reflect an inhibition of plaque- and tangle-bound cholinesterase activity rather than (or in addition to) the more conventional cholinomimetic effect associated with these substances. This may explain why equally positive results have not been obtained when other putative cholinomimetics such as pilocarpine and lecithin (not expected to have a direct impact on plaques and tangles) have been administered to these patients [4, 21, 24, 25]. A somewhat intermediate situation may exist in the cerebral cortex of very old nondemented individuals, in whom cholinesterase inhibitors would be expected to act predominantly as cholinomimetics. However, such inhibitors would also influence the cholinesterase activity of the plaques and tangles associated with aging.

The role of these enzymes in plaques and tangles is unknown. Perhaps the cholinesterase activity indicates that plaques and tangles have formed within axons and cell bodies that had been cholinesterase positive in the premorbid period. Although this hypothesis may be partially correct, several objections can also be raised [15]. For example, the normal brain contains very little cortical BChE activity, whereas many cortical plaques and tangles in patients with AD are BChE rich. Thus, the BChE within plaques and tangles could not be a marker for premorbidly BChE-positive neuronal structures. Furthermore, the optimal pH for the cholinesterases within normal axons (pH 8) is different from the optimal pH of the plaque- and tangle-bound cholinesterases (pH 6.8 to 7). It is therefore necessary to consider the possibility that the cholinesterase activity in plaques and tangles could be arising de novo in response to the overall degenerative process underlying AD. Perhaps the natural substrates for these enzymes in patients with AD include esters other than acetylcholine and butyrylcholine. It is conceivable that these plaque- and tangle-bound enzymes could even play a role in the pathogenesis of AD. In addition to their well-known esteratic activity, cholinesterases are also powerful proteases [2]. This proteolytic action could transform a circulating or neuronally formed amyloid precursor into the insoluble subunit found within plaques and tangles [11, 23]. Alternatively, the proteolytic-esteratic activity of cholinesterases could directly contribute to the degenerative changes of cytoskeletal proteins and lead to plaque and tangle formation. If these speculations were to find further support, cholinesterase inhibitors could assume a role in arresting the pathological processes of AD.

Both physostigmine and THA are potent cholinesterase inhibitors. The commonly employed dosages for physostigmine range from 0.5 to 3 mg. A subcutaneous physostigmine dose of 0.67 mg results in a peak plasma concentration of 5 ng/ml [26]. Our experiments show that cholinesterase inhibition in vitro begins at physostigmine concentrations of 0.06 ng/ml (corresponding to $10^{-7}$ M) and that this inhibition is complete at concentrations of 13 ng/ml. Therefore, the inhibition of cholinesterases within plaques and tangles occurs at physostigmine concentrations that can be reached in clinical trials. The plasma levels associated with pharmacological doses of THA are not yet known. However, assuming comparable absorption and distribution characteristics, it is likely that the plasma THA levels reached in clinical trials are at least as high as those necessary to inhibit brain cholinesterase activity. Our experiments also showed that a single exposure to physostigmine or THA can result in cholinesterase inhibition lasting for at least 15 hours. Although the plasma half-life of physostigmine is no longer than 20 minutes, its effect upon brain cholinesterase may thus last for much longer. Prior observations have shown that physostigmine and THA are both slightly more potent inhibitors of BChE than of AChE [10, 20]. The histochemical results reported here confirm this relationship. Additional observations are necessary to determine if these in vitro histochemical results are applicable to in vivo enzyme activity.

The clinical trials reporting the therapeutic effect of physostigmine and THA in patients with AD need further confirmation. Regardless of the outcome obtained in such trials, however, the histochemical obser-
Fig 3. All photomicrographs are from the second 88-year-old woman with Alzheimer's disease. All sections were adjacent to each other. The photographed area contains Layers 4 to 5 of the superior temporal auditory association cortex (Area 22). (A) Incubation for acetylcholinesterase (AChE) activity was carried out at pH 6.8. Numerous AChE-rich plaques (single arrow) and tangles (double arrow) are present. (B) Same incubation as in (A) but with the addition of tetrahydroaminoacridine (THA) at a concentration of $4 \times 10^{-7} \text{M}$. The THA greatly inhibits the AChE activity, leading to reduced staining of plaques (single arrow) and tangles (double arrow). (C) Same incubation as in (A) but with the addition of physostigmine at a concentration of $3 \times 10^{-5} \text{M}$. Almost all AChE staining is inhibited except for rare ghosts of plaques (arrow). (D) Incubation for butyrylcholinesterase (BChE) at pH 6.8. Numerous BChE-rich plaques (single arrow) and tangles (double arrow) are present. (E) Same incubation as in (D) but with the addition of $4 \times 10^{-7} \text{M}$ THA. Virtually all BChE activity is inhibited. Equimolar concentrations of THA (or physostigmine) are more potent for inhibiting BChE (E) than for inhibiting AChE (B). (F) Same incubation as in (D) but with the addition of $3 \times 10^{-5} \text{physostigmine.}$ All BChE activity remains inhibited. Although (B) and (E) show the effect of $4 \times 10^{-7} \text{M} \text{THA}$, equimolar concentrations of physostigmine had very similar effects. The $3 \times 10^{-5} \text{physostigmine}$ effects shown in (C) and (F) were also obtained with equimolar concentrations of THA. (× 160 before 12% reduction.)

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