Structural and Functional Changes in Proteins Induced by Free Radical–mediated Oxidative Stress and Protective Action of the Antioxidants *N-tert*-Butyl-α-phenylnitrone and Vitamin E[a]

D. ALLAN BUTTERFIELD,[b,d] TANUJA KOPPAL,[b] BEVERLY HOWARD,[b] RAM SUBRAMANIAM,[b] NATHAN HALL,[b] KENNETH HENSLEY,[b] SERVET YATIN,[b] KERRY ALLEN,[b] MICHAEL AKSENOV,[c] MARINA AKSENOVA,[c] AND JOHN CARNEY[c]

[b]*Department of Chemistry and Center of Membrane Sciences and*
[c]*Department of Pharmacology, University of Kentucky, Lexington, Kentucky 40506-0055, USA*

ABSTRACT: The free radical theory of aging proposes that reactive oxygen species (ROS) cause oxidative damage over the lifetime of the subject. It is the cumulative and potentially increasing amount of accumulated damage that accounts for the dysfunctions and pathologies seen in normal aging. We have prevously demonstrated that both normal rodent brain aging and normal human brain aging are associated with an increase in oxidative modification of proteins and in changes in plasma membrane lipids. Several lines of investigation indicate that one of the likely sources of ROS is the mitochondria. There is an increase in oxidative damage to the mitochondrial genome in aging and a decreased expression of mitochondrial mRNA in aging. We have used a multidisciplinary approach to the characterization of the changes that occur in aging and in the modeling of brain aging, both *in vitro* and *in vivo*. Exposure of rodents to acute normobaric hyperoxia for up to 24 h results in oxidative modifications in cytosolic proteins and loss of activity for the oxidation-sensitve enzymes glutamine synthetase and creatine kinase. Cytoskeletal protein spin labeling also reveals synaptosomal membrane protein oxidation following hyperoxia. These changes are similar to the changes seen in senescent brains, compared to young adult controls. The antioxidant spin-trapping compound *N-tert*-butyl-α-phenylnitrone (PBN) was effective in preventing all of these changes. In a related study, we characterized the changes in brain protein spin labeling and cytosolic enzyme activity in a series of phenotypically selected senescence-accelerated mice (SAMP), compared to a resistant line (SAMR1) that was derived from the same original parents. In general, the SAM mice demonstrated greater oxidative changes in brain proteins. In a sequel study, a group of mice from the SAMP8-sensitive line were compared to the SAMR1-resistant mice following 14 days of daily PBN treatment at a dose of 30mg/kg. PBN treatment resulted in an improvement in the cytoskeletal protein labeling toward that of the normal control line (SAMR1). The results of these and related studies indicate that the changes in brain function seen in several different studies may be related to the progressive oxidation of critical brain proteins and lipids. These com-

[a]This work was supported in part by NIH Grants (AG-10836 and AG-05119).
[d]Address correspondence to D. Allan Butterfield, Department of Chemistry and Center of Membrane Sciences, University of Kentucky, Lexington, KY 40506-0055. Tel: 606/257-3184; fax: 606/257-5876; e- mail: dabcns@pop.uky.edu

ponents may be critical targets for the beneficial effects of gerontotherapeutics both in normal aging and in disease of aging.

FREE RADICALS AND OXIDATIVE STRESS

The evolutionary process selected oxygen over other gases because of its ready availability, the high energy yield of oxidation, easy distribution in its gaseous state, solubility in biocomponents, and its efficient recycling using the processes of respiration and photosynthesis. Oxygen, however, is also the main source of damaging free radicals, which have been suggested to cause aging and ultimately the death of the organism.[1] There are other sources of free radicals, namely, ionizing radiations like X-rays, ultrasound, photochemical reactions, and biochemical and enzymatic processes; however, the human body is not exposed to all of these as frequently as it is to oxygen-derived radicals.

Oxidative injury is the result of an attack on cellular components by highly reactive, toxic oxygen moieties, collectively referred to as reactive oxygen species (ROS). Hydroxyl radicals, peroxyl radicals, superoxide anions, hydrogen peroxide, and nitric oxide are all a part of the ROS family. The half-lives of these free radicals generated in the cell vary from nanoseconds for the highly reactive hydroxyl radical to seconds for nitric oxide and peroxyl radicals. Also the reactivity of these radicals varies from the aqueous environment to those reacting deep within the membrane lipid bilayer. Oxy radicals, like hydroxyl radicals, have a very short life span, are extremely reactive, and hence attack the cellular components present in the vicinity of their production, whereas nitric oxide is very stable and relatively benign, except when it reacts, at diffusion-limited rates, with the superoxide anion to form peroxynitrite. Peroxynitrite is highly reactive and toxic to the cell, affecting several cellular components, leading to loss in structure and function.[2,3] Hence, the radical damage occurring in the cell is all pervasive. Intracellularly, mitochondria are a major source of free radicals.[4] Normal metabolism in healthy individuals uses the electron transport system in the mitochondria for energy production and in the process gives rise to a host of ROS. There are also various enzymatic and nonenzymatic metal-catalyzed systems capable of generating free radicals. To counteract these damaging free radical species, highly effective antioxidant systems have been developed that include enzymes like glutathione peroxidase, glutathione reductase, S-methyl transferase, superoxide dismutase (SOD), and catalase that can either act as repair agents or as antioxidant enzymes by eliminating precursors like hydrogen peroxide and superoxide from the cellular system. Also, there are proteins like hemoglobin, transferrin, and ceruloplasmin that bind ferrous and copper ions and prevent radical generation through Fenton chemistry; and proteases, ribonucleases, and lipases that preferentially degrade the modified components of proteins, DNA, and lipids, respectively. In addition, protection against free radical damage can be afforded by the inclusion of certain vitamins (vitamin C and vitamin E), carotenoids (β-carotene), flavanoids, and other antioxidants in the diet, which inhibit the initiation of the free radical processes or can act as chain-breaking antioxidants.[5]

In spite of the development of various antioxidant systems to counteract the damaging effects of ROS, with age, the cell succumbs to oxidative stress, which has been defined as an imbalance that is shifted towards the prooxidant system relative to the antioxidant systems in the body, leading to cell damage and ultimately cell death.[6] Oxidative stress is known to cause lipid peroxidation, protein oxidation, DNA fragmentation, impairment of cellular energy status, and disruption of ion homeostasis.[7–9] Free radical–mediated oxida-

tive stress has also been implicated in causing damage leading to the pathology of aging[10,11] and such age-associated disorders as stroke, amyotrophic lateral sclerosis, Parkinson's disease, and Alzheimer's disease (AD).[7,12–14] Models of aging, such as hyperoxia, have also been investigated.[15–17]

OXIDATIVE STRESS AND AGING

The free radical theory of aging[1] states that the progressive erosion of cellular components occurring due to free radical damage leads to aging and ultimately results in the death of the organism. Several studies report that the rate of metabolism is directly related to the rate of aging. Oxygen consumption and ROS production are closely related, and hence it is hypothesized that, in animals having high metabolic rates, the levels of ROS are also elevated due to increased oxidative stress. *In vivo* studies have shown that the level of oxidative stress increases during aging.[7,10,18–20] It has never been conclusively established whether this increase in oxidative stress is due to decreased antioxidant levels or due to an increase in production of prooxidant molecules in the cell. Thus oxidative stress can play an important role in aging either by affecting the efficiency with which the antioxidant defenses and/or repair mechanisms operate or by causing structural and functional changes within those molecules, or it can accelerate aging by altering the gene expression of the various cellular components.

Free radical oxidative stress with consequential protein oxidation and lipid peroxidation can lead to cell death. Our laboratory has been involved with factors associated with oxidative stress that alter the physical and chemical states of cortical synaptosomal membranes. Several *in vivo* and *in vitro* models of oxidative stress have been developed and studied for this purpose.[12,,21–23] The focus of this review is a summary of work done in our laboratory on changes in protein structure and function in three *in vivo* models of free radical–induced oxidative stress, namely, hyperoxia, ischemia–reperfusion injury (IRI), and accelerated senescence, and the protection offered by the free radical scavenger, *N-tert*-butyl-α-phenylnitrone (PBN), against these damages. This review also summarizes similar protein damage seen in an *in vitro* model of oxidative stress, that is, synaptosomes exposed to amyloid β-peptide, a peptide implicated in the pathology of AD, and the protective effects of the antioxidant vitamin E against the ensuing damage.

MARKERS OF MEMBRANE PROTEIN DAMAGE

Protein Conformational Changes

Alterations in protein conformations can lead to increased aggregation, fragmentation, distortion of secondary and tertiary structure, susceptibility to proteolysis, and diminution of normal function. The technique of EPR (electron paramagnetic resonance), in conjunction with protein- and lipid-specific spin labels, is used to study membrane protein and lipid conformational changes.[24] The power of EPR spin labeling methods derives from the extreme sensitivity of EPR, the information that can be obtained about motion and polarity of the local microenvironment near the paramagnetic center of the spin label, the relatively simple spectra that need to be analyzed, that opaque samples not

susceptible to light-scattering effects common to optical spectroscopy can be efficiently studied, and the fact that generally, only the spin label is paramagnetic, that is, the biological system is EPR silent and hence, does not interfere with the spectrum. The sulfhydryl-selective spin label MAL-6 (2,2,6,6-tetramethyl-4-maleimidopiperidine-1-oxyl), which covalently binds only to the -SH groups on the proteins, is the predominant spin label employed. MAL-6 is a stable paramagnetic nitroxide that generates an EPR spectrum on binding membrane proteins. Depending on whether the MAL-6 binds to -SH groups deeply within clefts of the protein or close to the protein surface, the spin label is either strongly (S) or weakly (W) immobilized, respectively. This difference in spin-label motion causes both a broad and a narrow low-field line. The ratio of the spectral amplitudes of the M_I low-field resonance line of the weakly immobilized site (W) to that of the strongly immobilized site (S), referred to as the W/S ratio, is a sensitive measure of changes in the physical state of the protein.[24] Decreased W/S ratios indicate increased protein–protein interaction and decreased segmental motion and/or conformational changes in the proteins that were labeled; the converse is also the case.[24]

Earlier studies in our laboratory using other conditions of free radical–induced oxidative stress, such as hydroxyl radical generation,[21] sepsis-associated lipopolysaccharide,[22] or menadione[23] have shown W/S ratios to be lowered in each case. Hence, the W/S ratio can be used as a valuable marker of protein alterations.

Protein Oxidation

Oxidation of proline, histidine, arginine, lysine, and other amino acid residues on proteins leads to the formation of carbonyl derivatives,[10] that is, protein carbonyls can act as a marker of protein oxidation. Protein carbonyl content has been shown to increase with age in several *in vitro* oxidative models, including the gerbil brain and human postmortem brain tissue.[20,25] The spectroscopic assay using dinitrophenylhydrazine (DNPH),[20] in which the carbonyl is reacted with DNPH to form the hydrazone, is one way of determining the levels of protein carbonyls. Other methods involving immunochemistry[26] and histofluorescence[27] have also been used to measure protein carbonyls.

Loss of Enzyme Activity

Oxidative modifications of active-site residues of proteins can lead to loss of enzyme function and activity. Glutamine synthetase (GS) and creatine kinase (CK) are two oxidatively sensitive enzymes.[10] Age-related decline in activities of GS and CK have been observed in gerbil brain and human brain tissue, with the more sensitive GS showing a greater decrease.[25,28,29] GS activity is determined using the cytosolic fraction isolated from homogenized brain neocortices following the assay as described.[30,31]

PROTECTIVE ACTION OF THE FREE RADICAL SCAVENGERS PBN AND VITAMIN E

Spin traps are molecules that have been used in EPR for trapping highly reactive, unstable radicals or radicals that are present in very low concentrations. The spin trap itself is nonparamagnetic but on reacting with the transient free radical species it forms an

EPR-active spin adduct. Spin traps are usually molecules with a nitrone moiety. In earlier studies administration of the free radical spin trap PBN was shown to reduce protein carbonyls of aged gerbils to levels comparable to those seen in young gerbils. This was also accompanied by reversal of memory loss exhibited by the aged animals.[25,32,33] PBN has also been shown to be effective in head trauma.[34]

Vitamin E, unlike PBN, does not rapidly traverse the blood brain barrier, but its efficacy as a free radical antioxidant lies in the fact that it is highly lipophilic and hence can reach the highly oxidation susceptible sites in the membrane lipid bilayer, namely the polyunsaturated fatty acids. Its phenolic head group scavenges the radicals, while the lipophilic side chain aligns with the lipid fatty acid chains to offer membrane stability. Also, molecules like vitamin C and glutathione are involved in recycling vitamin E to its reduced state, which is essential in its scavenging role. Free radical damage to the lipid bilayer can cause impairment of enzyme structure and function[35–37] and can lead to dysfunction in membrane permeability.[38] Studies conducted by other groups have shown the effectiveness of vitamin E in protecting against Aβ toxicity in PC-12 cells[39] and against Aβ-induced production and toxicity of the lipid peroxidation product, 4-hydroxynonenal (HNE), in cultured neuronal cells.[37]

PBN PROTECTION IN *IN VIVO* ANIMAL MODELS OF ISCHEMIA/REPERFUSION, HYPEROXIA, AND SENESCENCE

Stroke represents a major age-related neurodegenerative disorder and is the third leading cause of death in the United States. As a model of stroke, IRI has been shown to be associated with free radical oxidative stress.[40–42] As shown in FIGURES 1 and 2, using the protein-specific spin label MAL-6, the effects of 10-minute ischemia followed by a 1-hour and 14-hour reperfusion were investigated, demonstrating the protective action of PBN against IRI-induced protein damage.[41] For the PBN protection study the gerbils were pretreated with the nitrone 30 minutes prior to the injury. Results showed the W/S ratios comparable to those of the control values at both 1-hour and 14-hour reperfusion time points.[41] However, if instead of administering PBN preinjury, PBN were administered 6 hours postinjury, this nitrone would not attenuate the drastic change in W/S ratios, suggesting its most effective use at the initial stages of reperfusion when free radical damage can be intercepted.

Hyperoxia represents a model of accelerated oxidative stress and aging.[15,43] The hyperoxic conditions developed in our laboratory involved keeping gerbils in a 1 × 1.5 × 0.5 meter clear polycarbonate hyperoxia chamber with oxygen, at 1 atm pressure, constantly monitored at 90–100% for the given time period. Control animals were left outside the hyperoxia chamber for the same time period as the study. EPR studies using MAL-6, protein carbonyl measurements, and GS activity assays were measured according to previous literature methods.[20,24,31] Using the same gerbil model for hyperoxia as mentioned here, we previously had shown that both adult and aged rodents exhibit synaptosomal protein oxidation and that this damage reaches its peak at 24 hours in adult animals.[16] After an exposure to hyperoxic conditions for 48 hours, however, the adult rodents recover, as judged by the W/S ratios, whereas the aged gerbils continue to show further damage.[16] This result is consistent with the notion that with age the ability to recover from oxidative stress-induced damages also decreases. If this protein damage observed were indeed due to free radical–mediated oxidative stress, then free radical scavengers like PBN should

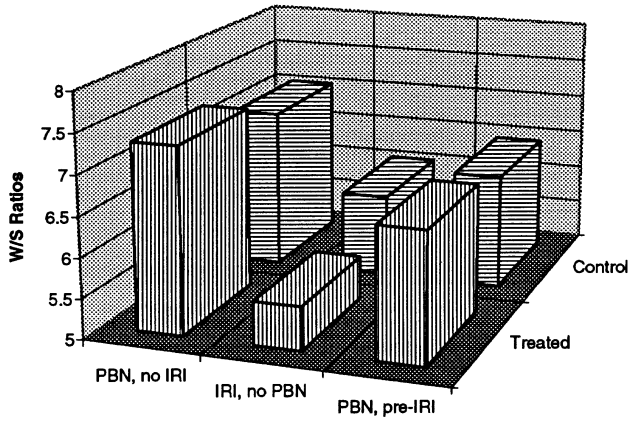

FIGURE 1. Average W/S ratios of MAL-6-labeled cortical synaptosomal membranes of adult gerbils injected with 300 mg/kg PBN, subjected to 10-minute ischemia without PBN pretreatment and 30 minutes preischemia PBN, and then given 1-hour reperfusion. Control animals were present for each group. $N = 5–7$, $p < 0.01$.

attenuate the extent of damage. Hence, PBN was administered intraperitoneally into adult gerbils at 10, 20, and 40 mg/kg body weight. After a 24-hour exposure to hyperoxia, the animals were decapitated, and the isolated synaptosomes were used for all the studies.[17]

The results obtained were in accordance with our predictions of a hyperoxia-induced free radical damage and the moderating action of PBN in preventing this damage (FIGURES 3 and 4). W/S ratios of MAL-6 covalently attached to cortical synaptosomal membrane proteins from animals in hyperoxia without PBN were much lower than those injected with PBN (FIG. 3). There was no difference in W/S ratios of MAL-6 attached to membrane proteins of animals with PBN injections and those left outside the hyperoxia chamber. All

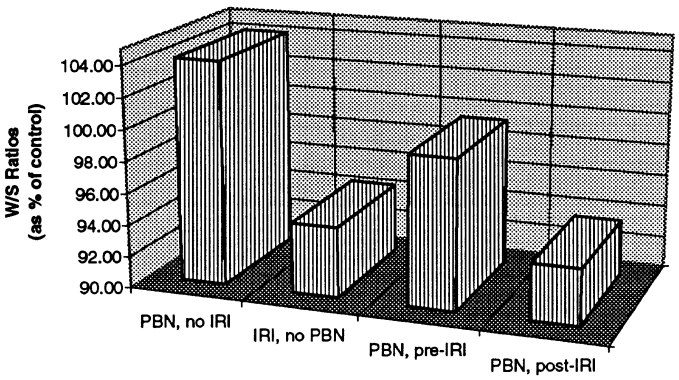

FIGURE 2. Effect of 300 mg/kg PBN given either 30-minute preischemia or 6-hour postreperfusion, on average W/S ratios of MAL-6 spin-labeled brain cortical synaptosomal membranes of adult gerbils. The animals were given 10-minute ischemia with 14 hours reperfusion, and the results are represented as a percent of the controls present for each group. $N = 5–7$, $p < 0.01$.

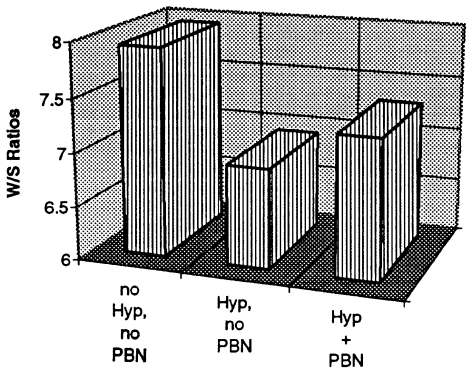

FIGURE 3. Effect of PBN (20 mg/kg) on average W/S ratios of MAL-6-labeled synaptosomal membranes of gerbils placed in 90–100% O_2 for 24 hours. The p value of the hyperoxic group versus the normoxic was found to be $p < 0.00001$, and versus the hyperoxic injected with PBN was $p < 0.0004$.

three doses of PBN provided effective prevention against protein oxidation, but 20 mg/kg body weight was found to be the optimum dose, based on the W/S ratios of MAL-6. Results seen on measuring the GS activity were similar to that obtained for the EPR study. Hyperoxia increased protein carbonyl levels and reduced GS activity and injection of animals with PBN-attenuated loss in GS activity (FIG. 4) but did not significantly lower the amounts of protein carbonyls formed. The nitroxide spin label Tempol [2,2,6,6-tetramethyl piperidine-1-oxyl-4-ol] also was effective in preventing protein oxidation.[17]

Senescence-accelerated prone mice (SAMP8) showed all signs of aging with respect to memory and behavior.[44,45] Because aging is thought to be associated with free radical oxi-

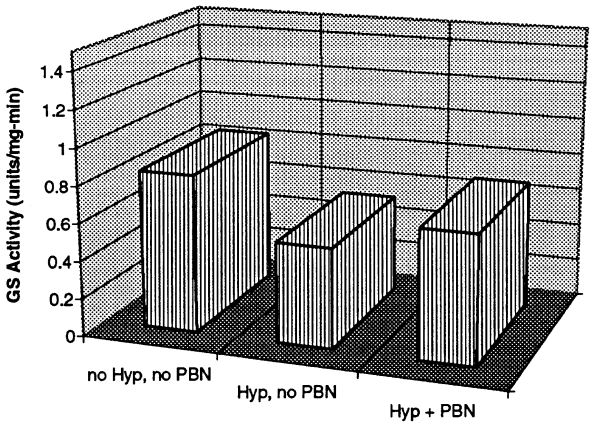

FIGURE 4. Effect of PBN treatment (20 mg/kg) on the GS activity of gerbils placed in 90–100% O_2 for 24 hours. The GS was isolated from the cytosol isolated from homogenized cortex of the gerbil brain. The p values for the hyperoxic group versus the control normoxic and the hyperoxic treated with PBN were less than 0.000005 and 0.0004, respectively.

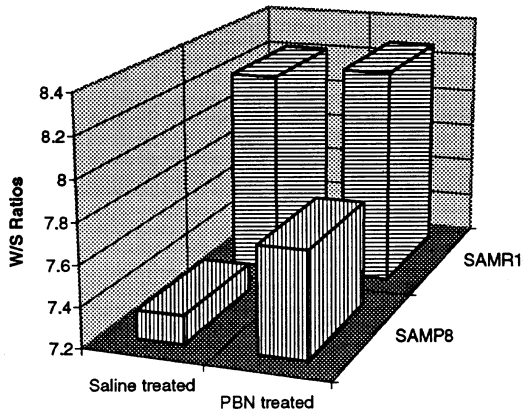

FIGURE 5. Graph represents average W/S ratios of MAL-6-labeled synaptosomes isolated from SAMP8 and SAMR1, injected i.p. with either saline or PBN (30 mg/kg), for 14 days. *p*-value for the SAMP8 saline versus PBN treated was found to be less than 0.001.

dative stress, we reasoned that PBN would modulate membrane protein oxidation in senescence-accelerated mice. The accelerated aging model was first developed by Takeda[46] as the senescence-accelerated mouse (SAM). The SAMP8 strain has a shorter life span and shows many typical signs of aging, whereas its genetic counterpart, the SAMR1 (senescence accelerated resistant), does not and lives longer.[47] PBN injected into the SAMP8 mice caused nearly a 50% increase in their life span.[48]

For our study, PBN was injected into the SAMP8 and SAMR1 mice daily, intraperitoneally, at 30 mg/kg body weight, for 14 days. Control SAMP8 and SAMR1 mice used in this study were given the same dose of saline injections for the same time period. Twenty-four hours after the final dose the animals were decapitated and examined for the various

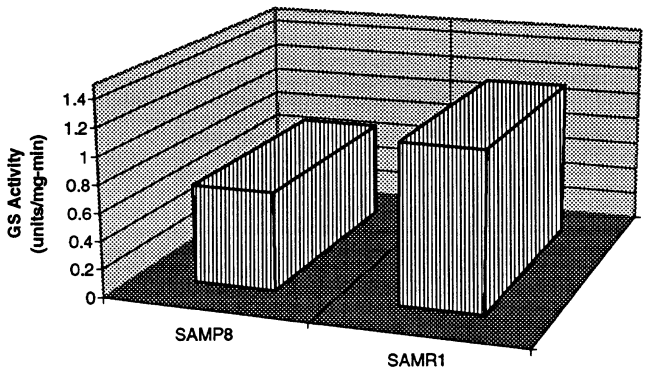

FIGURE 6. Graph represents GS activity of the cytosolic extract isolated from the cortices of the SAMP8 and and SAMR1 mice. p value < 0.05.

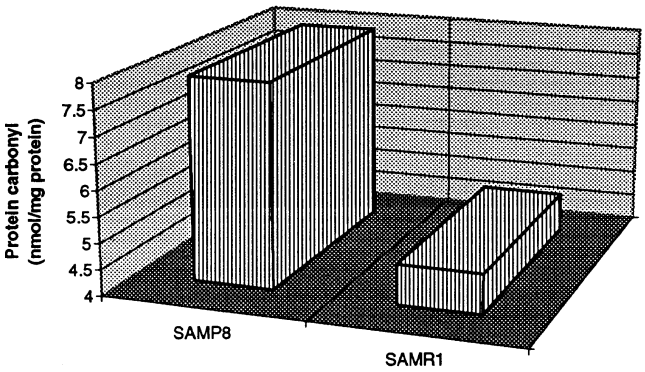

FIGURE 7. The graph shows the amounts of protein carbonyls present in the synaptosomes isolated from the cortices of the SAMP8 and SAMR1 mice.

studies, as described earlier. Consistent with oxidative damage to cortical synaptosomes with age, the W/S ratios of MAL-6 attached to membrane proteins from SAMP8 mice were significantly lower, protein carbonyls were higher, and GS activity was lower than those of the SAMR1 mice (FIGURES 5–7). By contrast, the SAMP8 mice injected with PBN showed higher W/S ratios, lower protein carbonyls, and higher GS activity when compared to those that were injected with only saline, and the values were also comparable to those obtained for SAMR1 (FIGURES 5–7), suggesting decreased oxidative stress. When these same strains of mice, treated with saline, were kept under hyperoxia for 24 hours, we observed a decrease in W/S ratios for both SAMP8 and SAMR1, indicating protein oxida-

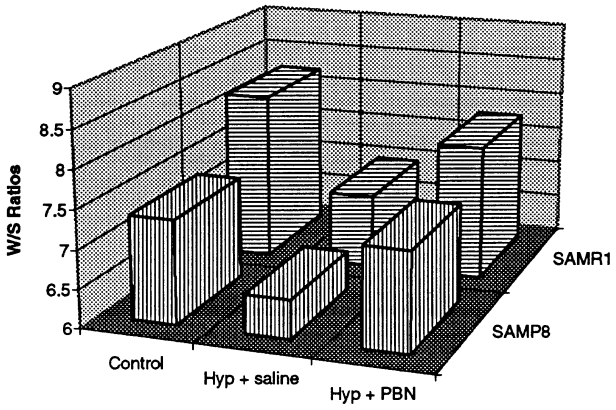

FIGURE 8. Effect of PBN treatment (30 mg/kg) on the W/S ratios of MAL-6-labeled synaptosomes isolated from SAMP8 and SAMR1 mice kept under 90–100% O_2 for 24 hours. Hyperoxic versus normoxic of SAMP8 and SAMR1 showed $p < 0.0001$ and $p < 0.001$, respectively. With respect to the hyperoxic but treated with PBN, the p values for SAMP8 and SAMR1 were less than 0.00001 and 0.002, respectively.

tion (FIG. 8). Again, treatment with PBN helped prevent lowering of the W/S ratios, to the extent that the mean values were comparable to those of the control mice (FIG. 8).

These protective results using PBN in the three *in vivo* models, IRI, hyperoxia, and accelerated senescence, suggest that damage during aging, consistent with the free radical theory of aging, is caused by free radical–mediated oxidative stress, and, hence, free radical scavengers may, if not prevent, then at least modulate or delay the onset of aging.

VITAMIN E AND Aβ (25–35)-INDUCED DAMAGE TO CORTICAL SYNAPTOSOMAL MEMBRANES

Aβ, a 39–43 amino acid–length peptide, cleaved from the transmembrane amyloid precursor protein (APP) and the major component of senile plaques (SP) in the brain of AD patients, is thought to be closely involved in the neurotoxicity of AD for a number of reasons. Genetic mutations on APP and presenilin genes, thought to be associated with APP processing, lead to AD; Down syndrome patients develop AD if they live long enough; and many of the pathological hallmarks of the AD brain are seen in APP overexpressing mice.[49] We and several other laboratories have demonstrated Aβ-associated free radical oxidative stress in neuronal systems.[7] To investigate structure and function of brain membrane systems exposed to Aβ, we have used cortical synaptosomes. Aβ (1–40) and Aβ (1–42) are the peptides found in the brain of AD patients. The region 25–35 of these peptides has been shown to be crucial for their toxicological properties.[50] Hence, for the purposes of understanding the molecular mechanics of the peptide action, we chose Aβ (25–35) for our studies.[51,52]

Aβ (25–35), obtained commercially from M. D. Enterprises, (Manhattan Beach, CA), was incubated with gerbil brain neocortical synaptosomal membranes at a final concentration of 1 mg/mL. A second aliquot was treated with Aβ (25–35) and vitamin E (final concentration 5 μM). Control samples were analyzed with each experiment. All the samples were incubated at 37°C for six hours, and all samples, except those for EPR studies, were frozen until analyzed. EPR spin labeling studies with MAL-6, protein carbonyls, cell sur-

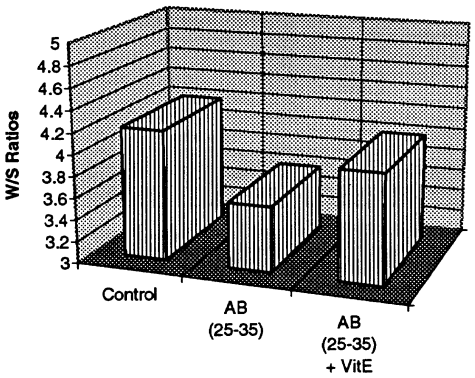

FIGURE 9. Effect of Aβ (25–35) with and without vitamin E on W/S ratio of MAL-6-labeled synaptosomes. Aβ (25–35) significantly reduces W/S ratio compared to control; $n = 3$, $p < 0.003$. Aβ (25–35) incubated with vitamin E restores the W/S ratio back to control values.

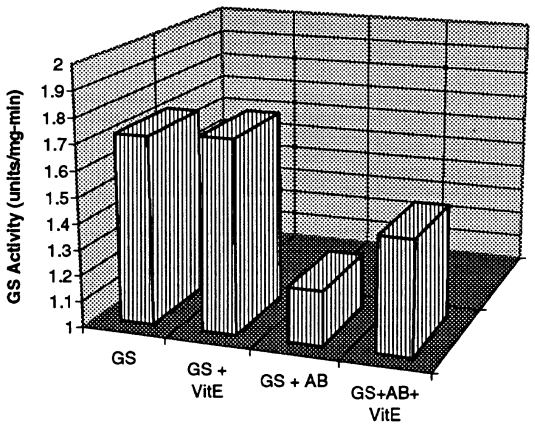

FIGURE 10. Effect of incubating GS with Aβ (25–35) in the presence and absence of vitamin E. Control values of GS activity, in the absence of any peptide, is chosen as 100%. Vitamin E alone does not alter GS activity. Aβ (25–35) significantly lowers GS activity ($n = 3$, $p < 0.005$). Vitamin E restores loss in activity caused by Aβ (25–35).

vival, and GS activity were measured according to methods mentioned earlier in this review.

The W/S ratios of MAL-6-labeled synaptosomal membranes incubated with Aβ (25–35) decreased from the control value, consistent with protein oxidation (FIG. 9). By contrast, samples having both Aβ (25–35) and vitamin E were protected against protein oxidation and showed W/S values similar to those of controls. GS activity dropped to 65% of the control on addition of Aβ (25–35) but was observed to be about 85% of control by addition of vitamin E (FIG. 10). Protein carbonyl levels, elevated by Aβ (25–35) treatment, were

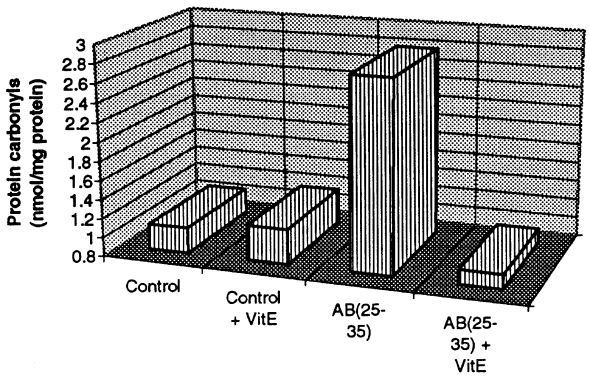

FIGURE 11. Protein carbonyl content of synaptosomes treated with Aβ (25–35) is significantly higher than untreated controls ($p < 0.01$; $n = 3$), suggesting that Aβ (25–35) caused oxidative modification of membrane proteins, resulting in a net increase in the carbonyl content. Samples treated with both the peptide and vitamin E were statistically identical to untreated controls.

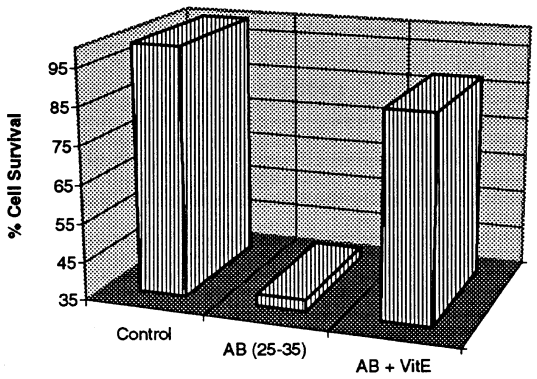

FIGURE 12. Loss in cell survival, as measured by the MTT reduction method, shows that Aβ (25–35) exposure resulted in the death of nearly 60% of the cells. Vitamin E was able to significantly protect from Aβ (25–35)-induced cell death and results in the survival of more than 90% of the cells.

comparable to control values in the presence of vitamin E (FIG. 11). In addition, toxicity to hippocampal neurons caused by Aβ (25–35) was partially prevented by vitamin E (FIG. 12).

Aβ causes lipid peroxidation,[29,53–56] and HNE, as one major lipid peroxidation product, is able to significantly modify the structure of brain membrane proteins.[35] Aβ forms HNE,[36] and both Aβ and HNE alter the function of ion-motive ATPases.[36,37] Free radical scavengers have been shown to prevent Aβ-induced formation of HNE in neuronal membranes.[36] Recently increased protein carbonyls, and decreased GS activity, in cell-free solution, following Aβ addition were demonstrated, and these changes were abrogated by PBN.[26] Taken together these vitamin E and PBN results are consistent with the notion that Aβ-associated free radicals and/or the oxidation products they produce are toxic to cells[7] and may be relevant to the known oxidative stress in AD brain.[57–59]

CONCLUSIONS

Oxidative stress in *in vivo* and *in vitro* developed animal models provide a good insight into the effects of various free radical insults that the human body has to withstand during the course of its life span. Since the free radical theory on aging was first formulated, there is a growing consensus that free radicals are largely accountable for the widespread damage seen in aging. Although the exact cause, sequence, and mechanisms underlying the damage have not yet been determined, progress has been made in developing and testing drugs and molecules that can attenuate this damage and increase the healthy life span of individuals. Tests conducted in animals have increased the awareness of people and led them to follow caloric restriction[60] and/or a diet rich in antioxidants, like vitamins and carotenoids. Studies with molecules like PBN have opened avenues for the use of nitrone-related molecules as potential therapeutic agents in the treatment of various oxidative stress disorders. The beneficial effects of such molecules have helped provide a better understanding of the action of free radical scavengers in mitigating cellular damage.

Aging is the single greatest risk factor for AD and is a significant factor in stroke. Our studies have provided insights into free radical damage and its modulation in *in vivo* and *in vitro* models of these age-related neurodegenerative disorders, but much more research needs to be performed, for example on the role of peroxynitrite-induced damage to brain membranes,[61] to completely understand the molecular basis of aging and age-related neurodegenerative disorders.

REFERENCES

1. HARMAN, D. 1992. Free radical theory of aging. Mutat. Res. **275:** 257–266.
2. RADI, R., J.S. BECKMAN, K.M. BUSH & B.A. FREEMAN. 1991. Peroxynitrite-induced membrane lipid peroxidation: The cytotoxic potential of superoxide and nitric oxide. Arch. Biochem. Biophys. **298:** 431–437.
3. SCHULTZ, J.B., R.T. MATTHEWS & M.F. BEAL. 1995. Role of nitric oxide in neurodegenerative diseases. Curr. Opin. Neurol. **8:** 480–486.
4. MIGUEL, J. & J.E. FLEMING. 1984. A two step hypothesis on the mechanism of *in vitro* cell aging: Cell differentiation followed by intrinsic mitochondrial mutagenesis. Exp. Gerontol. **19:** 31–36.
5. POULIN, J.E., C.COVER, M.R., GUSTAFSON & M.B. KAY. 1996. Vitamin E prevents oxidative modification of brain and lymphocyte band 3 during aging. Proc. Natl. Acad. Sci. USA **93:** 5600–5603.
6. SIES, H. 1985. *In* Oxidative stress. H. Sies, Ed.: 1–8. Academic Press. London.
7. BUTTERFIELD, D.A. 1997. β-Amyloid-associated free radical oxidative stress and neurotoxicity: Implications for Alzheimer's disease. Chem. Res. Toxicol. **10**(5): 495–506.
8. MECOCCI, P., U. MACGARNEY & M.F. BEAL. 1994. Oxidative damage to mitochondrial DNA is increased in Alzheimer's disease. Ann. Neurol. **36:** 747–757.
9. MATTSON, M.P., R.J. MARK, F. KATSUTOSHI & A.J. BRUCE. 1997. Disruption of brain cell ion homeostasis in Alzheimer's disease by oxyradicals, and signaling pathways that protect therefrom. Chem. Res. Toxicol. **10:** 507–517.
10. STADTMAN, E.R. 1992. Protein oxidation and aging. Science **257:** 1220–1224.
11. BUTTERFIELD, D.A. & E.R. STADTMAN. 1997. Protein oxidation processes in aging brain. Adv. Cell Aging Gerontol. **2:** 161–191.
12. COLE, P., N.C. HALL, J.M. CARNEY, O.J. PLANTE & D.A. BUTTERFIELD. 1996. Free radical oxidative stress and membrane alterations in ischemia-reperfusion injury. Recent Res. Dev. Neurochem. **1:** 99–109.
13. KITANI, K., A. AOBA & S. GOTO, EDS. 1996. Pharmacological Intervention in Aging and Age-associated Disorders. Ann. N.Y. Acad. Sci. **786:** 1–460.
14. JENNER, P. & C.W. OLANOW. 1996. Oxidative stress and the pathogenesis of Alzheimer's disease. Neurology **47:** 161–170.
15. STARKE-REED, P.E. & C.N. OLIVER. 1989. Protein oxidation and proteolysis during aging and oxidative stress. Arch. Biochem. Biophys. **275:** 559–567.
16. HENSLEY, K., B.J. HOWARD, J.M. CARNEY & D.A. BUTTERFIELD. 1995. Membrane protein alterations in rodent erythrocytes and synaptosomes due to aging and hyperoxia. Biochim. Biophys. Acta **1270:** 203–206.
17. HOWARD, B.J., S. YATIN, K. HENSLEY, K.L. ALLEN, J.P. KELLY, J. CARNEY & D.A. BUTTERFIELD. 1996. Prevention of hyperoxia-induced alterations in synaptosomal membrane-associated proteins by *N-tert*-butyl-α-phenylnitrone (PBN) and 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (Tempol). J. Neurochem. **67:** 2045–2050.
18. SOHAL, R.S., P.L. TOY & K.J. FARMER. 1987. Age-related changes in the redox status of the housefly, *Musca domestica*. Arch. Gerontol. Geriatr. **6:** 95–100.
19. SOHAL, R.S., A. MULLER, B. KOLETZKO & H. SIES. 1985. Effect of age and ambient temperature on n-pentane production in adult housefly, *Musca domestica*. Mech. Ageing Dev. **29:** 317–326.
20. OLIVER, C.N., B. AHN, E.J. MOERMAN, S. GOLDSTEIN & E.R. STADTMAN. 1987. Age-related changes in oxidized proteins. J. Biol. Chem. **262:** 5488–5491.

21. HENSLEY, K., N. HALL, W. SHAW, J.M. CARNEY & D.A. BUTTERFIELD. 1994. Electron paramagnetic resonance investigation of free radical induced alterations in neocortical synaptosomal membrane protein infrastructure. Free Radical Biol. Med. **17:** 321–331.
22. BELLARY, S.S., K.W. ANDERSON, W.A. ARDEN & D.A. BUTTERFIELD. 1995. Effect of lipopolysaccharide on the physical conformation of the erythrocyte cytoskeletal proteins. Life Sci. **56:** 91–98.
23. TRAD, C.H., W. JAMES, A. BHARDWAJ & D.A. BUTTERFIELD. 1995. Selective labeling of membrane protein sulfhydryl groups with methanethiosulfonate spin label. J. Biochem. Biophys. Methods **30:** 287–299.
24. BUTTERFIELD, D.A. 1982. Spin labeling in disease. *In* Biological Magnetic Resonance, Vol. 4. L.J., Berliner, Ed.: 1–78. Plenum Press. New York.
25. CARNEY, J.M., P.E. STARKE-REED, C.N. OLIVER, R.W. LANDRUM, M.S. CHENG & J.F. WU. 1991. Reversal of age-related increase in brain protein oxidation, decrease in enzyme activity, and loss in temporal and spatial memory by chronic administration of the spin trapping compound *N-tert*-butyl-α-phenylnitrone. Proc. Natl. Acad. Sci. USA **88:** 3633–3636.
26. AKSENOV, M.Y. M.V. AKSENOVA, J.M. CARNEY & D.A. BUTTERFIELD. 1997. Oxidative modification of glutamine synthetase by amyloid beta peptide. Free Radical Res. **27:** 267–281.
27. HARRIS, M.E., K. HENSLEY, D.A. BUTTERFIELD, R.E. LEEDLE & J.M. CARNEY. 1995. Direct evidence of oxidative injury by the Alzheimer's amyloid β-peptide in cultured hippocampal neurons. Exp. Neurol. **131:** 193–202.
28. SMITH, C.D., J.M. CARNEY, P.E. STARKE-REED, C.N. OLIVER, E.R. STADTMAN, R.A. FLOYD & W.R. MARKESBERRY. 1991. Excess brain protein oxidation and enzyme dysfunction in normal aging and in Alzheimer's disease. Proc. Natl. Acad. Sci. USA **88:** 10540–10543.
29. BUTTERFIELD, D.A., K. HENSLEY, M. HARRIS, M.P. MATTSON & J.M. CARNEY. 1994. β-Amyloid peptide free radical fragments initiate synaptosomal lipoperoxidation in a sequence-specific fashion: Implications to Alzheimer's disease. Biochem. Biophys. Res. Commun. **200:** 710–715.
30. ROWE, W.B., R.A. REMIZO, V.P. WELLNER & A. MEISTER. 1970. Glutamine synthetase. Methods Enzmol. **17:** 900.
31. MILLER, R.E., R. HADENBERG & H. GERSHAM. 1978. Regulation of glutamine synthetase in cultured 3T3-L1 cells by insulin, hydrocortisone, and dibutyryl cyclic AMP. Proc. Natl. Acad. Sci. USA **57:** 1418.
32. FLOYD, R.A. & J.M. CARNEY. 1991. Age influence on oxidative events during brain ischemia/reperfusion. Arch. Gerontol. Geriatr. **12:** 155–177.
33. CLOUGH-HELFMAN, C. & J.W. PHILLIS. 1991. The free radical trapping agent *N-tert*-butyl-α-phenylnitrone (PBN) attenuates cerebral ischemia injury in gerbils. Free Radical Res. Commun. **15:** 177–186.
34. SOUVIK, S., H. GOLDMAN, M. MOREHEAD, S. MURPHY & J.W. PHILLIS. 1994. α-Phenyl-*tert*-butylnitrone inhibits free radical release in brain concussion. Free Radical Biol. Med. **16**(6): 685–691.
35. SUBRAMANIAM, R., F. ROEDIGER, B. JORDAN, M.P. MATTSON, J.N. KELLER, G. WAEG & D.A. BUTTERFIELD. 1997. The lipid peroxidation product, 4-hydroxy-2-trans-nonenal, alters conformation of cortical synaptosomal membrane products. J. Neurochem. **69:** 1161–1169.
36. MARK, R.J., M.A. LOVELL, W.R. MARKESBERY, K. UCHIDA & M.P. MATTSON. 1997. Evidence that 4-hydroxynonenal mediates disruption of ion homeostasis and neuronal death by amyloid-β peptide. J. Neurochem. **68:** 255–264.
37. MARK, R.J., K. HENSLEY, D.A. BUTTERFIELD & M.P. MATTSON. 1995. Amyloid β-peptide impairs ion-motive ATPase activities: Evidence for a role in loss of neuronal Ca^{2+} homeostasis and cell death. J. Neurosci. **15:** 6239–6249.
38. ALVERADO, A., D.A. BUTTERFIELD, B.A. WATKINS, B.H. CHUNG & B. HENNIG. 1995. Lipid-induced alterations in membrane fluidity contribute to endothelial barrier dysfunction. Int. J. Biochem. Mol. Biol. **27:** 665–673.
39. BEHL. C., J.B. DAVIS, R. LESLEY & D. SCHUBERT. 1994. Hydrogen peroxide mediates amyloid β protein toxicity. Cell **77:** 817–827.
40. HALL, N.C., J.M. CARNEY, M.S. CHENG & D.A. BUTTERFIELD. 1995. Ischemia/reperfusion induced changes in membrane proteins and lipids of gerbil cortical synaptosomes. Neuroscience **64:** 81–89.
41. HALL, N.C., J.M. CARNEY, M.S. CHENG & D.A. BUTTERFIELD. 1995. Prevention of ischemia-reperfusion-induced alterations in synaptosomal membrane-associated proteins and lipids by *N-tert*-butyl-α-phenylnitrone and difluoromethylornithine. Neuroscience **69:** 591–600.

42. HALL, N.C., J.M. CARNEY, O.J. PLANTE, M. CHENG & D.A. BUTTERFIELD. 1997. Effect of 2-cyclohexene-1-one-induced glutathione depletion on ischemia/reperfusion-induced alterations in the physical state of brain synaptosomal membrane proteins and lipids. Neuroscience **77:** 283–290.
43. STADTMAN, E.R. & B.S. BERLETT. 1997. Reactive oxygen-mediated protein oxidation in aging and disease. Chem. Res. Toxicol. **10:** 485–494.
44. YAGI, H., T. KATOH, I. AGIGUTI & T. TAKEDA. 1988. Age-related deterioration of ability of acquisition in memory and learning in senescence-accelerated mouse: SAM-P/8 as an animal model of disturbances in recent memory. Brain Res. **474:** 86–93.
45. OHTA, A., T. HIRANO, H. YAGI & S. TANAKA. 1989. Behavioral characteristics of the SAM-P/8 strain in Sidman active avoidance task. Brain Res. **498:** 195–198.
46. TAKEDA, T., M. HOSOKAWA, S. TAKESHITA & M. IRINO. 1981. A new murine model of accelerated senescence. Mech. Ageing Dev. **17:** 183–194.
47. FLOOD, J. & J. MORLEY. 1992. Early onset of age-related impairment of aversive and appetite learning in SAM-P/8 mouse. J. Gerontol. **47:** 52–59.
48. EDAMATSU, R., A. MORI & L. PACKER. 1995. The spin trap tert-butyl-α-phenylnitrone prolongs the life span of the senescence mouse. Biochem. Biophys. Res. Commun. **211:** 847–849.
49. SELKOE, D.J. 1996. Amyloid β-protein and the genetics of Alzheimer's disease. J. Biol. Chem. **271:** 18295–18298.
50. PIKE, C.J., A.J. WALENCEWICZ-WASSERMAN, J. KOSMOSKI, D.H. CRIBBS, C.G. GLABE & C.W. COTMAN. 1995. Structure-activity analyses of β-amyloid peptides: Contributions of the β 25–35 region to aggregation and neurotoxicity. J. Neurochem. **64:** 253–265.
51. SUBRAMANIAM, R., T. KOPPAL, M. GREEN, S. YATIN, B. JORDAN & D.A. BUTTERFIELD. 1998. The free radical antioxidant vitamin E protects cortical synaptosomal membrane proteins from amyloid β-peptide (25-35) toxicity but not from hydroxynonenal toxicity: Relevance to the free radical hypothesis of Alzheimer's disease. Neurochem. Res. In press.
52. YATIN, S.M., M. AKSENOV & D.A. BUTTERFIELD. 1998. The antioxidant vitamin E modulates amyloid β-peptide–induced creatine kinase activity inhibition and increased protein oxidation: Implications for the free radical hypothesis of Alzheimer's disease. Neurochem. Res. In press.
53. KOPPAL, T., R. SUBRAMANIAM, J. DRAKE, M.R. PRASAD & D.A. BUTTERFIELD. 1998. Vitamin E protects against amyloid peptide (25-35)–induced changes in neocortical synaptosomal membrane lipid structure and composition. Brain Res. **786:** 270–273.
54. GRIDLEY, K.E., P.S. GREEN & J.W. SIMPKINS. 1997. Low concentrations of estradiol reduce beta-amyloid-induced toxicity, lipid peroxidation, and glucose utilization in human SK-N-SH neuroblastoma cells. Brain Res. **778:** 158–165.
55. DANIELS, W.M., S.J. VAN RENSBURY, J.M. VAN ZYL & J.J. TALJAARD. 1998. Melatonin prevents beta-amyloid-induced lipid peroxidation. J. Pineal Res. **24:** 78–82.
56. BRUCE-KELLER, A.J., J.G. BEGLEY, W. FU, D.A. BUTTERFIELD, D.E. BREDESEN, J.B. HUTCHINS, K. HENSLEY & M.P. MATTSON. 1998. Bcl-2 protects isolated plasma and mitochondrial membranes against lipid peroxidation induced by hydrogen peroxide and amyloid beta-peptide. J. Neurochem. **70:** 31–39.
57. SMITH, C.D., J.M. CARNEY, T. TATSUMO, E.R. STADTMAN, R.A. FLOYD & W.R. MARKESBERY. 1992. Protein oxidation in aging brain. Ann. N.Y. Acad. Sci. **663:** 110–119.
58. HENSLEY, K., N. HALL, R. SUBRAMANIAM, P. COLE, M. HARRIS, M. AKSENOV, M. AKSENOVA, P. GABBITA, J.F. WU, M.J. CARNEY, M. LOVELL, W.R. MARKESBERY & D.A. BUTTERFIELD. 1995. Brain regioinal correspondence between Alzheimer's diseaze histopathology and markers of protein oxidation. J. Neurochem. **65:** 2146–2156.
59. MARKESBERY, W.R. 1997. Oxidative stress hypothesis in Alzheimer's disease. Free Radical Biol. Med. **23**(1): 134–147.
60. GABBITA, S.P., K. HENSLEY, D.A. BUTTERFIELD & J.M. CARNEY. 1997. The effect of age and diet on mitochondrial respiration and lipid membrane status: An electron paramagnetic resonance investigation. Free Radical Biol. Med. **23:** 191–201.
61. KOPPAL, T., J. DRAKE, S. YATIN, B. JORDAN, S. VARADARAJAN, L. BETTENHAUSEN & D.A. BUTTERFIELD. 1998. Peroxynitrite-induced alterations in synaptosomal membrane proteins: Insight into oxidative stress in Alzheimer's disease. J. Neurochem. In press.