Aging- and Oxygen-induced Modifications in Brain Biochemistry and Behavior[a]

JOHN M. CARNEY,[b] CHARLES D. SMITH,[c]
ANN M. CARNEY,[b,e] AND D. ALAN BUTTERFIELD[d]

[b]*Department of Pharmacology*
The University of Kentucky
Lexington, Kentucky 40506

[c]*Department of Neurology*
The University of Kentucky
Lexington, Kentucky 40506

[d]*Department of Chemistry*
The University of Kentucky
Lexington, Kentucky 40506

INTRODUCTION

Reactive oxygen species (ROS) have been causally associated with a number of age-associated neurodegenerative diseases[1-3] and the process of brain aging itself.[4,5] A number of different reaction systems have been proposed to be involved in protein and lipid oxidation[6,7] (TABLE 1). In many cases, protein oxidation has been shown to be metal (Fe or Cu) catalyzed and site-directed. The metal-catalyzed oxidation is often the result of delocalized (loosely bound) iron and not the result of a net increase in tissue-free iron. Oxidation of particular proteins can be highly specific, in some instances restricted to single amino acids residing near metal-bearing catalytic sites (TABLE 2). Selective vulnerability to oxidation is also shown by polyunsaturated fatty acids found in the membrane of brain neurons. During periods of poor blood perfusion (e.g., stroke or head injury), or as a consequence of defects in the mitochondrial electron transport apparatus, there is a reduction in redox potential that results in a buildup in intracellular superoxide (O_2) and hydrogen peroxide (H_2O_2).[8] The brain contains many of the enzymatic and nonenzymatic systems (TABLE 1) required for the generation of oxygen free radical. In addition the brain contains relatively low antioxidant levels compared to the basal levels of ROS produced and the amount of available target material for ROS-mediated reactions.[9]

The generation of ROS is a normal byproduct of oxidative phosphorylation and metabolism.[10,11] Previous studies have demonstrated that there are normal background levels of H_2O_2 (10^{-6} M), $O_2^{\cdot-}$ (10^{-9} M) and hydroxyl free radical ($\cdot OH$ 10^{-9} M) in normal cells.[12] One of the consequences of ongoing ROS production is that there is a normal background level of protein oxidation in subjects of all ages. In

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[e]Current address St. Paul's School, Concord, New Hampshire.

TABLE 1. ROS Generating Systems that May Catalyze Oxidation of Protein

$Fe^{+2} + O_2$
$Fe^{+2} + H_2O_2$
Fe^{+3} + Ascorbate O_2
$NO + O_2^-$
Xanthine Oxidase/hypoxanthine/Fe^{+3}/O_2
NAD(P) H oxidase/WAD(P)H/Fe^{+3}/O_2
Cytochrome P_{450} reductor/cytochrome P_{450}/NADPH/ Fe^{+3}/O_2
SOD/O_2^-

Modified from Stadtman.[26]

certain pathologic conditions (e.g., Alzheimer's disease), and in normal aging, this level of cellular protein oxidation is increased.[13] Furthermore, changes in the level of ROS production may occur in conditions of either insufficient (hypoxia, ischemia) or excess (hyperoxia, hyperbaric oxygen) oxygen.

These findings are highly significant under the supposition that increased ROS activity, or the inability to restrain this activity, can result in pathology. The accumulation of protein oxidation products then becomes an index to the underlying ROS-mediated processes. An intriguing consequence of this line of reasoning is that intervention at the level of ROS production could ameliorate pathology. The present paper presents evidence that the production of oxygen radicals is involved in the early biochemical changes that ultimately result in significant behavioral deficits, and that interventions showing measurable decreases in indices of ROS-mediated oxidations also result in improvement in associated performance deficits.

MATERIALS AND METHODS

Glutamine Synthesis and Creatine Kinase Assays

Gerbil neocortical homogenate was prepared as previously described[14] from 3–4 month-old and 18–20 month-old male mongolian gerbils (Tumblebrook Farms, W. Brookfield, Mass.). The protein concentration of the supernatant (cytosolic) fraction of homogenate was determined by the Pierce BCA method.[15] Protein carbonyl levels were determined using the dinitrophenyl hydrazine procedure as previously described.[14] Glutamine synthesis (GS) activity was determined by the method of Rowe *et al.*[16] as modified by Miller *et al.*,[17] and corrected for nonspecific glutaminase

TABLE 2. Aminoacid Modification that May Occur During Protein Oxidation

Argine	→	glutamylsemialdehyde
Prolyline	→	glutamylsemialdehyde
Lysine	→	α aminoadipylsemialdehyde
Histidine	→	arparagine α aspartate
Cysteines	→	5-protein cross-links mixed disulfides
Tyrosine	→	dityrosyl cross-links

Modified from Stadtman.[26]

activity by comparison of activity in the presence and absence of adenosinediphosphate (ADP) and arsenate, creatine kinase (CK) activity was determined by the colormetric method (Sigma kit NO. 661).

Salicylate Hydroxylation

Generation of ROS was determined by hydroxylation of salicylate to form dihydroxybenzoic acid (DHBA). 2,3- and 2,5-DHBA were measured using high-pressure liquid chromatography (HPLC) with electrochemical detection as previously described.[18] Salicylate concentration was simultaneously measured with fluorescence detection (300-nm excitation, 412-nm emission), and DHBA was expressed as a ratio to salicylate recovered.

Behavioral Testing

Gerbils were tested for temporal and spatial memory using an eight-arm radial maze.[19] Experimentally naive gerbils were tested for their efficiency in patrolling behavior. Gerbils were placed into the central (start) chamber and then allowed to explore the maze. Errors were defined as reentry of previously entered arms of the maze. Both the time to explore all eight arms of the maze and the number of errors prior to exploring all arms of the maze were recorded for each subject. The observer was blind to the treatment groups.

Human Tissue Samples

Confirmed Alzheimer's and neurologically normal regional brain samples were obtained at autopsy within 12 hours of death. Brain regions were rapidly dissected and immediately frozen in liquid nitrogen until assay (FIG. 1). On the day of assay the samples were homogenized in protease inhibitor buffer as described earlier for gerbil brain tissue and the cytosolic fraction prepared. Previous studies[13] have demonstrated that postmortem intervals up to 12 hrs do not result in any time-dependent change in enzyme activity.

Statistics

Data are presented as the mean ± SE from protein oxidation and enzyme activity. The significance level of treatment effects and the reversal of phenyl-*tert*-butyl nitrone (PBN) effects were determined using analysis of variance and *post hoc* analysis. A $p < 0.05$ was considered significant.

RESULTS AND DISCUSSION

Brain Aging and Age-Related Disease

A great simplification in the theory of brain aging would occur if one or a few underlying cellular processes could be identified that would account for a substantial

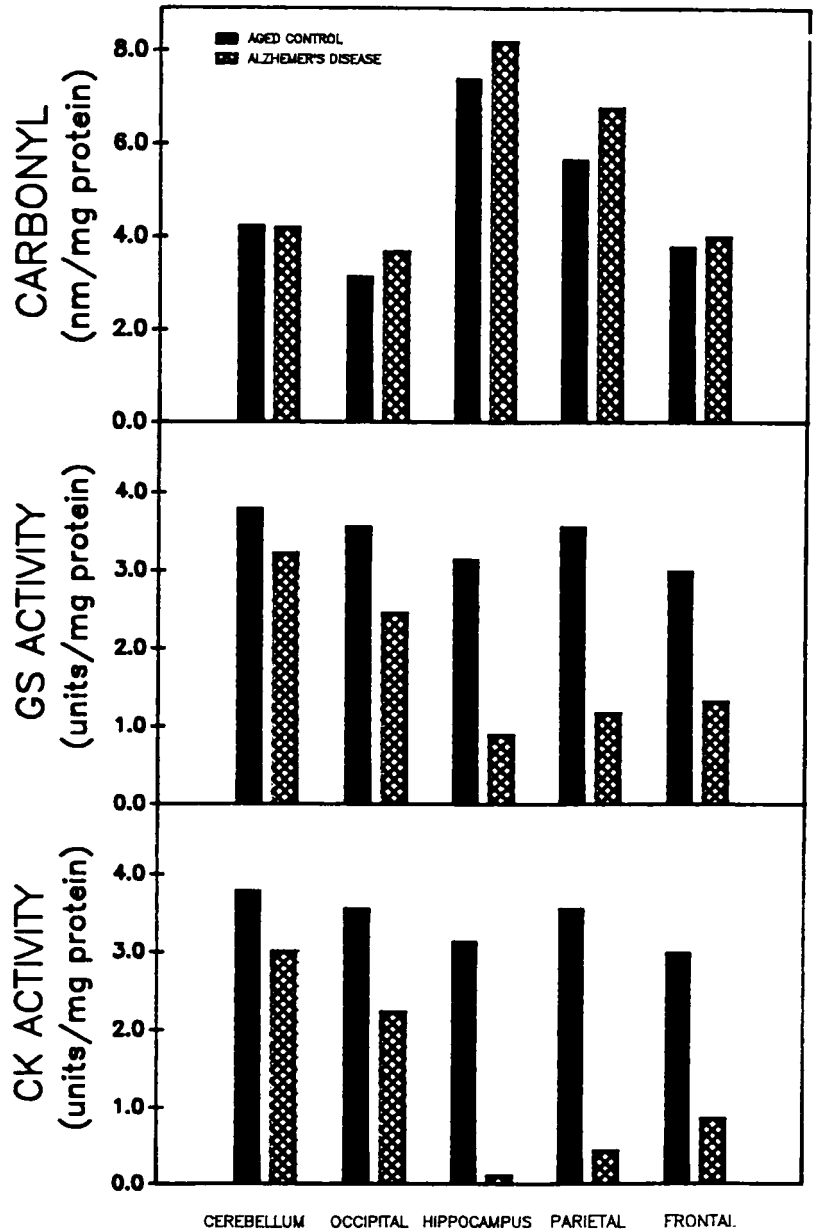

FIGURE 1. Regional differences in brain cytosolic protein oxidation, glutamine synthase (GS), and creatine kinase (CK) activities from selected brain regions of neurologically normal and Alzheimer's disease (AD) subjects. Brains were obtained at autopsy within 8 hr of the time of death and rapidly frozen in liquid nitrogen. *Solid bars* represent the average of two controls and the *cross-hatched bars* represent the average of four AD brains.

number of commonly observed aging phenomena. An increasingly secure candidate for such a process is the oxidation of normal cellular constituents mediated by ROS, for example, superoxide, hydrogen peroxide, hydroxyl free radical, and singlet oxygen.

Direct detection of these species *in vivo* has been difficult due to their intrinsically low tissue concentrations and the highly localized nature of their reactions. Indirect detection of specific reaction products, however, has proved fruitful. There is now substantial evidence that products of oxidation, such as protein carbonyl adducts, accumulate in the brain with age, and that the rate of oxidation increases similarly. Correlated with the increased level of oxidized protein there is a progressive decrease in enzyme activity. Among the various enzymes studied, several appear to be uniquely sensitive to oxidation (see TABLE 2).

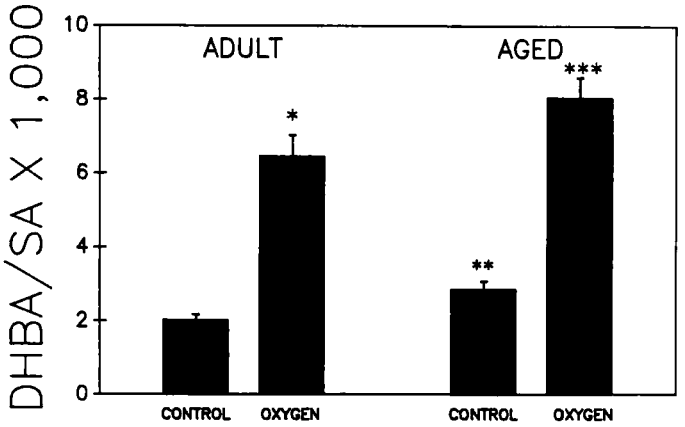

FIGURE 2. Effects of exposure to 100% oxygen for 6 hr on the production of oxy-radical species. Gerbils (3–4 months and 18–20 months old) were placed in a plexiglass exposure chamber that had been purged of air by pure oxygen. Environmental oxygen levels were monitored continuously and the oxygen content was maintained between 98% and 100% for the 6 hr of exposure. Sodium salicylate (100 mg/kg) was administered 1 hr before the end of the 6-hr period or 1 hr before obtaining the cortical brain samples in the control. Brain tissue was obtained immediately after the end of the 6-hr exposure. Data are expressed as the ratio of DHBA/salicylate × 1000 (see text for details of methods). Each *bar* is the mean (±S.E.) for 6 subjects per group (*$p < 0.01$, oxygen exposed adult vs. adult control; **$p < 0.05$, aged control vs. adult control; ***$p < 0.05$, aged oxygen exposed vs. adult oxygen exposed).

Animal studies have previously demonstrated an increase in brain protein oxidation and decreased enzyme activity.[19] We have extended the studies into two directions. The first is to determine the impact of hyperoxia on brain protein oxidation and enzyme activity. In these studies gerbils were exposed to 100% oxygen environment for 6 hr and then evaluated for oxidation products. Exposure to oxygen results in a significant increase in ROS as evidenced by significant increases in the DHBA/SA in the neocortex (FIG. 2). In addition to the overall effect of oxygen, aged gerbils were significantly more sensitive to the effect of oxygen compared to controls. The age-related increase in vulnerability of brain protein to oxidation reported here confirms previous reports on the enhanced effect of oxygen in aged subjects.[20] While the ultimate generator site is not identifiable by these studies, the facts that

mitochondrial genomic defects have been demonstrated to accumulate with age and that mitochondrial respiratory defects have been associated with cell damage[21] strongly implicate mitochondrial production of ROS following hyperoxia.

We feel the question now is not whether increased oxidation is connected with aging, but whether observed oxidative structural alterations are of the degree and kind to account for functional aging deficits. An important corollary question is whether certain age-related illnesses, for example, Alzheimer's disease (AD) and Parkinson's disease (PD), represent specific defects in the ability of brain cells to restrain ROS-mediated reactions, resulting in either diffuse (AD) or localized (PD) neuronal injury. The recent discovery that a genetic defect in the oxidation-protective enzyme superoxide dismutase is associated with familial amyotrophic lateral sclerosis (ALS) lends credence to this "oxidative" hypothesis. Thus in ALS the underlying global process of free-radical oxidation has been linked to a significant progressive neurodegenerative disease. Further, ALS demonstrates the principle that a specific and perhaps localized inability to ameliorate the effects of this process leads to a specific and localized injury to a subset of brain neurons, for example, the voluntary motor system, resulting in a specific functional deficit.

In the case of Alzheimer's disease, we have previously reported that there is a progressive increase in brain protein oxidation that occurs at an accelerated rate in the frontal cortex, compared to the occipital cortex.[12] This buildup of oxidized protein as a function of aging has been reported to occur in cultured human fibroblasts, red blood cell fractions, and in the brain in a number of animal studies.[13,22] We have extended our initial studies to investigate the regional distribution of protein oxidation and loss of enzyme activity in age-matched control and Alzheimer brains. Glutamine synthetase (GS) and creatine kinase (CK) were selected because these enzymes have been shown to be more affected in Alzheimer's disease than in aged-control subjects.[12] Protein carbonyl demonstrated marked regional differences across the five brain areas sampled in each individual. The highest levels of oxidized protein were found in the hippocampus and parietal cortex, while occipital cortex had the lowest levels. Activity of both GS and CK was inversely correlated with carbonyl content. In particular, hippocampal cortex had levels of CK activity that in some cases were not detectable under our assay conditions. In contrast to the almost undetectable levels of CK activity in areas that show high levels of oxidized protein, Western blot analysis demonstrated that there was a substantial level of immunoreactive CK in hippocampal and parietal cortex, although slightly lower than in aged controls. This was also the case for GS. These findings support the hypothesis that site-specific oxidation can inactivate an enzyme without targeting that enzyme for proteolysis. These findings support other work showing that aging is associated with decreased proteolytic function in brain and other tissues.[20]

A question then arises as to the *source* of ROS that produce protein oxidation. There is wide agreement that mitochondria are a major source of oxygen free radicals.[8] If the normal level of cellular respiration uses 10^{10} oxygen molecules per second, then we would expect 2×10^8 oxygen radicals to be generated every second by the mitochondria. The generation of oxygen free radicals by mitochondria has been directly demonstrated by Nohl *et al.*[23] using the NRT compound DMPO (5,5[1]-dimethyl-1-pyroline-*N*-oxide).

We speculate from the studies just cited that the principle of local vulnerability to the generalized process of ROS-mediated oxidation may have special relevance to the brain. The brain has a low level of antioxidant activity (or potential) relative to overall oxygen consumption. It is estimated that 2% of cerebral oxygen utilization

generates partially reduced oxygen species, some of which are highly reactive. In addition, there may be a special role for the brain astrocyte in ameliorating these ROS-related reactions. GS, a heme-centered enzyme found exclusively in astrocytes, is essential for the production of the ROS-reductant glutathione in the brain. Recent evidence suggests that GS is itself particularly vulnerable to oxidative modification. Since GS is responsible for glutamate turnover as well as glutathione production, inactivation of GS may both reduce antioxidant potential generally and potentiate injury locally in brain areas vulnerable to glutamate toxicity, for example, the CA1 region of the hippocampus. Local variations in the distribution of available ROS-potentiating catalyst transition metals (e.g., iron and copper) could further complicate the map of local oxidative vulnerability as the brain ages.

The question of ROS-mediated effects in nervous system aging is particularly intriguing because there already exists evidence that drug interventions designed to

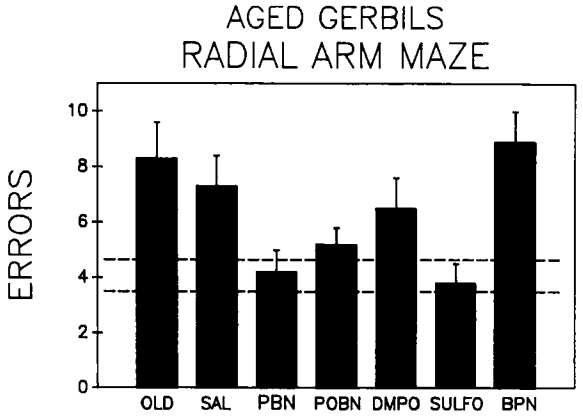

FIGURE 3. Reduction in radial arm maze errors following 14 days of twice daily injection of 10 mg/kg NRT, compared to control and saline-treated groups. Each histogram represents the mean (±S.E.) of 12 aged gerbils. *Dashed lines* indicate one S.E. for the adult (3–4 month-old) gerbils under the same testing conditions. The abbreviations for NRT's are as follows: PBN, *N-tert*-butyl-alpha-phenylnitrone; POBN, DMPO, SULFO, 2'-sulfonyl-*N-tert*-butyl-alpha-phenylnitrone; BPN, butyl-alpha-phenylnitrone.

reduce free radical oxidation ameliorate both structural and functional deficits in experimental animal models of aging. We evaluated the ability of a series of free-radical trapping compounds to reduce the age-related increase radial-arm maze errors. We have demonstrated that a PBN could significantly decrease the level of age-associated errors when it was administered for 14 days.[19] A comparison of a series of related compounds demonstrated a similar effect in many, but not all, compounds that had similar trapping abilities (FIG. 3). PBN, POBN, and *a*-sulforyl PBN were all effective in decreasing the error rates to those seen in young adult gerbils. DMPO was intermediate in effect and PBN had no beneficial effect. Thus, it appears that the capacity to form stable adducts with radicals is not sufficient in itself to result in improvements in temporal/spatial memory, as determined by radial maze performances.

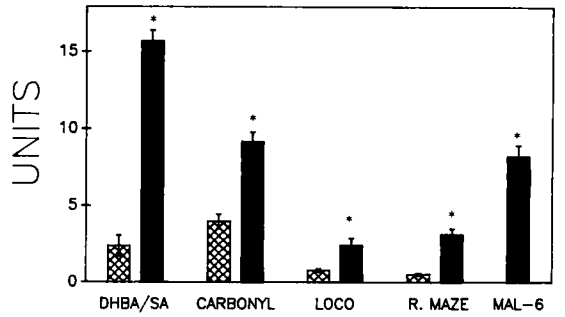

FIGURE 4. Postischemic changes in free-radical production (DHBS/SA), protein oxidation (CARBONYL), damage to cytoskeletal matrix (MAL-6), and behavior. *Cross-hatched bars* represent the mean (±S.E.) for groups of 6 adult gerbils. For the purpose of cross comparisons, the data are scaled in arbitrary units across the different measures. (* = $p < 0.01$).

Ischemia/Reperfusion Injury, Protein Oxidation, and Behavioral Dysfunction

The study of ischemia/reperfusion can serve to illuminate many of the issues discussed previously. The use of multiple endpoints in the cascade of reperfusion injury has made it possible to characterize the role of ROS in enzyme damage, pathology, and behavioral dysfunction. Free radical production following ischemia/reperfusion injury has been demonstrated both directly and indirectly in a number of *in vivo* and *in vitro* systems.[24] In a series of studies we used salicylate hydroxylation (DHBA/SA), protein oxidation (carbonyl), and loss of GS activity as biochemical indicators of ROS-mediated activity. The time course of the induced changes demonstrated the etiological importance of early oxygen radical production in the process of IRI.

In a subsequent series of studies we have expanded our research to include an evaluation of the cytoskeletal proteins known to become oxidatively modified during IRI (FIG. 4). As has been previously reported, IRI results in significant increases in

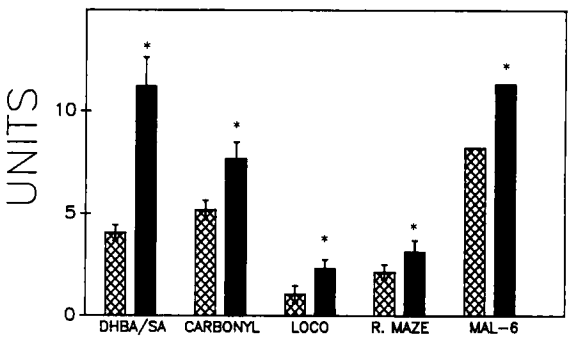

FIGURE 5. Postischemic changes in biochemical and behavioral indices of damage in adult and aged gerbils. Data are expressed as described in FIGURE 4. *Cross hatched bars* represent the changes in measures following ischemia in adult gerbils. *Solid bars* represent the change in aged gerbils. (* = $p < 0.05$ aged vs. adult.)

both neocortical DHBA/SA and soluble protein fraction carbonyl content. Paralleling the increased level of oxidized soluble protein, there is a significant loss of MAL-6 binding sites on cytoskeletal protein. Similar changes in MAL-6 binding have been observed in aged red cells and are interpreted to indicate a loss of or damage to the weak binding sites on the surface of cytoskeletal proteins. These changes in ROS production and protein oxidation were paralleled by changes in the level of locomotor activity and radial maze errors.

We have previously reported that aged gerbils are differentially sensitive to IRI with respect to locomotor activity, early gene expression, and metabolic recovery from ischemia.[25] FIGURE 5 demonstrates the consistent, age-related enhanced vulnerability to ROS following IRI. Aged gerbils demonstrated significantly greater cortical DHBA/SA and protein carbony 1 hr after reperfusion. Consistent with this, there was a significantly greater loss of MAL-6 binding sites in cytoskeletal samples from aged, compared to adult, gerbils 60 min after reperfusion. Maze performances and locomotor activity both demonstrated enhanced IRI vulnerability of aged gerbils.

SUMMARY

In summary, the work presented here has shown accumulation of oxidized protein with age in an animal aging model. In gerbil brain, this accumulation is associated with (1) decreased activity of oxidatively sensitive enzymes creatine kinase and glutamine synthetase; (2) decreased function of particular cytoskeletal proteins; and (3) decreased performance in a radial-arm maze task. Manipulations shown to increase the presence of reactive oxygen species in the brain increase oxidized protein, decrease the index enzyme activities and cytoskeletal protein defects, and worsen performance deficits. Moreover, intervention designed to quench ROS-mediated reactions decrease oxidized protein levels, and nearly normalize index enzyme activities and associated behavioral deficits. The precise connections between the performance deficits and protein measures are probably highly complex and likely to remain obscure for now. Currently, the behavioral measures serve as a marker for the functional consequences of the protein alterations.

Our studies in humans have shown oxidized protein accumulation with age and a differential decrease of glutamine synthetase activity in the frontal lobe in AD. Further preliminary results in AD autopsy material show a striking correlation between the distribution of index enzyme inactivation and the known intensity distribution of AD pathology. These findings support the hypothesis that inability to restrain age- or pathology-related increases in local ROS activity can result in AD.

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