# [7] Chemical Modifications of Deposited Amyloid-β Peptides

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## Introduction

Alzheimer's disease is a dementia characterized by the extracellular accumulation of amyloid in the cortical regions of the brain and in the walls of the cerebral and leptomeningeal blood vessels. This amyloid is composed primarily of insoluble fibrillar amyloid-β (Aβ), peptides of 40 to 42 amino acids derived by proteolysis of the type I transmembrane amyloid precursor protein.[1] The more insoluble 42 residue peptide is the major form of Aβ present in cortical neuritic deposits, or plaques, whereas the more soluble 40 residue peptide is predominant in the walls of the cerebral vasculature.[2,3] Deposition of amyloid fibrils precedes the onset of clinical manifestation of dementia by several decades, during which time the Aβ is subjected to a variety of degradation reactions. Aminopeptidases remove a variable number of N-terminal amino acid residues, yielding a heterogeneous mixture of shorter Aβ peptides, particularly in the amyloid

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associated with cortical neuritic plaques.[2,3] Spontaneous degradation reactions occurring over time include the isomerization of aspartyl residues,[2] the racemization of aspartyl and seryl residues,[2,4] and the cyclization of N-terminal glutamyl residues.[5–7] Accumulation of these chemical modifications may play a pathological role in Alzheimer's disease because they appear to contribute to the increased insolubility and resistance to enzymatic degradation observed in Aβ fibrils isolated from the brain.[4,8,9] This article describes the techniques utilized in our laboratory to characterize the chemical modifications observed in the Aβ peptides of Alzheimer's disease brains.

## Characterization of Isomerized and Racemized Residues in Aβ Peptides

The purification of amyloid core protein from neuritic plaques and amyloid from vascular walls is described elsewhere in this volume.[9a]

Monomeric Aβ (2 mg) purified from the cerebral cortex or leptomeningeal vessels from Alzheimer's disease patients or the synthetic Aβ1–40 or Aβ1–42 (obtained from either California Peptide Inc., Napa Valley, CA, or Bachem, Torrance, CA) is dissolved in 1 ml of 80% (v/v) glass-distilled formic acid and dialyzed (Spectrapor No. 6 membrane, molecular weight cutoff 1000 flat width 18 mm, Spectrum Medical Industries, Inc., Los Angeles, CA) against 2 liter of distilled water for 3 hr, followed by 2 liter (× 2) of 100 m*M* ammonium bicarbonate under continuous stirring at room temperature. The dialyzed Aβ peptides are digested by trypsin (TPCK treated; Worthington Biochemical Corp., Freehold, NJ) at an approximately 1:50 enzyme:substrate ratio for 14 hr at 37°. Proteolysis is terminated by lyophilization. The tryptic peptides (1 mg) are dissolved in 500 μl of 10% (v/v) glass-distilled formic acid and are separated by high-performance liquid chromatography (HPLC) on an LKB $C_{18}$ reversed-phase column (Spherisorb ODS2, 5-μm bead size, 4.6 × 250-mm column, Pharmacia-LKB, Sweden). Prior to loading onto the column, the insoluble tryptic

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core (particularly prevalent in the digests of AβN-42 peptides) is removed by centrifugation at 12,000*g* for 10 min in a 1.5-ml microcentrifuge tube. The column is eluted at a constant flow rate of 0.7 ml $min^{-1}$ at room temperature with a linear gradient of 0–20% acetonitrile/0.1% trifluoroacetic acid over 90 min, followed by a linear gradient of 20–60% acetonitrile/0.1% trifluoroacetic acid for an additional 30 min. The column effluent is monitored at 214 nm and fractions are collected every 30 sec. The insoluble tryptic core, composed mainly of the hydrophobic peptide Aβ29–42, can be digested further by cyanogen bromide in formic acid.[2] The residues in this peptide, however, are not susceptible to the degradation reactions investigated in this article.

Only four peptides should be generated by tryptic digestion of Aβ at its one Arg and two Lys residues, and this is observed when synthetic Aβ is digested as described earlier (Fig. 1a). Peak C contains Aβ1–5, peak I corresponds to Aβ6–16, and peak J is Aβ17–28. When Alzheimer's disease brain-derived neuritic plaque Aβ is digested and the peptides are separated by HPLC, however, a more complicated chromatograph is obtained (Fig. 1b). Amino acid analysis (Table I) and mass spectrometry reveal that peaks A and B have the same mass and composition as peak C and that peaks G and H have the same mass and composition as peak I. The same new peaks are observed when vascular Aβ is subjected to this analysis, although there is less of them than in neuritic plaque Aβ. When these peptides are subjected to gas-phase automatic amino acid sequencing, only peptides in peaks C and I undergo complete Edman degradation. Peptides in peaks A and B are resistant to the Edman reaction, whereas peaks G and H release residue 6 (His) in the first cycle but no amino acids in the subsequent cycles. These results are consistent with the formation of isomerized aspartyl (isoaspartyl) residues in positions 1 and 7 via a spontaneous intramolecular reaction (Fig. 2). Isoaspartyl residues are bonded into the peptide backbone through their β-carboxyl group; although not released by Edman sequencing chemistry, they become normal aspartic acid upon acid hydrolysis. Aspartyl residues are also subject to racemization, resulting in D-aspartyl derivatives (Fig. 2). Both racemized and isomerized residues have the same mass as the L-aspartyl residue. Formation of the succinimide intermediate that produces these damaged forms from L-aspartyl (and in other proteins L-asparaginyl) residues is one of the most common spontaneous reactions affecting proteins as they age.[10–14] These residues are present at low levels

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[13] J. Najbauer, J. Orpiszewski, and D. W. Aswad, *Biochemistry* **35,** 5183 (1996).
[14] H. T. Wright, *in* "Deamidation and Isoaspartyl Formation in Peptides and Proteins" (D. W. Aswad, ed.), p. 229. CRC Press, Boca Raton, FL, 1995.

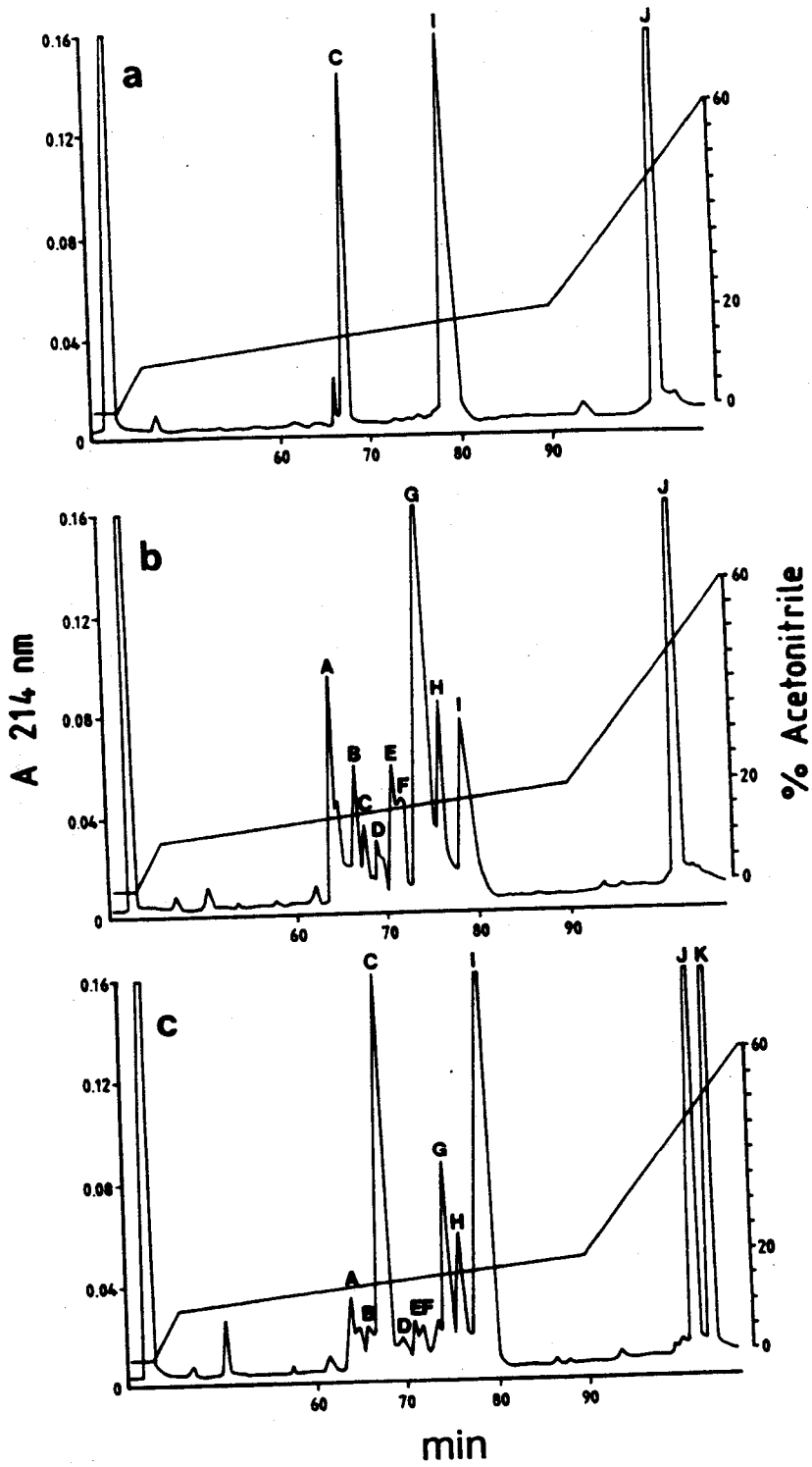


FIG. 1. Chromatographic profiles of neuritic plaque and vascular Aβ tryptic peptides separated on $C_{18}$ reversed-phase HPLC. (a) Synthetic $A\beta_{1-42}$ yields peaks C (residues 1–5), I (residues 6–16), and J (residues 17–28). (b) Neuritic plaque Aβ from Alzheimer's disease brain produces many additional tryptic peptides. (c) Vascular Aβ from leptomeningeal deposits yields lesser quantities of the modified peptides. Adapted with permission from A. E. Roher *et al., J. Biol. Chem.* **268,** 3072 (1993).

TABLE I
TRYPTIC FRAGMENTS OF PARENCHYMAL Aβ

| Tryptic fragment | Predicted sequence | Peak | Observed sequence |
|---|---|---|---|
| Tp1 | $^{1}$DAEFR$^{5}$ | A | $^{1}$DAEFR$^{5}$ |
| | | | $^{2}$AEFR$^{5}$ |
| | | B | $^{1}$DAEFR$^{5}$ |
| | | | $^{10}$YEVHHQK$^{16}$ |
| | | C | $^{1}$DAEFR$^{5}$ |
| Tp2 | $^{6}$HDSGYEVHHQK$^{16}$ | D | $^{10}$YEVHHQK$^{16}$ |
| | | E, F | $^{9}$GYEVHHQK$^{16}$ |
| | | | $^{8}$SGYEVHHQK$^{16}$ |
| | | G | $^{6}$HDSGYEVHHQK$^{16}$ |
| | | H | $^{6}$HDSGYEVHHQK$^{16}$ |
| | | I | $^{6}$HDSGYEVHHQK$^{16}$ |
| Tp3 | $^{17}$LVFFAEDVGSNK$^{28}$ | J | $^{17}$LVFFAEDVGSNK$^{28}$ |
| Tp4 | $^{29}$GAIIGLMVGGVVIA$^{42}$ | | $^{29}$GAIIGLMVGGVVIA$^{42}$ |

in most proteins, but are particularly abundant in cells with limited protein turnover, such as erythrocytes[15] and eye lens.[16] The presence of a single L-isoaspartyl residue has been observed to alter the structure and function of a number of proteins assayed *in vitro,* suggesting that the accumulation of this damage might be detrimental in living organisms.[17] Isoaspartyl residues are also resistant to enzymatic degradation[18–20] and appear to confer this resistance along with increased insolubility to Aβ.[8]

*Determination of Aspartyl Isomerization*

Several routine methods used in protein characterization, including those just described, can provide indirect evidence suggesting the presence of isoaspartyl residues. This evidence can be missed, however, if the normal and isoaspartyl forms of the peptide are not first isolated from each other. For example, the decrease in amino acid recovery with each cycle of Edman sequencing can make it difficult to detect the additional loss due to the presence of a small amount of an isoaspartyl residue at a particular position. Peptides that differ solely by the bond formed by a single amino acid can be difficult to separate. It is fortunate that the tryptic digestion of Aβ

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[16] P. N. McFadden and S. Clarke, *Mech. Ageing Dev.* **34,** 91 (1986).
[17] E. Kim, J. D. Lowenson, D. C. MacLaren, S. Clarke, and S. G. Young, *Proc. Natl. Acad. Sci. U.S.A.* **94,** 6132 (1997).
[18] E. D. Murray, Jr., and S. Clarke, *J. Biol. Chem.* **259,** 10722 (1984).
[19] B. A. Johnson and D. W. Aswad, *Biochemistry* **29,** 4373 (1990).
[20] J. D. Lowenson and S. Clarke, *J. Biol. Chem.* **267,** 5985 (1992).

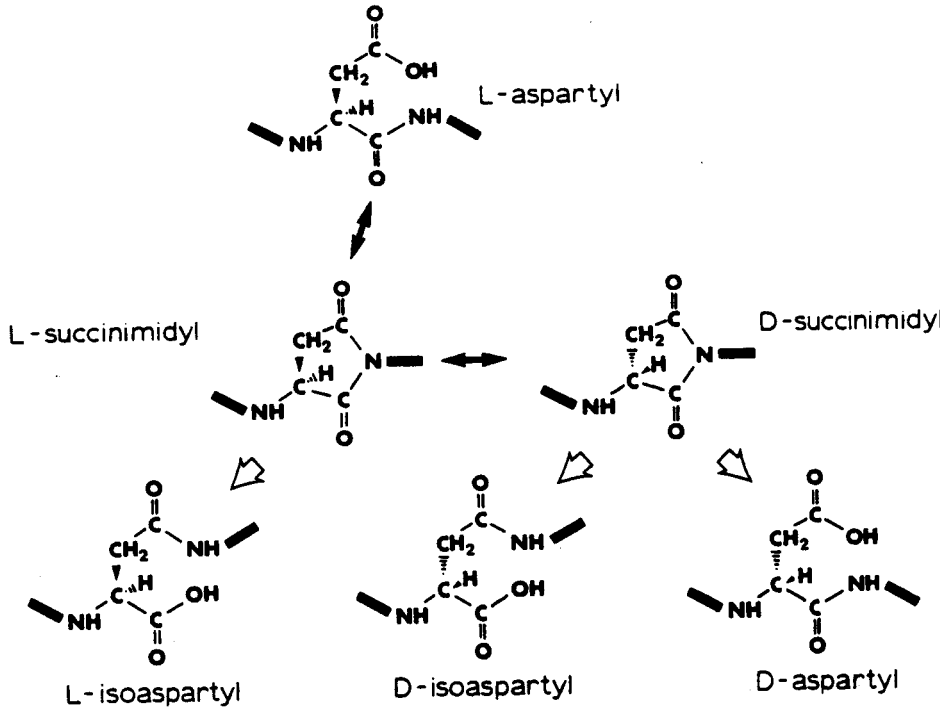


FIG. 2. Spontaneous reactions leading to the formation of altered aspartyl residues in polypeptides. At pH 7.4 and 37°, L-aspartyl residues in peptides and proteins can degrade to the L-succinimidyl form with $t_{1/2}$ values as short as 41 days for an Asp-Gly sequence and 168 days for an Asp-Ser sequence.[11] In similar peptides, $t_{1/2}$ values of 19.5 hr for racemization of the L-succinimide and 2.3 hr for hydrolysis of the succinimide to the normal and isoaspartyl forms have been observed.[18] Although not shown, each of these reactions is reversible. The attachment of the residue to the peptide backbone is represented by bold lines. Reproduced with permission from A. E. Roher *et al., J. Biol. Chem.* **268,** 3072 (1993).

produces peptides that are small and balanced with hydrophobic and hydrophilic residues, allowing the aspartyl and isoaspartyl forms of the peptide to elute differently from a reversed phase HPLC column. The acidic pH of the 0.1% trifluoroacetic acid running buffer accentuates the p$K_a$ difference between the free $\beta$-carboxyl group of the normal aspartyl residue and the more acidic free $\alpha$-carboxyl of the isoaspartyl residue.

The best way to quantitate L-isoaspartyl residues directly is to use the L-isoaspartate (D-aspartate) *O*-methyltransferase (EC 2.1.1.77) as an analytical probe. This enzyme methylates the $\alpha$-carboxyl group of L-isoaspartyl residues and, with approximately 1000-fold lower affinity, the $\beta$-carboxyl group of D-aspartyl residues, but does not recognize L-aspartyl residues in peptides or proteins.[20] Because methylation of D-aspartyl residues can, in some cases, be misinterpreted as L-isoaspartyl methylation,[20] additional characterization, including the racemization analysis described later, may be necessary. When *S*-adenosyl-L-[*methyl*-$^{14}$C]methionine ([$^{14}$C]AdoMet)

or its [$^3$H]methyl analog is used as the methyl donor, L-isoaspartyl residues are radiolabeled by this methyltransferase. The methylation reaction is terminated by the addition of 50 μl of 0.2 *M* sodium hydroxide, 1% (w/v) sodium dodecyl sulfate (SDS), which also converts the base-labile methyl esters on the L-isoaspartyl residues to [$^{14}$C]methanol. In a "vapor diffusion assay," an aliquot (50–70 μl) of each base-treated incubation mixture is spotted on a 2 × 8-cm piece of thick filter paper (Bio-Rad, Richmond, CA) that had been prefolded in an accordion pleat.[21] This is wedged quickly into the neck of a 20-ml scintillation vial containing 10 ml of Safety-Solve II counting fluor (RPI), which is immediately capped tightly and allowed to equilibrate at room temperature for 2 hr. During this time, the volatile [$^{14}$C]methanol diffuses into the fluor, whereas the non-volatile [$^{14}$C]AdoMet remains on the filter paper. The filter paper is then removed and the vials are counted.

The volatile radioactivity obtained from a no-substrate blank containing methyltransferase, [$^{14}$C] AdoMet, and buffer is subtracted from each Aβ sample incubated for the same length of time. Each assay also includes a positive control in which a saturating quantity of a stock methyltransferase substrate [e.g., chicken ovalbumin (grade V from Sigma) or a synthetic L-isoaspartyl-containing peptide] is incubated under the same conditions as the Aβ samples to measure the maximum number of methyl groups that could be transferred during the assay.

In a typical assay, intact Aβ (150 pmol, as determined by amino acid analysis) or Aβ tryptic peptides (10 pmol) are incubated at 37° for various times in 20 μl of 0.1 *M* sodium citrate, pH 6, containing 3.2 units of methyltransferase and 10 μ*M* [$^{14}$C]AdoMet (ICN, Costa Mesa, CA, 52 mCi/mmol). The methyltransferase in this example was purified from human erythrocyte cytosol[21] to about 5000 units mg$^{-1}$ [1 unit: 1 pmol methyl groups transferred/min to saturating (1 m*M*) chicken ovalbumin, a good substrate with an apparent $K_m$ of 35 μ*M*]. The reaction is buffered to pH 6 because, although the methyltransferase is less active at this pH, the methyl esters produced are more stable and therefore less likely to spontaneously demethylate.[22] The concentration of [$^{14}$C]AdoMet, at 5-times its $K_m$ for the methyltransferase, will not limit the rate of the reaction. As is illustrated in Fig. 3, solubilized Aβ1–42 from neuritic plaques accepts seven-fold more methyl esters than synthetic Aβ1–42, demonstrating that it contains at least one methylatable L-isoaspartyl residue. The stoichiometry of methylation is only 1 methyl per 21 Aβ molecules, but the continued increase in methylation with time indicates that all the available L-isoaspartyl residues have

[21] J. M. Gilbert, A. Fowler, J. Bleibaum, and S. Clarke, *Biochemistry* **27,** 5227 (1988).
[22] E. D. Murray, Jr., and S. Clarke, *J. Biol. Chem.* **261,** 306 (1986).

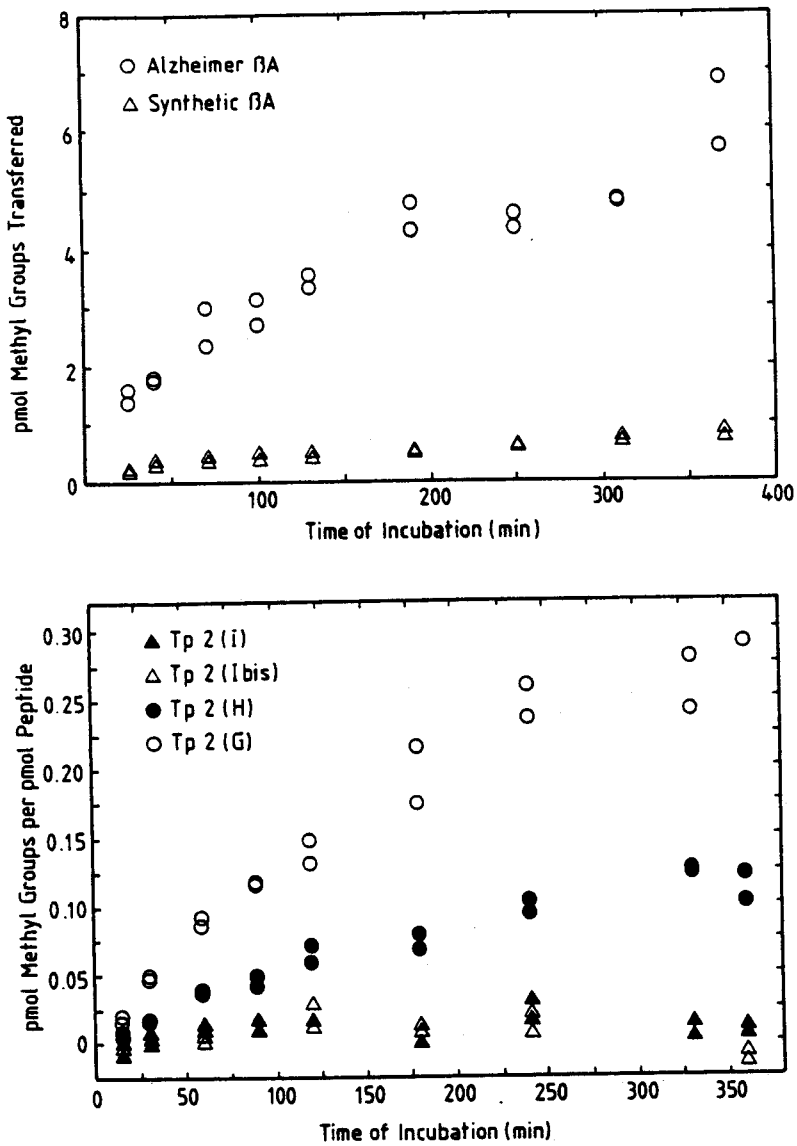


FIG. 3. Detection of altered aspartyl residues in Aβ and Aβ tryptic peptides by methylation with L-isoaspartate (D-aspartate) *O*-methyltransferase. Neuritic plaque or synthetic Aβ1–42 (150 pmol; top) and the tryptic peptides Aβ6–16 (10 pmol; bottom) are incubated with 3.2 units of methyltransferase and 10 $\mu M$ [$^{14}$C]AdoMet at 37° for the times indicated. Reproduced with permission from A. E. Roher *et al., J. Biol. Chem.* **268,** 3072 (1993).

not been methylated (Fig. 3). An assay of the tryptic peptides in peaks G, H, I, and I-bis (a trailing shoulder of peak I) shows that G and H contain L-isoaspartyl residues, whereas I and I-bis do not (Fig. 3). As with intact Aβ, the absolute number of L-isoaspartyl residues cannot be determined from these results because even 360 min of incubation time is not long enough to fully methylate the peptide. This assay, however, combined with

the mass spectrometry, sequencing, and composition analyses, indicates that peak G consists solely of the L-isoaspartyl-containing form of Aβ6–16, and thus the amount of this damage in the Aβ can be obtained from the UV absorbance on the HPLC chromatograph.

If an L-isoaspartyl-containing peptide or protein cannot be purified to homogeneity, the quantity of L-isoaspartyl residues can still be determined using the methylation assay as long as enough enzyme is present, the affinity of the substrate for the methyltransferase is high enough, and the reaction is allowed to proceed to completion. If the first two conditions are met, a time course of methylation should demonstrate saturation of the available substrate within 2 hr. If longer incubations are attempted, it should be determined whether the enzyme remains active and that the incubation is free from active proteases.

In the assay of Aβ described earlier, 3.2 units of methyltransferase is not enough to fully methylate the L-isoaspartyl residue in peak G within a time period during which the enzyme remains active. Other L-isoaspartyl-containing peptides of similar size and sequence to Aβ6–16 have $K_m$ values of 1–16 $\mu M$,[23] but the concentration of peptide in this assay is just 0.33 $\mu M$, and therefore the reaction proceeds at a rate well below its maximal velocity. Simply adding more enzyme, the concentration of which is just 0.16 units $\mu l^{-1}$, dilutes the peptide further, counteracting the increase in enzyme activity. To circumvent this problem we have begun using recombinant human L-isoaspartyl (D-aspartyl) *O*-methyltransferase expressed in *Escherichia coli*,[24] which, when purified to a specific activity of more than 13,000 units $mg^{-1}$ and a concentration of up to 58 units $\mu l^{-1}$, can provide as much as 350-fold higher activity in the same incubation volume, allowing faster methylation of even poor substrates. It should be noted, however, that while the recombinant methyltransferase provides high activity combined with a very low background of endogenous methylation due to its purity, even crude erythrocyte cytosol can be used to identify and, in some cases, quantitate L-isoaspartyl residues. Blood is drawn from a finger tip, disodium EDTA (1 mg $ml^{-1}$ blood) is added to prevent clotting, and the erythrocytes are washed five times in 10 volumes of phosphate-buffered saline to remove L-isoaspartyl-containing plasma proteins. These cells are lysed by freezing in 5 volumes of 5 m*M* sodium phosphate, 1 m*M* EDTA, pH 7.4, to which 25 $\mu M$ phenylmethylsulfonyl fluoride is added just prior to use. On thawing, the membrane ghosts are removed by centrifugation at 16,000*g* for 10 min at 4°. The cytosolic methyltransferase is only about 5–10 units $mg^{-1}$ protein, but at 0.07–0.17 units $\mu l^{-1}$ is suitable for some

[23] J. D. Lowenson and S. Clarke, *J. Biol. Chem.* **266,** 19396 (1991).
[24] D. C. MacLaren and S. Clarke, *Prot. Express. Purif.* **6,** 99 (1995).

analyses. For example, both recombinant methyltransferase (11.5 units, 0.2 $\mu$l) and crude erythrocyte cytosol (1.4 units, 18.68 $\mu$l) can stoichiometrically methylate 4.2 pmol of the high-affinity substrate VYP-isoD-HA, which has a $K_m$ of 0.29 $\mu M$,[23] within 30 min (Fig. 4, top). The cytosolic methyltransferase, however, can only methylate half of the 3.2 pmol of L-isoaspartyl residues expected in 50 pmol of ovalbumin during a 40-min incubation (Fig. 4, bottom),[2] but still provides a good estimate of the amount of damaged residues. When using crude cytosol, care must be taken to assay and subtract endogenous methylation, ascertain that the substrate is not being degraded by erythrocyte proteases, and avoid the use of 2-mercaptoethanol, which is methylated by an erythrocyte thiol methyltransferase and can interfere with the vapor diffusion assay.

There are, however, some limitations in the use of methyltransferase to detect L-isoaspartyl residues. For example, the methyltransferase cannot be used to confirm that A$\beta$1–5 peak A (Fig. 1B) contains an L-isoaspartyl residue. Because this residue is at the N terminus, its affinity for the enzyme is too low to be measured given the amount of peptide available. In this case, the physical data presented earlier, as well as the racemization data given later, strongly support the presence of the L-isoaspartyl residue. As further support, the peptide L-isoAsp-Ala-Glu-Phe-Arg can be synthesized and shown to coelute with A$\beta$1–5 peak A. Furthermore, the soluble carbodiimide EDC could be used to attach a CBZ-derivatized amino acid to the amino terminus of the tryptic peptide, which would probably make it a much better methyltransferase substrate.[23,25] In fact, simply acetylating the N-terminal amino group of A$\beta$6–16 peak G, in which the L-isoaspartyl residue is penultimate to the N terminus, with acetic anhydride caused it to be methylated 2.5-fold faster than the unacetylated form (unpublished data, but see Ref. 15).

Peptides containing two aspartyl and/or asparaginyl residues provide another complication for the L-isoaspartyl methyltransferase assay. If two sites on a peptide have become damaged, a fraction of peptides in the population should contain two L-isoaspartyl residues, while many more will contain one such residue, with a different aspartyl derivative at the other site. These peptides can be distinguished by methylating them as described earlier and then separating them by reversed-phase HPLC. Methylated peptides are significantly more hydrophobic than their unmethylated forms. New peaks eluting later than the control peptide are collected and their radioactivity is quantitated in a scintillation counter. The amount of each peptide collected is calculated from its absorbance monitored with a UV

[23] P. Galletti, D. Ingrosso, C. Manna, F. Sica, S. Capasso, P. Pucci, and G. Marino, *Eur. J. Biochem.* **177,** 233 (1988).

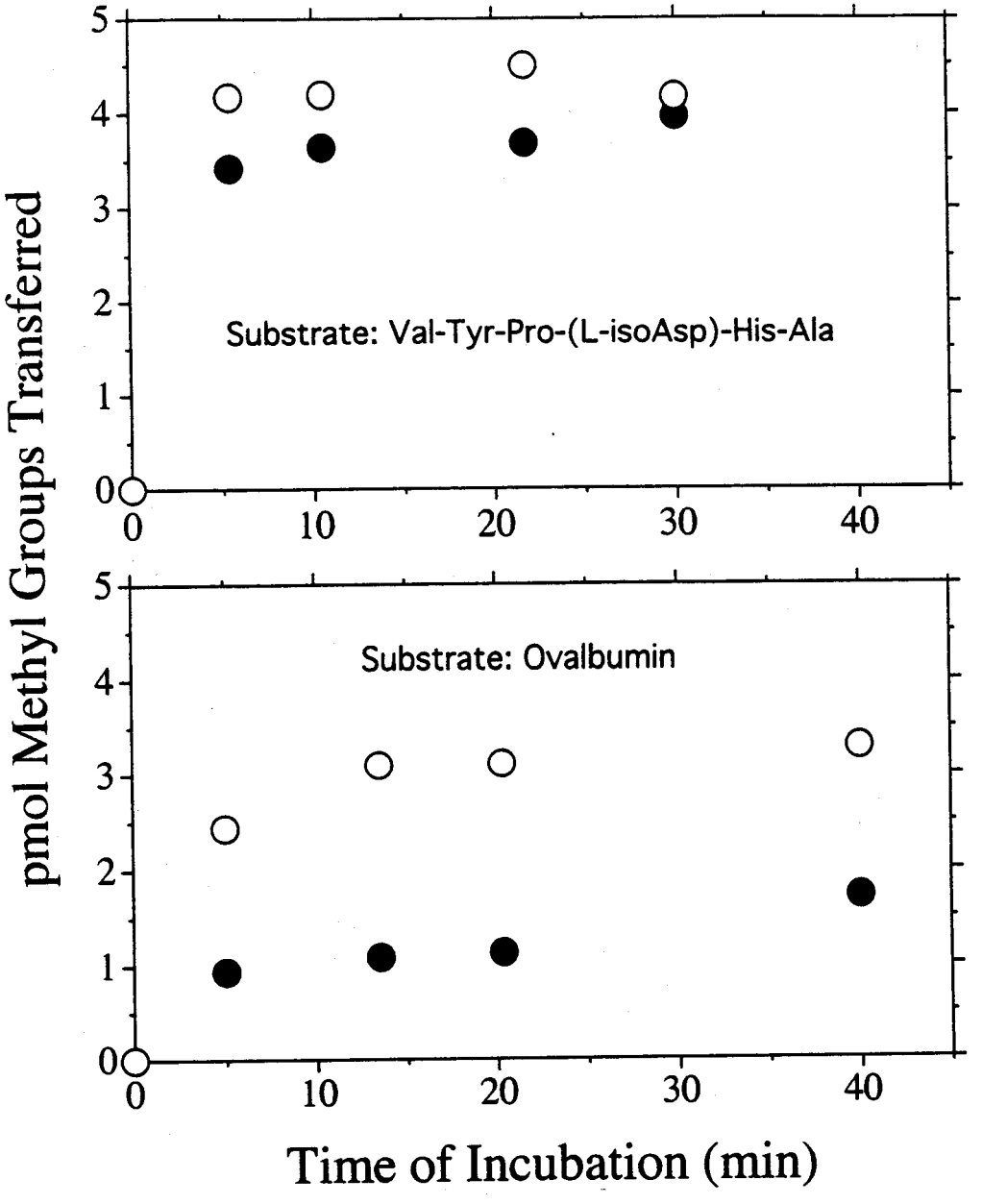


FIG. 4. Quantitation of L-isoaspartyl residues: Comparison of recombinant human L-isoaspartate (D-aspartate) *O*-methyltransferase, and crude human erythrocyte cytosol. The synthetic peptide Val-Tyr-Pro-(L-isoAsp)-His-Ala (4.2 pmol; top) or chicken ovalbumin (50 pmol; bottom) is methylated with 10 $\mu M$[$^{14}$C]AdoMet and 11.5 units of recombinant methyltransferase (○) or 1.4 units of crude erythrocyte cytosol methyltransferase (●). Just over 1 in 16 ovalbumin molecules contain a methylatable L-isoaspartyl residue, so about 3.2 pmol methyl esters are expected from 50 pmol ovalbumin.

detector. Peptides containing two L-isoaspartyl sites will show 2 pmol of methyl groups per pmol of peptide and should be the most hydrophobic peptides in the population.

*Determination of Aspartyl Racemization*

Although a link between isomerization and racemization of aspartyl residues had previously been observed *in vitro* with synthetic peptides,[10] analysis of A$\beta$ from Alzheimer's disease brain provided the first evidence that these reactions also occurred at the same site *in vivo*. This has since been observed in aged eye lens crystallin,[26] and a mechanism by which the structure of the succinimide intermediate accelerates racemization[10] has been supported by *ab initio* theoretical calculations.[27]

To quantitate the abundance of D-aspartyl residues, A$\beta$ tryptic peptides are dried down in 6 × 50-mm glass tubes that have been heated previously to 240 ° for 24 hr to destroy any contaminating amino acids. These peptides are hydrolyzed with vaporized HCl *in vacuo* at 108° for 3 hr using a PicoTag workstation (Waters, Milford, MA). Hydrolysis for 3 hr is long enough to release all aspartyl and asparaginyl residues, but short enough to limit the racemization resulting from these harsh conditions.[28] D-Aspartyl, D-isoaspartyl, and D-asparaginyl residues are each released as D-aspartate by hydrolysis. The hydrolysates are resuspended in double distilled water, and the amino acids are derivatized with *N*-acetyl-L-cysteine and orthophthalaldehyde.[29] The resulting fluorescent diastereomers are separated isocratically on a 3.9 × 150-mm reversed-phase Resolve $C_{18}$ column (Waters) equilibrated in a mixture of 95% buffer A (50 m*M* sodium acetate, pH 5.4) and 5% buffer B (80% methanol, 20% buffer A)[28] and monitored with a Gilson Model 121 fluorometer (Middleton, WI. The excitation and emission filters used have windows of 305–395 and 430–470 nm, respectively. Under these conditions, the derivatives containing D-aspartate and L-aspartate elute at about 6.3 and 7.3 min, respectively. Their fluorescence color constants are determined with D- and L-aspartate standards. Comparison of the D-aspartate present in an L-aspartate standard before and after undergoing the acid hydrolysis used on the peptides, as well as the analysis of acid-hydrolyzed synthetic A$\beta$, indicates that no more than 2% of observed D-aspartate is due to racemization during the procedure. The method of

[26] N. Fujii, Y. Ishibashi, M. Fujino, and K. Harada, *Biochim. Biophys. Acta* **1204,** 157 (1994).
[27] J. L. Radkiewicz, H. Zipse, S. Clarke, and K. N. Houk, *J. Am. Chem. Soc.* **118,** 9148 (1996).
[28] L. S. Brunauer and S. Clarke, *J. Biol. Chem.* **261,** 12538 (1986).
[29] D. W. Aswad, *Anal. Biochem.* **137,** 405 (1984).

D'Aniello *et al.*[30] is reported to give a much lower racemization background but requires several enzymatic steps that may be difficult with very small samples.

The results of this analysis show that whereas peaks A and G contain primarily L-aspartate, peaks A-bis (the trailing shoulder of peak A) and H (the trailing shoulder of peak G) each contain more than 50% D-aspartate (Fig. 5). Combined with the blockage to sequencing, this indicates that these peptides contain D-isoaspartyl residues. As seen in Fig. 1B, neither peptide is well resolved from the more abundant L-isoaspartyl-containing analogs. In fact, the finding that half of the peptide in peak H contains an L-isoaspartyl residue explains why this material is methylated half as well as peak G (Fig. 3). The D-aspartate fraction in peak I-bis is twice that in peak I, indicating that this poorly resolved shoulder peak is the D-aspartyl-containing $A\beta_{6-16}$ contaminated with the L-aspartyl form (Fig. 5). Peak C from both plaque Aβ (Fig. 5 "C") and vascular Aβ (Fig. 5 "M-C") contains very little D-aspartate, confirming its identity as L-aspartyl-Aβ1–5. Interestingly, the elution pattern of L-isoaspartyl-, D-isoaspartyl-, L-aspartyl-, and D-aspartyl-Aβ6–16 peptides on reversed phase HPLC closely matches that observed with a synthetic tetrapeptide[31] and an 18 residue tryptic peptide[32] containing isomerized and racemized residues, suggesting that this pattern is independent of peptide length and sequence. The relative elution positions of the Aβ1–5 peptides are more difficult to interpret due to N-terminal heterogeneity of the Aβ. Although L-isoaspartyl-, D-isoaspartyl-, and L-aspartyl-Aβ1–5 elute in peaks A, A-bis, and C, respectively, peak B apparently contains both L-isoaspartyl and D-isoaspartyl residues, and D-aspartyl-Aβ1–5, the least abundant of the peptides, has not been identified.

Taken together, our analyses show that about 75% of the aspartyl residues in positions 1 and 7 in Aβ from dense neuritic plaques are in the isoaspartyl configuration and that about 25% of both the aspartyl and the isoaspartyl residues have racemized to the uncommon D form.[2] Aβ purified from vascular amyloid deposits has fewer damaged residues (Fig. 1C): only 20–25% of the residues in positions 1 and 7 are in the isoaspartyl configuration,[3] suggesting that this Aβ is younger than the dense neuritic plaque material or else is in a microenvironment that limits succinimide formation.

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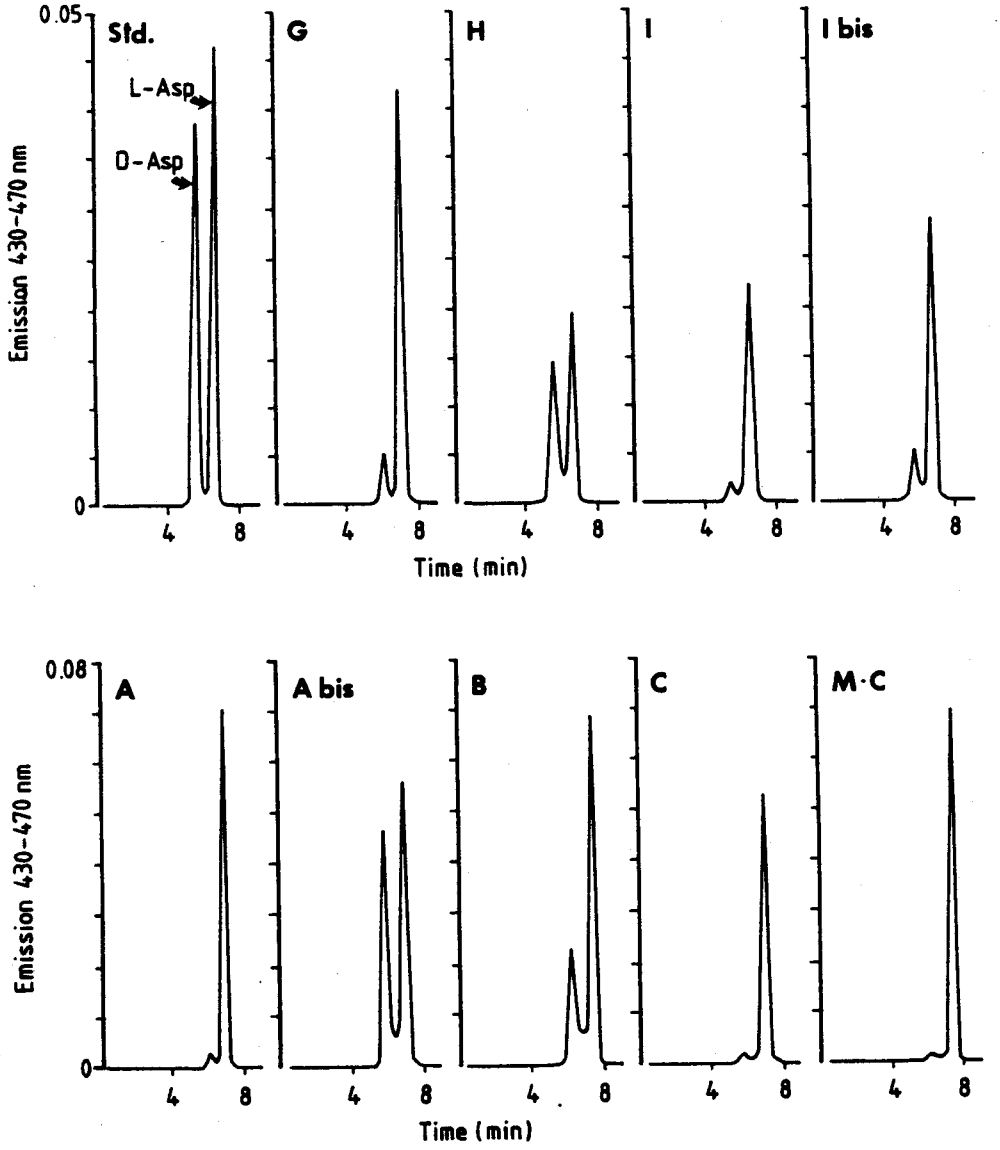


FIG. 5. Stereoconformational determination of $Asp_1$ and $Asp_7$ in A$\beta$ tryptic peptid Aspartyl residues, released from the indicated neuritic plaque A$\beta$ tryptic peptides by m acid hydrolysis, are derivatized with *N*-acetyl-L-cysteine and phthalaldehyde and resolved reversed-phase HPLC. (Top) Analysis of Asp in neuritic plaque A$\beta$1–6 samples G, H, I, a I-bis and D L-aspartate standard. (Bottom) Analysis of $Asp_1$ in neuritic plaque A$\beta$1–5 samp A, A-bis, B, and C and meningeal A$\beta$1–5 sample C (M-C). Reproduced with permiss from A. E. Roher *et al., J. Biol. Chem.* **268,** 3072 (1993).

*Racemization of Seryl Residues in Aβ*

Like aspartyl residues, racemized seryl residues have been observed in long-lived proteins.[33,34] Some synthetic Aβ derivatives containing a D-seryl residue are found to be more resistant to proteolytic degradation.[35] We measured the level of serine racemization in Aβ using the same method as for aspartyl residues. With the system described in this article, derivatized L-serine and D-serine elute at about 11 and 12 min, respectively. Hydrolysis of synthetic Aβ1–42 produces 1.6% D-serine, so this is the maximum D-serine that can be generated by the experimental procedures. Hydrolysis of Aβ1–42 from dense neuritic plaques, however, releases 6.2% D-serine, indicating that at least 4.6% of the two seryl residues in this Aβ is racemized. The serine in position 8 contains the same amount of the D form as does Aβ1–42, indicating that this residue and serine-26 are probably equivalently racemized.

## Characterization of Aβ 3-Pyroglutamyl Residues

When glutamate-3 is exposed at the amino terminus of Aβ by proteolysis of the first two residues, it can undergo conversion into a pyroglutamyl residue (3pE). This modification prevents further proteolytic degradation of the Aβ-3pE by aminopeptidases, thus promoting the accumulation of this peptide. Immunocytochemical studies[7] and mass spectrometric analyses of complex mixtures of Aβ peptides[5,6,36] provided the first indication of pyroglutamyl in some of the Aβ. Tryptic digestion followed by separation of the resulting N-terminal peptides by HPLC permitted the quantitation of Aβ-3pE levels in Alzheimer's disease brains.[36]

Amyloid is purified from neuritic plaques and leptomeningeal vascular deposits,[2,36] and the Aβ isolated by FPLC and digested by trypsin as described earlier. The resulting tryptic peptides are separated by HPLC on an LKB $C_{18}$ reversed-phase column (Spherisorb ODS2, 3-μm bead size, 4.6 × 100-mm column, Pharmacia-LKB, Sweden) using a linear gradient at a flow rate of 0.7 ml $min^{-1}$ of 5–8.5% acetonitrile/0.05% trifluoroacetic acid over 70 min at room temperature. The column effluent is monitored at 214 nm and fractions are collected every 30 sec. This shallow gradient allows for a better separation of the N-terminal peptides ending at residue

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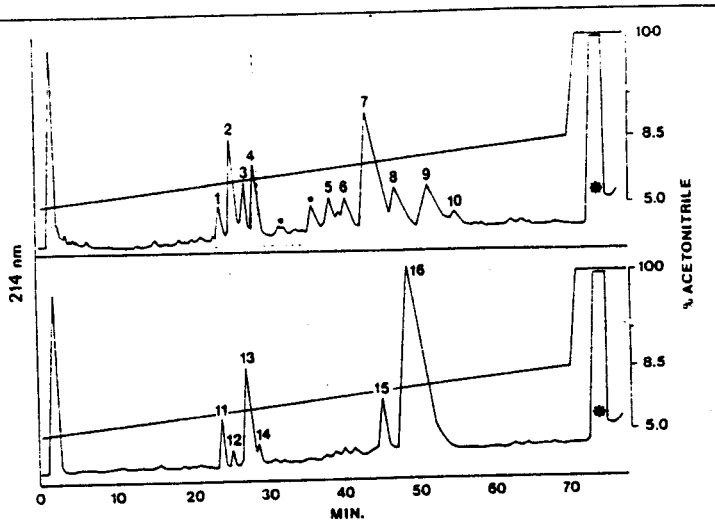


FIG. 6. Purification of Aβ 3-pyroglutamyl peptide on $C_{18}$ reversed-phase HPLC. The N-terminal peptide 3pE-Aβ3–5 elutes as peak 4 in a tryptic digest of neuritic plaque core Aβ (top) and as peak 14 in a tryptic digest of vascular Aβ (bottom). The tryptic peptide in peak 4 coelutes exactly with the synthetic peptide pyroglutamylphenylalanylarginine (broken line). Reproduced with permission from Y.-M. Kuo *et al.*, *Biochem. Biophys. Res. Commun.* **237,** 188 (1997).

TABLE II
Aβ N-TERMINAL TRYPTIC PEPTIDES FROM NEURITIC PLAQUE CORE AND VASCULAR AMYLOID

| HPLC peak[c] | Sequence | Aβ residues | Neuritic plaque Aβ peptides (%) A[a] | B[a] | C[a] | Average |
|---|---|---|---|---|---|---|
| 1 | AEFR | 2–5 | 9 | 7 | 9 | 8 |
| 2 | (isoD)AEFR | 1–5 | 24 | 13 | 22 | 20 |
| 3 | DAEFR | 1–5 | 11 | 7 | 11 | 10 |
| 4 | pEFR | 3pE-5 | 46 | 62 | 45 | 51 |
| 5 | GYEVHHQK | 9–16 | 5 | 4 | 6 | 5 |
| 6 | SGYEVHHQK | 8–16 | 5 | 7 | 7 | 6 |
| | | | Vascular Aβ peptides (%) | | | |
| | | Aβ residues | D[b] | E[b] | F[b] | Average |
| 11 | AEFR | 2–5 | 23 | 19 | 20 | 20 |
| 12 | (isoD)AEFR | 1–5 | 5 | 7 | 5 | 6 |
| 13 | DAEFR | 1–5 | 60 | 64 | 65 | 63 |
| 14 | pEFR | 3pE-5 | 12 | 10 | 10 | 11 |

[a] A, B, and C represent three independent neuritic plaque amyloid preparations.
[b] D, E, and F represent three independent vascular amyloid preparations.
[c] See Fig. 6.

Arg-5. Peptide 4 from neuritic plaque Aβ and an equivalent peptide from vascular Aβ (Fig. 6) elute with the same retention time as synthetic pyroglutamylphenylalanylarginine (obtained from California Peptide Inc.) at 28.7 min. Amino acid analysis confirmed that these peptides are tripeptides composed of glutamate, phenylalanine, and arginine (Aβ3–5). However, their molecular weight obtained by mass spectrometry, being 18 mass units lower than expected for this tripeptide, confirms the loss of water caused by cyclization of the N-terminal glutamyl residue.

## Conclusion

The procedures described in this article demonstrate that few of the Aβ peptides present in dense neuritic plaques in Alzheimer disease brain are untouched by one or more of several spontaneous chemical modifications. Only 10% of the peptides possess the normal L-aspartyl residue expected at the N terminus, whereas 20% start with an L-isoaspartyl residue, 51% start with a pyroglutamyl residue, and the rest have suffered various amounts of proteolysis (Table II). Only 20% of the residues in position 7 are in the normal L-aspartyl configuration, with the rest being isomerized and/or racemized. Racemization also effects 4–6% of the serine residues. If these modifications arise randomly through the Aβ population in a stable amyloid deposit, fewer than 2% of the peptides should remain unaltered. Even if chemical modification is not random (i.e., younger peptides are undamaged, whereas older peptides are multiply modified), no more than 10% of the Aβ will be unaltered at the N terminus. Aβ from vascular amyloid deposits is less damaged, and thus perhaps younger, than that in neuritic deposits, but modification of 37% of these peptides at the N-terminus is still significant (Table II). It is therefore important to take these modifications into account when studying the role of Aβ in the development and progression of Alzheimer's disease.