Isolation and Chemical Characterization of Alzheimer’s Disease Paired Helical Filament Cytoskeletons: Differentiation from Amyloid Plaque Core Protein

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Abstract. The paired helical filaments (PHFs) of Alzheimer’s disease were purified by a strategy in which the neurons and amyloid plaque cores of protein (APCP) were initially isolated. This was achieved by several steps of isocratic sucrose centrifugations of increasing molarity and a discontinuous isotonic Percoll density gradient. After collagenase elimination of contaminating blood vessels, lysis of neurons was produced by SDS treatment. The released PHF cytoskeletons were separated from contaminating APCP and lipofuscin by sucrose density gradient. A final step consisted in the chemical purification of highly enriched PHFs and APCP components via a formic acid to guanidine hydrochloride transition. PHFs and APCP were fractionated by size exclusion HPLC and further characterized and quantitated by automatic amino acid analysis. We also present some of the morphological and immunochemical characteristics of PHF polypeptides and APCP. Our studies indicate that apart from differences in localization and morphology, PHF and APCP significantly differ in (a) chemical structure (peptide and amino acid composition); (b) epitope specificity (antiubiquitin, antitau, antineurofilament); (c) physicochemical properties (structural conformation in guanidine hydrochloride); and (d) thioflavine T fluorescence emission. These parameters strongly suggest important differences in the composition and, probably, in the etiopathology of PHF and APCP of Alzheimer’s disease.

Alzheimer’s disease (AD) is a primary degenerative dementia characterized by a progressive loss of memory, perception, orientation, reasoning, and judgement that escalates with time until all the intellectual and social functions of the individual are lost (61, 65, 68). Two outstanding histopathological lesions found mainly in the cerebral cortex of the Alzheimer degeneration are the neuritic (senile) plaques and the intracellular neurofibrillary tangles (NFTs) (30, 31, 62, 63). Neuritic plaques are a collection of dystrophic neurites surrounding an extracellular deposition of filamentous structures, known as the amyloid plaque core protein (APCP), whose major component has been recently isolated and characterized (13, 36, 37). Apparently these filaments are produced by the polymerization of a short polypeptide chain (the β-amyloid protein), derived from the processing of a much larger precursor (28, 33, 49, 60), which resembles a cell surface receptor and whose mRNA is expressed in neuronal cells (3, 9, 14, 21, 73). The gene responsible for this putative receptor has been mapped to human chromosome 21 (15, 50, 58). However, the cells that ultimately produce these anomalous extracellular amyloid filaments have not been unequivocally identified (36, 42, 52, 62, 63, 67, 69–71). Neurofibrillary tangles represent an intracytoplasmic collection of paired helical filaments (PHFs), made of two helically intertwined 10-nm-diam filaments, with a 70-nm periodicity (30, 31, 62, 63). The origin and precise chemical nature of these filaments are still a highly debated issue (1, 12).

Even though NFTs and neuritic plaques are the major histopathological lesions of AD, they are not restricted only to this disease. Tangles are formed in brains of people with several other neurological disorders including the Parkinson dementia complex of Guam, Down syndrome, and as the result of trauma in the aftermath of boxing (11, 22). Furthermore, tangles and plaques also appear to be components of the brain of normal elderly individuals (64). However, because of the extensive damage that these lesions cause to the central nervous system, tangles and plaques may play an important role in the dementing process of AD. © The Rockefeller University Press. 0021-9525/88/12/2703/14 $2.00

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1. Abbreviations used in this paper: AD, Alzheimer’s disease; APCP, amyloid plaque core protein; DAB-S, disaggregation buffer containing 10 mM Tris-HCl, pH 7.4, 0.25 M sucrose, 2 mM EDTA, 200 mg/ml PMSF, 500 µg/µl leupeptin, 700 µg/ml pepstatin, 50 µg/liter Gentamicin sulfate, and 250 µg/liter Amphotericin-B; MAP, microtubule-associated protein; NFT, neurofibrillary tangles (light microscopic description); PHF, paired helical filament (electron microscopic appearance); TBS, Tris-buffered saline (10 mM Tris-HCl, pH 7.4, 0.15 M NaCl, and 0.02% Tween-10).
Studies using large pedigrees from a form of familial AD seem to suggest a genetic origin for this type of AD (57). Using restriction fragment length polymorphisms and linkage analysis, the primary genetic defect in these individuals was localized a few centimorgans away from the β-amyloid locus in chromosome 21 (59, 66). This suggests that, while the β-amyloid protein is important for the disease state, it is not the primary defect at least in this form of familial AD. In addition, it has not been shown that either familial AD or sporadic AD (the latter representing the majority of the AD cases) is caused by the same genetic defect.

It has been reported by some investigators that PHFs of AD are chemically identical to the extracellular β-amyloid protein of the neuritic plaque (36). This has become a controversial issue since PHFs seem to carry epitopes shared by normal cytoskeletal elements and ubiquitin. Moreover, after depolymerization of PHF cytoskeletons rearranged into their native configuration. By contrast, APCP repolymerized from homogeneous 10-nm filaments.

Helical Filaments

Materials and Methods

Isolation of Neuronal Cells

Brains from eight patients who died as the result of AD were obtained within an average period of 8 h postmortem. The right cerebral hemispheres were immediately frozen and stored at −70°C until the moment of use. The left cerebral hemispheres were histologically processed for diagnostic and morphometric studies. The clinical diagnosis of primary degenerative dementia of Alzheimer type was confirmed in every instance not only by routine neuropathologic examination but also by accepted quantitative criteria. Each case met the diagnostic criteria for neuritic plaques and neurofibrillary tangles recommended by the National Institutes of Health Neuropathology Panel (29). Additionally, each case fulfilled the rigorous morpomorphic criteria published from the Dementia Study Laboratory, University of Western Ontario (4, 5). A sample of the dramatic histopathologic severity of lesions in these eight hemispheres is provided in Table 1.

| Age | Sex | Tangle Index/mm² | Grana Index/mm² | Neuronal Density/mm² |
|-----|-----|------------------|-----------------|---------------------|
| 63  | F   | 159.8            | 317.1           | 3,500               |
| 78  | M   | 27.9             | 403.4           | 3,898               |
| 74  | M   | 168.1            | 452.1           | 5,857               |
| 77  | F   | 93.3             | 292.1           | 2,548               |
| 76  | M   | 136.1            | 426             | 5,551               |
| 84  | M   | 80.2             | 251             | 5,185               |
| 69  | M   | 113.6            | 223.5           | 3,806               |
| 74  | M   | 101.8            | 322.1           | 4,995               |

* Of these 24 parameters quantified in the eight cases, 23 exceed the minimum severity required (tangles >20/mm²; granulovacuoles >55/mm²; neurons <5,600/mm²).

In one case, two of the three lesions exceed the minimum, but only one of three need do so to confirm the diagnosis (4).
PHF cytoskeletons, lipofuscin granules, and a small amount of unidentified insoluble cell debris. These pellets were resuspended in 50 ml of 1.5% SDS and submitted to a linear sucrose density gradient prepared in 50 mM Tris-HCl, pH 8.0, 1.5% SDS and formed by 2 ml each of 2.1, 2.0, 1.7, 1.4 M sucrose and 1.5 ml each of 1.3 and 1.2 M sucrose, respectively. A total of 48 gradient tubes (14 × 89 mm) were prepared. A 1-ml aliquot of the above SDS-insoluble material was loaded at the top of each of the tubes and centrifuged at 200,000 g for 1 h at 20°C in an SW 41 Ti rotor (Beckman Instruments, Inc.). The resulting interfaces were aspirated with a peristaltic pump and the sucrose removed by dilution and three washes with 0.5% SDS in 25 mM Tris-HCl, pH 8.0. The 2.1/2.0- M interface contained more than 90% pure PHF cytoskeletons; the remaining 10% consisted of lipofuscin granules and a negligible amount of APCP. The 2.0/1.7-M interface was composed of 80% PHF cytoskeletons. The 1.7/1.4-M interface, on the other hand, contained 80% APCP, 20% PHF cytoskeletons (of smaller size and sparser concentration) and lipofuscin granules. The 1.3/1.4-M interface was composed of APCP and lipofuscin.

Since the largest quantity of PHF cytoskeletons localized at the 2.0/1.7-M sucrose interface, we further enriched these structures by resuspending them in 1.0 M sucrose, 0.5% SDS, followed by centrifugation at 200 g for 15 min in conical 1.5-ml reactive vials. A small brown pellet, mainly lipofuscin plus some PHF cytoskeletons and some large APCP aggregates, collected at the bottom of the vial. The supernatant was recentrifuged at 600 g for 15 min, yielding a small brown pellet. The pellet was discarded and a large pale brown intermediate layer plus a whitish turbid supernatant were subsequently recovered by centrifugation at 1,500 g. These latter two fractions contain more than 95% PHF cytoskeletons, which differed only in their sizes and degree of filament aggregation.

Further purification of the PHF proteins was obtained by dissolving a pellet of 150 μl of PHF cytoskeletons in 2.2 ml of concentrated (89%) formic acid, allowed to stand at room temperature for 20 min, followed by centrifugation at 1,500 g for 10 min. The supernatant was collected and centrifuged at 12,000 g for a further 10 min. The two centrifugations permitted the elimination of a small amount of remaining lipofuscin granules, mainly those entrapped in the network of PHF cytoskeletons. The supernatant containing the solubilized PHF proteins was then dialyzed (dialysis tubing cut off 1,000 D) against two changes (500 ml each) of 6 M guanidine-hydrochloride (GHCI), 125 mM Tris-HCl, pH 8.0, 5 mM DTT. When the first dialysis buffer reached 3.0, a white flocculent precipitate formed in the bag. After 15 min, the precipitate was collected through a dialysis filter made up to 50 ml with 50 mM Tris-HCl, pH 8.0. To acidify the solution, 400 μl of concentrated HCl was added before use. The fixed specimens were rinsed with water and stained for 10 min. After rinsing away the excess fluorochrome with water, the slides were mounted using Bacto FA fluid (Difco Laboratories, Inc.) and observed in a fluorescence microscope (excitation filter, 370 nm). When necessary, the quantity and quality of the purified PHF cytoskeletons and APCP preparations were determined by transmission electron microscopy, using Formvar-coated grids stained for 4 min with 1% aqueous uranyl acetate and observed on a 100 CX microscope (JEOL USA, Electron Optics Div., Peabody, MA) at 60 kV.

**Light Microscopic Immunocytochemistry**

**Purity of Neuronal Preparation.** Determination of the glial contamination in our neuronal preparations was made by immunocytochemistry using a monoclonal antibody against galactocerebroside and polysialic antibodies against myelin basic protein and glial fibrillary acidic protein. A cell suspension (100 μl) was washed with 10 mM PBS (Na2HPO4, pH 7.2, 0.15 M NaCl) and resuspended in 100 μl of 4% paraformaldehyde. After 15 min, the suspension was washed three times with PBS and incubated with 200 μl of either normal mouse or rabbit serum (1:20 dilution) containing 3% BSA. This was followed by three washes with PBS and the addition of the above-mentioned antibodies diluted 1:50 in PBS. After a 1-h incubation, the specimens were washed three times with PBS, 200 μl of FITC-labeled goat anti-mouse or goat anti-rabbit serum (1:100 dilution) was added accordingly, incubated 45 min at room temperature, washed three times with PBS, and mounted with a drop of Bacto FA fluid (Difco Laboratories, Inc.).

**PHF Preparations.** Diluted aliquots of PHF cytoskeletons recovered from the final centrifugation steps were spread on clean glass slides and dried at 37°C for 1 h. The slides were washed twice with PBS and incubated with normal goat serum (diluted 1:5 with PBS) for 5 min. After three 15-min washes with PBS, the slides were incubated with primary antibody (1:100 for antiubiquitin and antiantau; 1:50 for antineurofilament) for 30 min at room temperature. Slides were again washed three times with PBS and incubated with the secondary antibody conjugated to FITC (goat anti-rabbit IgG for ubiquitin and tau or goat anti-mouse IgG for neurofilament protein—all diluted 1:50) for 30 min at room temperature. After incubation, the slides received three washes with buffer, gently blotted and mounted in Bacto FA medium (Difco Laboratories, Inc.) for fluorescence microscopy. In an additional series of experiments, PHF preparations were double stained with rhodamine- and fluorescein-labeled secondary antibody (TRITC-goat anti-rabbit IgG against tau and FITC-goat anti-mouse IgG against neurofilament) using the same protocol as above.

**Immunostaining of PHF Proteins**

Immunoreactivity of PHF proteins was assessed by immunostaining on a dot blot as follows: 2-μl aliquots of the proteins to be investigated were spotted directly onto nitrocellulose filters (BA85; Schleicher & Schuell, Inc., Keene, NH) and washed twice with 20 ml of Tris-buffered saline (10 mM Tris-HCl, pH 7.4, containing 0.15 M NaCl and 0.02% Tween-10, TBS) to remove any residual GHCl. The filters were then blocked with a solution containing 2% ovalbumin in TBS for 1 h. Proteins bound on the filters were assayed for their immunoreactivity to antibodies directed against ubiquitin, tau, and neurofilament proteins with secondary antibodies conjugated with biotinylated anti-rabbit IgG for ubiquitin and tau and anti-mouse IgG for neurofilament protein.
Amino Acid Analysis

Amino acid analysis of proteins was carried out in 6 M HCl, containing 10% phenol, in melting point tubes at 108°C for 24, 48, and 72 h. Alternatively, hydrolysis was performed at 150°C for 75 min on a PicoTag workstation (Waters Instruments, Inc., Rochester, MN). After removal of the acid at a speedvac centrifuge, the specimens were derivatized to form phenylthiocarbamyl amino acids and separated by HPLC on a Novapack C18 column (Waters PicoTag; Waters Instruments, Inc.).

HPLC

300-μl aliquots of the PHF peptides, soluble in 6 M GHCl, were centrifuged at 250,000 g for 20 min (TL 100.2 rotor, Beckman Instruments, Inc., Palo Alto, CA) and submitted to size exclusion chromatography on a TSK 3000 SW column (600 x 7.5 mm; Beckman Instruments, Inc., Alex Division, San Ramon, CA) with a mobile phase of 6 M GHCl containing 5% formic acid.

The material precipitating in 6 M GHCl, containing the APCP peptides, was separated from the soluble PHF in the supernatant by centrifugation. The resulting pellet was twice washed with 6 M GHCl, resolubilized by the addition of 8 vol of 88% formic acid and dialyzed (membrane cut off of 1,000 D) against four changes (500 ml each) of 70% formic acid for 4 h at 2°C. The optically transparent specimen was centrifuged at 250,000 g for 20 min at 4°C. Aliquots of 300 μl were chromatographed on a Superose 12 size exclusion column (300 x 10 mm; Pharmacia Fine Chemicals, Piscataway, NJ) using as mobile phase 70% formic acid.

Results

The purification scheme used in the present investigation is succinctly summarized in Fig. 1. We found that, in spite of having remained frozen at -70°C for an average period of 60 mo, the AD brain tissue used in this study yielded neurons which, morphologically, did not differ from those isolated in our laboratory from fresh tissue processed immediately postmortem. We believe that an important step in the conservation of cell morphology was the sectioning and initial processing of the brain cortex in isotonic sucrose while still frozen. In addition, the incorporation of enzyme inhibitors in the freshly made disaggregation buffers helped to prevent undesirable proteolysis of cells. Gentle filtration of the brain tissue through a series of decreasing aperture stainless steel meshes, instead of the use of nozzle aspiration or syringe pressure forcing, minimized the unavoidable mechanical trauma to the cells. An example of the quality of preservation of the neurons obtained up to this point is illustrated in Fig. 2. A series of preliminary studies established that the best routine for the enrichment of the cellular population consisted of the bulk elimination of myelin and cell debris, derived from the mincing of the tissue and subsequent filtration, by centrifugation in 0.8 M sucrose. These materials floated at the top of the centrifuge bottle or loosely accumulated at the surface of the bottom pellet and were eliminated by careful and fast decantation. Increasing the sucrose concentration to 1.3 M, followed by centrifugation, resulted in the formation of a large aggregation of material at the top of the bottle. Because of its compact consistency, this material was removed by vacuum aspiration. Microscopic examination showed it to consist of glial cells, blood vessels, cell processes, and other cell debris. When the sucrose concentration was increased to 1.9 M, the enriched neuronal population floated on the surface of the medium as a semisolid pellet that was easily scooped out with a spatula, whereas the pellet at the bottom of the tube contained numerous free nuclei. Percoll density gradient centrifugation further enriched the neuronal fraction.

The use of antibodies against the oligodendrocyte antigens (myelin basic protein and galactocerebrosides) and against the astroglial marker (glial fibrillary acidic protein) revealed the presence of two glial cells per 3,000 neurons in the 44/34% Percoll interface. The 34/27 and 27/20% Percoll interfaces contained 2 and 10% glial cells, respectively. The materials retained at the top of the gradient (20% Percoll and above) and at the bottom of the tube were discarded. However, small blood vessel fragments of various sizes still contaminates the enriched neuronal interfaces. Since our objective was to further concentrate the neuronal population containing the PHFs of AD, we decided to use collagenase. This enzyme completely eliminated the remaining blood vessels. Unfortunately, a number of the neuronal processes were detached from their...
Neurons isolated from frozen brain tissue by the technique outlined in Materials and Methods during the early preparative stages before collagenase digestion. Neurons in the top and bottom panels were stained with carbocyanine D272, and those in the middle panels with the carbocyanine D378. These cationic lipophilic dyes also permitted the identification and visualization of the detailed morphology of neurites, including varicosities and appendages. Bar, 60 μm.

Figure 2.
Figure 3. Thioflavine T-stained PHF cytoskeletons obtained from 2.1/2.0-M sucrose interface shown in Fig. 3. This layer contained more than 90% PHF cytoskeleton (assessed by fluorescence and electron microscopy). A small amount of lipofuscin granules (arrows) and some small specks of APCP (arrowhead) still contaminated the specimen. Acid thioflavine T staining was chosen (over thioflavine S or Congo red) because when the specimens were excited at 370 nm, the APCP fluoresced in blue (emission, 420 nm), and the PHF cytoskeletons in green-yellow (emission, 520 nm). The autofluorescence of lipofuscin was also differentiated (emission, 450 nm). Hence, besides their morphology, all those structures were clearly distinguished by fluorescence microscopy. Bar, 20 μm.

The amino acid compositions of these polypeptides are given in Table III. Fractions 2 and 3 appear to be highly related in composition and Mr, suggesting that fraction 2 may be a dimer of fraction 3. These molecules are rich in Gly, Glu, Ser, and Asp. Although fractions 1 and 4 also resemble one another, having Glu, Gly, Asp, and Ser as their more abundant amino acids, nevertheless, important differences must exist between these two proteins. Fraction 1 is the only one in the entire set which proved positive for ubiquitin, tau, and neurofilament by immunodot blot technique. Amino acid analysis also permitted the direct calculation of the molar ratio among the five components. Fraction 4 appears to contain most of the protein (64%) followed by fraction 1 (18%).

Size exclusion chromatography of formic acid/6 M GHCl/formic acid–solubilized APCP peptides also rendered five major fractions (Fig. 9 B) with an apparent Mr of 200,000, 33,000, 10,000, 5,000, and an undetermined Mr, for peak 5. The amino acid compositions of fractions 3 and 4 revealed a close relationship, which suggested fraction 3 was a dimer of fraction 4 (Table IV). Furthermore, it is fraction 4 (representing 57% of the total APCP) that appears to correspond to the sequenced 42–amino acid peptide of the β-amyloid (13, 36, 37). By contrast, fractions 1 and 2 (representing 15 and 4%, respectively) resembled one another more closely than they did either fraction 3 or 4. Among others, the most striking differences are in the values of Thr, Pro, Val, and Arg. In addition, the peptides obtained from the APCP size exclusion chromatography were negative when stained with antibodies to ubiquitin, tau, and neurofilament by immunodot blot.

**Discussion**

In a recent review entitled ‘Alzheimer’s Disease: Its Proteins and Genes,” Glenner (12) stressed that in spite of the fact that the PHFs of this degenerative disease have been the subject of intense investigation for over 15 years, their chemical nature still “remains ill defined.” Until now, two principle reasons have precluded a more definitive advance in this area: (a) the inability to obtain appropriately pure specimens of PHF in quantities adequate to allow further biochemical characterization, and (b) the problem of PHF insolubility. In this paper we have presented a protocol which described the isolation of neuronal PHF of very high purity and in sufficient quantity to permit further biochemical characterization. Such studies will help to determine the intrinsic composition of these structures.

With the exception of Iqbal et al. (25) who, in 1974, attempted to purify PHF from isolated neurons, previous methodologies (26, 27, 36, 53, 54) aimed at obtaining purified PHF fractions used, among others, early homogenization of the brain by detergents in the presence of reducing agents and, in some cases, the use of proteolytic enzymes or even high temperature treatment. We devised a technique that avoided these potentially detrimental procedures (6, 23, 45). The preservation of intact neurons up to the final Percoll
fractionation helps to avoid initial contamination of PHF by extracellular APCP and other cellular debris.

In spite of being frozen at -70°C for five years, cellular integrity was well preserved (Fig. 2). Several factors used in the present protocol may have contributed to their preservation. Among them was the initial slicing and mincing of the brain at low temperature in an isotonic medium containing protease inhibitors, as well as the very gentle sieving of the tissue through decreasing size stainless steel meshes. Although the cells were treated with hypertonic sucrose solutions, they were exposed to these conditions for the minimum time necessary followed by immediate return to isotonic medium. We recognize, however, that mechanical trauma and the necessary chemical steps, as well as the inability of the cells to regenerate, were an important source of neuronal damage during the isolation procedure. Nevertheless, from an initial 2,000 g of cortex, we recovered 50 ml of cells (settled at unit gravity), of which 90% were neurons, from our Percoll interfaces and a final volume of ~150 µl of purified PHF cytoskeletons.

In a previous series of experiments, we used the fluorescence-activated cell sorter (FACS) as the final step in the purification of PHF cytoskeletons. FACS purification, as also experienced by other laboratories (24, 40), was only partially successful. When reacted under acidic conditions, thioflavine T distinctly labeled PHF cytoskeletons and APCP, while the autofluorescent lipofuscin remained unlabeled. Separation of these elements occurred in the FACS when excited at 370 nm. To label these structures, however, it was necessary to remove the detergent (SDS). This caused a significant aggregation of cores and tangles, which was very difficult to dissociate even after the SDS was reincorporated in the buffer. We estimated that the maximum purity of PHF cytoskeletons attainable through FACS amounted to no more than 70%. By using the technique presented in this paper, the degree of purity in our best PHF preparations was 95% or more. At EM level, the 5% contamination consisted of some granules of lipofuscin entrapped within the isolated PHF cytoskeleton network, and a negligible amount of APCP in the form of small but very prominent protein aggregates. Moreover, electron microscopy of the isolated PHF cytoskeletons showed them to be homogeneously composed of PHF.
Figure 6. Amyloid plaque protein cores stained with thioflavine T. These cores were obtained from the 1.7/1.4-M sucrose density gradient interface. Thioflavine T staining, when excited at 370 nm, distinguishes two discrete types of amyloid cores: intensely glowing silver blue (arrowhead) and deep dark blue (arrow). We suggest that these differences in staining characteristics may be due to small chemical inequality either in protein composition or post translational modification (such as glycosylation and phosphorylation). A second possibility is that they simply represent differences in filament concentration. However, in a random sample of cores, like the one shown in this figure, a continuous distribution of fluorescent tones is rarely seen. Bar, 10 μm.

as established by filament measurements of 20–24-nm diameter and 70-nm axial periodicity.

As the data presented in Table II clearly show, the amino acid composition of PHF substantially differed from APCP and lipofuscin molecules. The relative amounts of eight amino acids (Pro, Thr, Met, Arg, Lys, Phe, Ser, and Ala) differed by more than 40%, when the values of PHF and APCP were compared. The most represented amino acids in the PHF were Gly, Glu, Lys, and Asp, while in the APCP they were Gly, Val, Glu, and Ala. These results strongly suggest a different protein composition and quite possibly a different pathogenesis as well. When compared to the PHF amino acid compositions published by others (36, 54), important deviations, which could have resulted from different degrees of contamination, were apparent. The issue of whether or not identity exists between the polypeptide composition of the intraneuronal PHFs and the extracellular APCP has not been settled. On the one hand, Masters et al. (36) and Guiroy et al. (19) support the concept of identity based on amino acid sequence data between AD-PHF and AD-APCP and between the AD-PHF and PHF obtained from patients who died of Parkinson dementia complex of Guam. This latter degenerative disease is purportedly free of neuritic plaques and, therefore, should not contribute a potential APCP contamination during the isolation procedures. On the other hand, highly purified or enriched PHFs seem to carry an assortment of antigenic epitopes that were encountered in other proteins like ubiquitin (43, 48, 56), tau (18, 35, 44, 56, 72), neurofilaments (2, 10, 39, 41, 47) and other microtubule-associated proteins (34, 47). The purification scheme described here permitted the conservation of some of these epitopes, which suggests they may be “intrinsic” components of AD-PHF. Moreover, to the best of our knowledge, antibodies raised against either PHF or APCP do not show cross-reactivity (54). This also suggests nonidentity between the two types of filaments.

The results of the chemical depolymerization studies showed that lipofuscin granules seem to be chemically stable on brief exposure to concentrated formic acid and, therefore, can be eliminated by centrifugation. By contrast, PHF and APCP are apparently depolymerized by formic acid and are probably disrupted to random coils. Upon removal of formic acid by dialysis against 6 M GHC1, PHF polypeptides remained in solution whereas APCP repolymerized. It could be argued that the very hydrophobic nature of the APCP induces the regeneration of its supromolecular structure, even in the presence of a hydrophilic strong electrolyte. This important thermodynamic behavior suggests major physicalchemical differences between the structures of APCP and PHF.

Similarly, the amino acid composition of the APCP and PHF proteins obtained after fractionation by size exclusion chromatography demonstrate significant differences. In support of these compositional deviations, which have been highlighted in Results, are the stoichiometric differences within and between the fractions separated by the two chromatographic steps. The most representative APCP fraction is fraction 4, accounting for 57% of the entire sample, with an apparent $M_r$ of ~5,000. With some minor deviations, this peak closely corresponds to the β-amyloid protein. On the other hand, the most representative fraction in the PHF preparation, with an estimated 64% of the total sample, appears after the 1,700-$M_r$ marker. Either this peptide has an anomalous retention time in the TSK 3000 SW column (Beckman Instruments, Inc., Altex Division) or this fraction is...
Table II. Amino Acid Compositions of PHF Cytoskeletons, APCP, and Lipofuscin*

| Amino acid | PHFs† (mol/100 residues) | APCP‡ (mol/100 residues) | Lipofuscin (mol/100 residues) |
|------------|-------------------------|--------------------------|-----------------------------|
| Cys        | ND                      | 0.8§                     | 16.3§                       |
| Asp        | 9.0                     | 8.1§                     | 8.2§                        |
| Met        | 1.1                     | 2.7                      | 1.5                         |
| Thr†       | 5.0                     | 1.4                      | 4.1                         |
| Ser†       | 8.8                     | 4.8                      | 6.4                         |
| Glu        | 10.7                    | 9.6                      | 7.8                         |
| Pro        | 4.0                     | 1.1                      | 2.7                         |
| Gly        | 12.7                    | 15.5                     | 12.5                        |
| Ala        | 5.1                     | 8.8                      | 6.3                         |
| Val‡       | 8.8                     | 14.3                     | 8.1                         |
| Ile§       | 5.2                     | 6.5                      | 4.3                         |
| Leu        | 6.6                     | 6.1                      | 7.7                         |
| Tyr        | 2.6                     | 1.7                      | 2.3                         |
| Phe        | 3.3                     | 6.3                      | 5.1                         |
| His        | 3.5                     | 5.0                      | 0.9                         |
| Lys        | 9.3                     | 5.2                      | 3.3                         |
| Arg        | 4.3                     | 2.1                      | 2.5                         |
| Trp        | ND                      | ND                       | ND                          |

* Average values from determinations at 24, 48, and 72 h of hydrolysis, except as noted.
§ Highly significant differences between PHF and APCP; Chi-square = 33.780, P = 0.002.
 Quantitated as cystic acid and methionine sulfone after performic acid oxidation.
 Extrapolated to 0 h hydrolysis value.
 ‡ 72 h hydrolysis value.

composed of 2–3 peptides of similar size. If the former alternative applies, one could assume a peptide with a minimum length of 31–33 amino acid residues, as calculated from the peak’s amino acid analysis molar ratios. Next in representation is the PHF fraction 1 (18%). This peak, which elutes with an Mr of ~200,000, resembles fraction 4 in amino acid composition. Therefore, the possibility of this being an aggregated form of the major fraction 4 exists. However, fraction 1 is the only one in the entire set of TSK 3000 SW-separated peptides that is immunologically positive for ubiquitin, tau, and neurofilament epitopes. Last is the high degree of similarity that exists among PHF fractions 2, 3, and 5, with peak 5, again, possibly representing an anomalously retained peptide.

Of particular interest was the proteolytic signal protein ubiquitin (76 amino acids long), which appeared to be intimately associated and perhaps covalently linked to the PHF polypeptides, since it continued to be present even after the repolymerization procedure. Mori et al. (43) have isolated from PHF preparations peptides that correspond to two internal sequences of ubiquitin.

The primary goal in devising a technique that would permit large-scale purification of brain cells from suitable AD postmortem material was that, in the absence of an animal

Figure 7. Immunochemical reactivity of PHF cytoskeletons derived from the 2.0/1.7-M sucrose interface, stained by indirect immunofluorescence with FITC-conjugated second antibody. (A) Anti-ubiquitin; (B) antitau; (C) antineurofilament (8D8 antibody). Bars, 30 μm.
Table III. Amino Acid Composition of Alzheimer’s Disease Paired Helical Filament Proteins

|                | Peak 1 M, 200,000 | Peak 2 M, 9000 | Peak 3 M, 4500 | Peak 4 M, -- | Peak 5 M, -- |
|----------------|-------------------|----------------|----------------|-------------|-------------|
|                | mol %             | mol %          | mol %          | mol %       | mol %       |
| Cys            | ND                | 8.2            | 8.4            | 10.5        | 8.3         |
| Asp            | 4.2               | 3.1            | 3.6            | 3.1         | 3.7         |
| Thr            | 10.5              | 13.3           | 13.9           | 10.6        | 12.9        |
| Ser            | 21.0              | 17.2           | 17.6           | 24.3        | 17.2        |
| Glu            | 4.0               | 3.1            | 3.4            | 3.3         | 3.8         |
| Gly            | 15.5              | 22.0           | 21.6           | 16.1        | 20.4        |
| Ala            | 7.2               | 7.9            | 7.6            | 6.5         | 8.2         |
| Val            | 5.7               | 6.0            | 5.0            | 5.9         | 4.9         |
| Met            | 1.3               | 2.7            | 2.7            | 2.6         | 2.3         |
| Tyr            | 1.6               | 2.0            | 1.2            | 1.6         | 1.6         |
| Phe            | 4.7               | 3.0            | 3.1            | 3.1         | 3.5         |
| His            | 2.8               | 2.8            | 2.8            | 2.5         | 3.1         |
| Arg            | 7.2               | 7.9            | 7.6            | 6.5         | 8.2         |
| Trp            | 5.7               | 6.0            | 5.0            | 5.9         | 4.9         |

between the disappearance of microtubules and neurofilaments and the appearance of PHF cytoskeletons, which appear to carry epitopes of the former structures. We believe that these two phenomena are related (16). Great emphasis has been put upon the characterization of PHF, partly because, with the exception of those seen in aging, they are alien to the normal neuron and because they appear in such profusion. However, of equal importance is the question of model, access to pathological neurons would allow for a more selective investigation of the cellular pathophysiology of AD at different stages of its evolution. Of immediate particular interest was the suggestion of a reciprocal relationship

Table IV. Amino Acid Composition of Alzheimer’s Disease Amyloid Plaque Core Proteins

|                | Peak 1 M, 200,000 | Peak 2 M, 33,000 | Peak 3 M, 10,000 | Peak 4 M, 5,000 | Peak 5 M, -- |
|----------------|-------------------|-------------------|-------------------|-----------------|-------------|
|                | mol %             | mol %             | mol %             | mol %           | mol %       |
| Cys            | ND                | ND                | ND                | ND              | ND          |
| Asp            | 8.7               | 9.6               | 8.2               | 8.1             | 8.0         |
| Thr            | 4.7               | 4.4               | 0.8               | 0.3             | 2.6         |
| Ser            | 8.1               | 6.8               | 5.2               | 4.8             | 6.7         |
| Glu            | 10.8              | 12.9              | 10.8              | 9.9             | 9.8         |
| Pro            | 4.6               | 3.9               | 0.7               | 0.2             | 4.5         |
| Gly            | 12.3              | 11.6              | 16.5              | 16.1            | 21.6        |
| Ala            | 7.9               | 9.4               | 9.3               | 9.1             | 9.5         |
| Val            | 7.5               | 7.5               | 14.2              | 14.5            | 7.6         |
| Met            | 2.3               | 2.0               | 2.1               | 2.7             | 1.8         |
| Ile            | 4.4               | 4.2               | 6.0               | 6.0             | 4.3         |
| Leu            | 8.0               | 8.7               | 5.8               | 5.7             | 5.3         |
| Tyr            | 3.2               | 2.9               | 2.7               | 2.6             | 3.5         |
| Phe            | 3.7               | 3.8               | 5.9               | 6.8             | 3.5         |
| His            | 3.0               | 2.7               | 5.9               | 6.8             | 3.6         |
| Lys            | 4.4               | 4.1               | 3.7               | 4.5             | 2.5         |
| Arg            | 6.4               | 5.5               | 2.2               | 1.9             | 4.6         |
| Trp            | ND                | ND                | ND                | ND              | ND          |

Figure 8. Electron micrographs of stained APCP. APCPs are formed by the compact aggregation of filaments, measuring 10 nm in diameter, which appear flexible. (A) A dense amyloid core purified by the present protocol. (B) After treatment with concentrated formic acid for 2 h at room temperature, the specimen was diluted with 50 vol of water and lyophilized. APCPs were resuspended in 50 mM Tris-HCl, pH 7.5, 0.2% SDS. An aliquot of this specimen was sonicated and a drop was deposited onto a Formvar-coated copper grid. After 3 min, excess buffer was drained away by filter paper. The grid was washed three times with water and stained with 1% aqueous uranyl acetate for 4 min. The repolymerized APCP appeared as a network of 10-nm filaments morphologically indistinguishable from those shown at the periphery in A. Bars, 1.0 μm.
The yield in nmol for each of the fractions permitted the calculation of their relative fractional representation as 18, 4, 6, 64, and 8%, respectively. Immunodot blot analyses indicated that only the protein eluted in peak 1 contained ubiquitin, tau, and neurofilament light chain, while phosphorylase B, BSA, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, hemoglobin α-chain, insulin, glucagon, and neurotensin were absent from it.

The chromatography was developed at a flow rate of 0.5 ml/min and monitored at 280 nm. The column was calibrated, using the same denaturing solvents as described above, with 10 different proteins and peptides of known Mr: myosin, phosphorylase B, BSA, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, hemoglobin β-chain, insulin, glucagon, and neurotensin. 300-μl aliquots of solubilized PHFs were centrifuged at 250,000 g for 20 min before loading onto the column. PHFs resolved into five different peaks with Ms of ~200,000 (peak 1), 9,000 (peak 2), 30,000 (peak 3), 4,500 (peak 4), and 1,700 (peak 5). The amino acid compositions of these fractions is given in Table III.

The amino acid compositions of these fractions is given in Table III. The amino acid compositions of these fractions is given in Table III. The amino acid compositions of these fractions is given in Table III.
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