The Alzheimer's disease (AD) brain exhibits region-specific patterns of amyloid plaque deposition, neurofibrillary tangle (NFT) accumulation, and neuron death. The limbic system and association areas of the neocortex show the most pronounced histopathological alterations in AD, whereas cortical somatosensory and cerebellar neurons are relatively spared (Pearson et al., 1985; Henderson and Finch, 1989; Braak and Braak, 1994). Recent models of AD attempt to link disease progression with an inflammatory component combined with increased oxidative stress (Rogers et al., 1996). Classical hallmarks of inflammation such as edema and neutrophil infiltration are not acknowledged characteristics of the AD brain, although numerous correlates of inflammation are present. Acute-phase reactants such as C-reactive protein, major histocompatibility complex glycoproteins, complement, monocyte chemoattractants, interleukin-1, and interleukin-6 are elevated in AD brain in spatial association with neuritic plaques (Griffin et al., 1989, 1995; McGeer et al., 1991, 1992; Mecocci et al., 1993; Balazs and Leon, 1994; Chen et al., 1994; Hensley et al., 1995; Lovell et al., 1995; Tabcheh et al., 1993; Savenkova et al., 1994; Domigan et al., 1995; Marquez and Dunford, 1995; Eiserich et al., 1996, 1998; Jacob et al., 1996). Reactive microglia, functionally similar to monocytes, are increased in the AD brain and concentrate near senile plaques (McGeer et al., 1987; Haga et al., 1989; Itagaki et al., 1989; Carpenter et al., 1993; MacKenzie et al., 1995).

Enhanced oxidative stress in the AD brain is manifested by increases in protein carbonyl content and lipid and DNA oxidation products and by inactivation of sensitive enzymes (Oliver et al., 1987; C. Smith et al., 1991, 1992; Mecocci et al., 1993; Balazs and Leon, 1994; Chen et al., 1994; Hensley et al., 1995; Lovell et al., 1995; M. Smith et al., 1996; Butterfield et al., 1997; Lyras et al., 1997; Sayre et al., 1997). Correlation between oxidative and inflammatory biomarkers has not been achieved in the AD brain, although the activation of an inflammatory response might, in large part, explain AD brain oxidation. For instance, activated microglia release superoxide (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$) (Colton et al., 1994), whereas astrocytes and microglia stimulated with appropriate cytokines or $\beta$-amyloid peptides (AB) express inducible nitric oxide synthase (iNOS) and generate nitric oxide-derived species, including peroxynitrite (ONOO$^-$) (Beckman et al., 1994; Goodwin et al., 1995; Li et al., 1996; Hensley et al., 1997).

In the present study HPLC with electrochemical array detection (HPLC-ECD) was used to quantify discrete tyrosine oxidation products expected to form during an inflammatory response. Tyrosine (Tyr), 3-nitrotyrosine (3-NO$_2$-Tyr), and 3,3'-dityrosine (diTyr) were determined simultaneously in the protein digests of brain specimens obtained from AD and neuropathologically normal subjects. The presence of 3-NO$_2$-Tyr is thought to indicate NOS-derived peroxynitrite (Hensley et al., 1997; M. Smith et al., 1997; Yi et al., 1997), whereas diTyr formation and protein cross-linking are associated with peroxidase activity and neutrophil or macrophage activation (Heinecke et al., 1993; Salman-Tabcheh et al., 1993; Savenkova et al., 1994; Domigan et al., 1995; Marquez and Dunford, 1995; Eiserich et al., 1996, 1998; Jacob et al., 1996; Malencik and Anderson, 1996; Malencik et al., 1996; Michon et al., 1997). DiTyr and 3-NO$_2$-Tyr were elevated markedly in the AD brain, especially in the hippocampus. Moreover, uric acid, a proposed endogenous antioxidant and ONOO$^-$ scavenger, was decreased in AD in a manner consistent with the increases of 3-NO$_2$-Tyr and diTyr. These findings indicate a relationship between the inflammatory state and oxidative damage in the AD brain.

MATERIALS AND METHODS

CNS tissue. Specimens were obtained at postmortem from five cognitively normal subjects and 11 AD patients who met National Institute of Neurological and Communicative Disorders and Stroke--Alzheimer's Disease and Related Disorders Association (NINCDS--ADRDA) Work Group Criteria for AD (McKhann et al., 1984; Henderson and Finch, 1989).
1989). All AD subjects met accepted criteria for the histopathological diagnosis of AD (Mirra et al., 1993; National Institute on Aging, 1997). Normal subjects were members of a volunteer group who underwent annual neuropsychological testing. Normal individuals had no history of dementia, neurological disease, or systemic diseases likely to affect the brain. The mean age ± SD of normal subjects was 78 ± 6 years (three male, two female) and of AD subjects was 78 ± 8 years (seven male, four female). The mean postmortem interval ± SD was 3.0 ± 1.6 hr for AD and 2.6 ± 0.5 hr for normal subjects. The mean duration ± SD of clinically defined AD was 10 ± 6 years (uncertain in 2 of 11 cases). Specimens were frozen in liquid N₂ and stored at −80°C until processing for HPLC-ECD. Tissue from four brain regions was collected from each individual: hippocampus and parahippocampal gyrus (HIP), inferior parietal lobule (IPL), superior and middle temporal gyri (SMTG), and cerebellum (CBL). Ventricular cerebrospinal fluid (VF) was removed from the lateral ventricles before the brain was removed from the cranial vault. VF was centrifuged at 10,000 × g for 10 min and frozen at −80°C.

Tissue preparation before HPLC-ECD analysis. Brain specimens (300–500 mg) were homogenized with a Dounce-type homogenizer in 10 mM sodium acetate (NaOAc), pH 6.5, and protein concentration was determined by the Lowry method (Lowry et al., 1951). VF was not homogenized. A protease digestion strategy was used to liberate tyrosine residues. Samples (previously homogenized orVF) were mixed with freshly prepared solutions of S. griseus protease (Pronase) to yield 5.0 mg/ml brain protein and 1 mg/ml Pronase in a volume of 0.25 ml. Similar samples were prepared without Pronase for the purpose of determining unbound (free) concentrations of analytes. Pronase-treated samples were incubated for 18 hr at 50°C, after which they were treated with a 10% volume of 60% trichloroacetic acid (TCA) for 10 min at 14,000 × g at 4°C. Samples that were not treated with Pronase were subjected immediately to TCA precipitation and centrifugation. Supernatants were removed and passed through a 0.4 μm polyvinylidene difluoride membrane. Filtrates were frozen at −80°C until analysis.

HPLC-ECD analytical protocols. Routine HPLC-ECD was performed on an ESA (Chelmsford, MA) model 5600 CoulArray instrument equipped with eight detector cells operating in the oxidative mode at specified potentials, as previously described (channel/potential = 1, 180 mV; 2, 240 mV; 3, 350 mV; 4, 600 mV; 5, 700 mV; 6, 750 mV; 7, 830 mV; 8, 900 mV) (Hensley et al., 1997). The working electrode was porous carbon, whereas the reference and counter electrodes were palladium wire. Analyte separation was conducted on a TOSOHAGAS (MONTGOMERYVILLE, PA) reverse-phase ODS 80-T M C-18 analytical column (4.6 mm inner diameter × 25 cm; 5 μm particle size). A two-component gradient elution system was used, with component A of the mobile phase being 50 mM NaOAc, 50 mM citric acid, and 0% methanol, pH 3.1, and component B being similar to A except with 20% methanol (MeOH). A gradient elution profile was used as follows: 0–20 min, 0% MeOH; 20–30 min, linear ramp to 10% MeOH; 30–40 min, isotropic 10% MeOH; 40–50 min, linear ramp to 15% MeOH; 50–60 min, isotropic 15% MeOH; 60–70 min, linear ramp to 20% MeOH; 70–90 min, isotropic 20% MeOH. All experiments except dityrosine were obtained from Sigma (ST. LOUIS, MO). An automated injection protocol was used wherein each sample was included as part of a three-injection series. Injection one consisted of a mixture of standards, injection two was the actual sample to be analyzed (60 μl), and injection three was the standard mixture combined with (spiked into) the sample. Components of the standard mixture were adjusted as necessary to approximate mean regional concentrations in tissue preparations. Peak assignment and quantitation were performed by an individual blind to sample identity (Hensley et al., 1997). Specific spike–recovery and stability experiments were performed to confirm that tyrosine derivatives were recovered quantitatively from brain tissue after protease treatment and that artifactual oxidation, nitration, or chlorination did not occur during TCA precipitation and subsequent manipulations.

HPLC-ECD confirmatory protocols. To validate further the assignment of HPLC peaks, we chromatographed selected samples on a 12 channel ECD, using an ion-pairing mobile phase designed to induce retention time shifts among closely eluting analytes. Cell potentials were specified as follows (channel/potential): 1, 200 mV; 2, 300 mV; 3, 400 mV; 4, 525 mV; 5, 600 mV; 6, 625 mV; 7, 650 mV; 8, 675 mV; 9, 700 mV; 10, 750 mV; 11, 825 mV; 12, 900 mV. Component A of the two-component mobile phase was 58 mM lithium phosphate, 0% methanol, and 3 mg/l lithium dodecysulfate (LDS), pH 3.2, and component B consisted of 58 mM Li₂PO₄, 20% methanol, and 3 mg/l LDS, pH 3.2. The gradient profile began with a 15 min isocratic elution (100%), followed by a linear ramp to 20% methanol (100% B) at 80 min run time. Under these conditions the 3-NO₂-Tyr eluted at 73 min, whereas diTyr eluted at 86 min. As a final test for authenticity of the 3-NO₂-Tyr peak, samples were treated with 10 mM sodium hydroxylate to reduce 3-NO₂-Tyr to 3-amino tyrosine (3-NH₂-Tyr; Hensley et al., 1997). Although 3-NH₂-Tyr elutes near the solvent front under most chromatographic conditions (Hensley et al., 1997), this analyte is shifted to a convenient retention time, using the LDS gradient (40 min), and oxidizes at a characteristically low potential (150 mV).

Dityrosine synthesis and characterization. 3,3′-Dityrosine was synthesized from tyrosine and H₂O₂, using horseradish peroxidase as a catalyst according to described methods (Malencik et al., 1996). Purity and identity were verified by HPLC and by gas chromatography–mass spectrometry after derivatization with propanol and heptafluorobutyryl anhydride (Heinecke et al., 1993).

Nitrite and nitrate assays. Nitrite (NO₂⁻) was assayed by the Griess diazotization reaction (Green et al., 1982). Nitrate (NO₃⁻) was assayed by the same method after treatment with nitrate reductase and NADPH, as described (Gilliam et al., 1993).

Data analysis. Tyrosine derivatives were expressed as a ratio to Tyr: uric acid was expressed as a micromolar concentration in defined and constant sample volumes. Concentration variations were assessed by two-way ANOVA, using disease state and brain region as the primary and secondary factors. Student’s t tests were used post hoc to determine individual p values and the significance of correlations. A p value <0.05 was considered significant.

RESULTS

Validation of sample preparation

The Pronase digestion scheme used in this study quantitatively liberates tyrosine from albumin with ~5% excess tyrosine released via autohydrolysis of the protease (Hensley et al., 1997; Shigenaga et al., 1997). Pronase digestion combined with HPLC-ECD analysis has measured successfully the 3-NO₂-Tyr and 3,4-DOPA in glial cells treated with interleukin-1β (Hensley et al., 1997) and in zymosan-treated macrophages (Shigenaga et al., 1997). Proteolytic digestion eliminated the need for organic extraction and delipidation steps that would be necessary if an acid hydrolysis of proteins were attempted and avoided the charring of homogenates during acid hydrolysis.

Tyrosine, diTyr, and 3-NO₂-Tyr were stable at 50°C in 10 mM NaOAc, pH 6.5, for at least 18 hr. NO₃⁻ and NO₂⁻ (50 μM each) could be incubated with tyrosine (100 μM) and 6% TCA for 18 hr at 50°C or at room temperature for 5 d with <0.01% yield of 3-NO₂-Tyr. DiTyr and 3-NO₂-Tyr spikes were recovered quantitatively from tissue preparations incubated under protein digestion conditions. Therefore, artifacts arising from nitration, chlorination, and cross-linking of tyrosine during sample preparation were concluded not to be a concern. Tyrosine concentrations in protein digests did not vary significantly between normal and AD groups in any tissue that was studied.

NO₂⁻ and NO₃⁻ analyses

Mean NO₂⁻ concentration was decreased and mean NO₃⁻ concentration was increased in VF from AD subjects relative to normal subjects, although statistical significance was achieved only with respect to NO₂⁻ [mean ± SEM [NO₂⁻] = 2.65 ± 0.37 μM (normal) versus 1.74 ± 0.20 μM (AD), p < 0.05; mean ± SEM [NO₃⁻] = 2.60 ± 0.66 μM (normal) versus 3.95 ± 0.67 μM (AD), not significant (NS)]. The sum of [NO₃⁻] + [NO₂⁻] did not differ between AD and normal groups [5.12 ± 0.48 μM (normal) versus 5.68 ± 0.62 μM (AD)]. Because of these trends the [NO₃⁻]/[NO₂⁻] ratio tended to increase in AD [1.33 ± 0.59 (normal) versus 2.69 ± 0.55 (AD), NS]; NO₂⁻ and NO₃⁻ levels in tissue homogenates were below the detection limits of the Griess assay (1 μM).
3-Nitrotyrosine and 3,3'-dityrosine in brain

Figure 1 illustrates the resolution of tyrosine derivatives in a typical HPLC-ECD chromatogram of an AD brain protein digest (SMTG region) chromatographed by the same mobile phase as the sample shown in Figure 1, with the inclusion of LDS in the mobile phase. A, Original sample (full scale = 25 μA current) showing ECD response at 200 mV cell potential (channel 1). The peak marked in A (asterisk) coeluted near 3-NH₂-Tyr but differed markedly with respect to oxidation potential (see Results). B, Original sample at 700 mV cell potential (channel 9) illustrating the peak assigned to 3-NO₂-Tyr. C, D, Regions of the chromatogram shown in A and B, respectively, after treatment of the sample with hydrosulfite to partially reduce the 3-NO₂-Tyr to 3-NH₂-Tyr (labeled).

**Figure 2.** HPLC-ECD chromatogram of a protein digest from an AD brain (SMTG region) chromatographed by using the same column as the sample shown in Figure 1, with the inclusion of LDS in the mobile phase. A, Original sample (full scale = 25 μA current) showing ECD response at 200 mV cell potential (channel 1). The peak marked in A (asterisk) coeluted near 3-NH₂-Tyr but differed markedly with respect to oxidation potential (see Results). B, Original sample at 700 mV cell potential (channel 9) illustrating the peak assigned to 3-NO₂-Tyr. C, D, Regions of the chromatogram shown in A and B, respectively, after treatment of the sample with hydrosulfite to partially reduce the 3-NO₂-Tyr to 3-NH₂-Tyr (labeled).

**Figure 3.** Quantitation of tyrosine oxidation products in various regions of the AD and normal human brains. Error bars indicate SEM; *p < 0.05.

3-Nitrotyrosine and 3,3'-dityrosine in brain

Figure 1 illustrates the resolution of tyrosine derivatives in a typical HPLC-ECD chromatogram of an AD brain protein digest (SMTG region). Figure 2 illustrates 3-NO₂-Tyr and hydrosulfite-reduced 3-NH₂-Tyr peaks identified in a sample prepared from the same SMTG tissue, chromatographed by using the LDS mobile phase described in Materials and Methods. DiTyr and 3-NO₂-Tyr coeluted with authentic standards on both gradients, with appropriate voltammetric characteristics. Furthermore, the 3-NO₂-Tyr peak was partially reduced to the amino derivative with hydrosulfite (~30% conversion; it should be noted that complete hydrosulfite reduction of dilute 3-NO₂-Tyr is a practical impossibility owing to kinetic issues and reversibility of the reaction (Hensley et al., 1997)). 3-NH₂-Tyr was not found in samples that were not treated with hydrosulfite, although small amounts of a closely eluting peak were observed that oxidized at markedly higher oxidation potential than authentic 3-NH₂-Tyr (~300 mV; electrochemical response dominant on channel 2; Fig. 2).

Both 3-NO₂-Tyr and diTyr were elevated significantly in AD hippocampus and cortical regions, although neither was elevated in the cerebellum (Fig. 3). 3-NO₂-Tyr and diTyr concentrations covaried across brain regions (r = 0.58 if regressed point-by-point, r = 0.96 by regression of mean regional values; p < 0.01 in either case; Fig. 4). The relative difference between subject groups is most striking in the HIP region, where diTyr content increased almost fivefold and 3-NO₂-Tyr content increased almost eightfold in AD (see Fig. 3). Interestingly, the absolute concentrations of diTyr and 3-NO₂-Tyr were five- to 10-fold
greater in SMTG than in HIP, IPL, or CBL regardless of disease state (Figs. 3, 4). Nonetheless, the relative differences in analyte levels between AD and normal SMTG were less pronounced (two- to fivefold) than corresponding HIP and IPL perturbations (Figs. 3, 4). The trends illustrated in Figures 2 and 3 were reproduced if data were expressed as absolute concentrations of analyte in protein digest or as a ratio to milligrams of protein digested. Analysis of free analytes before protease treatment indicated a negligible contribution of unbound analytes to the levels measured in the digests. The subtraction of unbound analytes resulted in 10–20% decrease in analyte/Tyr ratios in all cases, with no alteration of statistically significant groupings, as shown in Figure 1. Additionally, 3-NO₂-Tyr and diTyr were not observed if Pronase was incubated in the absence of brain protein.

Tyrosine derivatives in VF
VF contained 0.01–0.5 μM free diTyr and 3-NO₂-Tyr, and these values increased only two- to fivefold with protease digestion. Free diTyr content increased 3.7-fold in AD VF relative to normal VF and increased twofold in protein digests (Fig. 5). 3-NO₂-Tyr content increased in both free and protein digest fractions (2.3- and 1.4-fold, respectively), although this increase was not statistically significant (Fig. 5). Protein concentration within VF was similar between AD and normal groups [0.47 ± 0.15 mg/ml (normal) versus 0.41 ± 0.03 mg/ml (AD)].

Uric acid analysis
Uric acid concentrations were highly variable. Nonetheless, mean values were decreased 40–50% in AD brain specimens relative to normal specimens (Fig. 6). Uric acid levels also were decreased in VF from AD subjects (Fig. 6). Although region-specific variations in uric acid were not observed, AD was found to be a significant factor in brain uric acid content (p < 0.05 by ANOVA). Additionally, an inverse correlation was observed between VF [NO₃⁻] /[NO₂⁻] ratio and uric acid/protein ratio (r = 0.52; n = 16; p < 0.05).

DISCUSSION
HPLC-ECD analysis of the AD brain indicates disease-related patterns of protein diTyr and 3-NO₂-Tyr accumulation and uric acid loss. The hippocampus, the most severely affected region of the AD brain among those presently investigated (Pearson et al., 1985; Price et al., 1991; Braak and Braak, 1994), showed the greatest relative alterations in diTyr and 3-NO₂-Tyr. Neocortical regions that also are affected in AD exhibited lesser relative changes in these analytes. The cerebellum, which virtually is unmarred by the landmark histopathological correlates of AD (senile plaques and neurofibrillary tangles), is unaffected by protein nitration and cross-linking.
Several previous studies used HPLC-ECD to measure 3-NO2-Tyr in brain of experimental animals and normal humans (Schulz et al., 1995; Maruyama et al., 1996). However, a recent communication by Kaur and colleagues warns that 3-NO2-Tyr determination by reverse-phase HPLC is subject to artifacts arising from an unidentified coeluting species with electrochemical similarity to 3-NO2-Tyr but that differs from 3-NO2-Tyr in spectroscopic characteristics (Kaur et al., 1998). The chromatographic conditions described by Kaur and colleagues used an isocratic mobile phase with high methanol content (10%) and flow rate (1 ml/min) such that authentic 3-NO2-Tyr standards eluted with a retention time of ~10 min, as did several other tested compounds, including kynurenine (Kaur et al., 1998). The chromatographic protocol described in our study was sufficient to resolve 3-NO2-Tyr from all other electrochemically active compounds we have tested, including kynurenine (retention time ~79 min under LDS-free conditions). Even using low methanol (0%) and a slow flow rate (0.6 ml/min), we find numerous electrochemically active compounds elute within the first 15 min of the chromatographic run, with prominent peaks being poorly resolved. It is most likely that the unidentified peak described by Kaur and colleagues (1998) consists of many coeluting substances.

The current study is the first to quantify 3-NO2-Tyr and to detect or quantify diTyr in the AD brain. 3-NO2-Tyr has been detected previously by immunochemical means in AD cortex, where it is associated with NFT-bearing neurons (Good et al., 1996; M. Smith et al., 1997; Su et al., 1997). HPLC-ECD and immunochemical techniques yield complementary data. Whereas immunochemical staining suggests cellular localization of analytes, the disadvantage is that specific analytes are not resolved and determined quantitatively. Interestingly, Maruyama and colleagues report an HPLC-ECD analysis of free 3-NO2-Tyr in normal human brains wherein they observe threefold greater levels of 3-NO2-Tyr in cerebrum than cerebellum (Maruyama et al., 1996). The yield of 3-NO2-Tyr reported by Maruyama and colleagues in cerebrum: ([3-NO2-Tyr]/[Tyr] = 1.62 × 10−3), closely approximates the quantities we observe, as does the finding that cerebellum is relatively immune to protein nitration. Previous analyses of NO2− and NO3−, two other putative indices of NO generation, have been contradictory. Kuiper and colleagues (1994) report decreased NO2− and unchanged NO3− in AD CSF, whereas Navarro and colleagues (1996) and Milstien and colleagues (1994) independently report no alteration in CSF NO2− plus NO3−. NO2− rather than NO3− is thought to be the major breakdown product of ONOO− so that the ratio [NO2−]/[NO3−] may be a marker for ONOO− generation rather than either analyte considered independently (Pfeiffer et al., 1997). In the present study a strong trend was seen for increased VF [NO2−]/[NO3−] ratio in AD, which correlated inversely with uric acid levels and which paralleled a trend toward increased 3-NO2-Tyr content.

Tyrosine dimerization as well as nitration can be affected by ONOO− (MacMillan-Crow et al., 1998), so the same oxidant could be responsible for the accumulation of both 3-NO2-Tyr and diTyr. Alternatively, efficient synthesis of diTyr results from exposure to peroxidase enzymes. DiTyr standards used in this study were synthesized in 50% yield by the treatment of tyrosine with H2O2 and horseradish peroxidase (Malencik et al., 1996), although myeloperoxidase catalyzes the same reaction (Marquez and Dunford, 1995; Jacob et al., 1996). Conceivably, the induction of NOS in the AD brain could correlate with the expression or recruitment of various peroxidases. Cyclooxygenase-2 (COX-2), a membrane-localized peroxidase involved in arachidonic acid metabolism and often expressed simultaneously with iNOS during inflammation, is expressed in AD brain (Lukiw and Bazan, 1997). It is possible that COX isoforms may be capable of diTyr synthesis, although this hypothesis has not been investigated systematically.

The decrease in uric acid levels in AD may be related to increased tyrosine nitration in certain brain regions. Uric acid efficiently scavenges ONOO− in vitro (Whiteman and Halliwell, 1996; our unpublished observations) and inhibits tyrosine nitration in cultured neurons challenged with Aβ, iron salts, or NO generators (Mattson et al., 1997; Keller et al., 1998). Similarly, 1–100 μM concentrations of uric acid protect cultured neurons from iron and Aβ-induced apoptosis (Mattson et al., 1997). We estimate endogenous brain uric acid concentration to be 10–50 μM (after correction for dilution that occurs during the processing of tissue), within the apparent neuroprotective dose for this substance (Mattson et al., 1997). It is therefore possible that the uric acid decline in AD brain reflects, or possibly contributes to, AD-related neurodegeneration. One previous study reports decreased uric acid in AD CSF (Tohgi et al., 1993), whereas another reports increased uric acid in AD CSF (Degrell and Niklasson, 1988). Measurements of serum uric acid in AD are similarly contradictory (Maesaka et al., 1993; Ahlskog et al., 1995). Interestingly, uric acid reportedly protects rodents against motor dysfunction and tyrosine nitration in the experimental allergic encephalomyelitis model of multiple sclerosis (Hooper et al., 1997), suggesting that clinical symptoms of specific neurological disorders might respond to alterations in this compound.

Clearly, further research is needed to elucidate the chemistries involved in protein oxidation in the aging human brain, particularly with respect to determining which types of oxidative stress are most involved in the pathogenesis of AD. The present study suggests that oxidizing agents generated by inflammation-associated enzyme systems may be a significant contributor to protein oxidation within the AD brain. The degree to which this type of oxidative stress is involved in other neurodegenerative conditions remains to be determined.

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