Ozone, Sulfur Dioxide, and Ultraviolet B Have Similar Effects on mRNA Accumulation of Antioxidant Genes in *Nicotiana plumbaginifolia* L.1


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We have studied the expression of antioxidant genes in response to near ambient conditions of O3, SO2, and ultraviolet B (UV-B) in *Nicotiana plumbaginifolia* L. The genes analyzed encode four different superoxide dismutases (SODs), three catalases (Cat1, Cat2, and Cat3), the cytosolic ascorbate peroxidase (cyt APx), and glutathione peroxidase (GPx). The experimental setup for each treatment was essentially the same and caused no visible damage, thus allowing direct comparison of the different stress responses. Our data showed that the effects of O3, SO2, and UV-B on the antioxidant genes are very similar, although the response to SO2 is generally less pronounced and delayed. The effects of the different stresses are characterized by a decline in Cat1, a moderate increase in Cat3, and a strong increase in Cat2 and APx. Remarkably, SOD and cyt APx expression in the ozone-sensitive *Nicotiana tabacum* L. cv PB6 revealed that induction of the cytosolic copper/zinc SOD and cyt APx occurs only with the onset of visible damage. It is proposed that alterations in mRNA levels of catalases and GPx, but not of SODs and cyt APx, form part of the initial antioxidant response to O3, SO2, and UV-B in *Nicotiana*.

As a result of anthropogenic activities, concentrations of O3 and SO2 in the lower atmosphere have increased considerably in industrialized parts of the world. Upward trends of surface UV-B radiation have been recognized and attributed to a depletion in stratospheric ozone (Lefohn, 1992). During peak episodes, atmospheric concentrations of these factors may reach levels that are toxic to plants and to which plants respond by triggering various defense mechanisms. One such response that has been invoked in several abiotic stress conditions is the antioxidant defense system. This system consists of low-mol-wt antioxidants such as ascorbate, GSH, \( \alpha \)-tocopherol, and carotenoids (reviewed by Alscher and Hess, 1993), as well as of protective enzymes such as SODs, Cats, and peroxidases. SODs convert superoxide radicals into H2O2 and O2 and can be divided into three classes according to their metal co-factor: Cu/Zn, Mn, and Fe (reviewed by Bowler et al., 1994). Cats dismutate H2O2 into water and O2, whereas peroxidases reduce H2O2 to water. Different forms of peroxidases can be distinguished based on the co-substrates that are oxidized, e.g. antioxidants or phenolic compounds (reviewed by Campa, 1991). H2O2, in the presence of O2, and trace amounts of transition metals, can give rise to the highly toxic hydroxyl radical (Halliwell, 1987). Therefore, rapid detoxification of both O2 and H2O2 is primordial to prevent oxidative damage. In plants, APx’s are functionally associated with SOD in the cytosol and in the chloroplast, whereas Cats are mainly involved in the scavenging of peroxisomal H2O2 (Gillham and Dodge, 1986).

The importance of this defense system during environmental adversity is evidenced by recent studies showing that in transgenic plants overproduction of SOD in the chloroplasts leads to a better recovery after chilling (Sen Gupta et al., 1993) and freezing stress (McKersie et al., 1993) and reduces leaf necrosis in the case of O3 stress (Van Camp et al., 1994b). Furthermore, plant varieties with enhanced tolerance to photoinhibition, chilling, drought, O3 or SO2 were shown to contain increased levels of enzymic and nonenzymic antioxidants. Some of these plants displayed cross-tolerance to several stresses (for a review, see Van Camp et al., 1994a), indicating that a similar antioxidant response operates against different stress factors. However, to date this has not been confirmed by expression studies. Even using the same stress condition, contradictory results have been obtained concerning the expression of antioxidant enzymes (for a review, see Bowler et al., 1992). Many of these inconsistencies can be ascribed to the use of different plant species and experimental conditions for stress treatment.

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Abbreviations: APx, ascorbate peroxidase; Cat, catalase; chl, chloroplastic; cyt, cytosolic; GPx, glutathione peroxidase; SOD, superoxide dismutase.
In this study, we have compared the effect of O₃, SO₂, and UV-B on the antioxidant defense system in Nicotiana plum-baginifolia L. During the exposure period, concentrations of these stress factors were applied in daily cycles, according to outside conditions. Levels of UV-B radiation and O₃ used for stress treatment correspond to ambient levels in Western Europe and Northern America, whereas for SO₂ levels similar to outside concentrations in highly polluted areas were applied. In the case of O₃ stress, Nicotiana tabacum L. cv PBD6 was included in our analysis to compare the antioxidant response of an O₃-sensitive (N. tabacum cv PBD6) and an O₃-tolerant tobacco species (N. plumbaginifolia). Gene expression in response to environmental stress was studied at the level of steady-state mRNA abundance because this gives a more precise estimate of antioxidant gene activation than enzyme activity. We have monitored the mRNA levels of the following nuclear-encoded enzymes: mitochondrial MnSOD, chl FeSOD, chl and cyt Cu/ZnSOD, cyt APx, GPx, and three CAts. With the exception of chl APx, for which no molecular probe is available, this study includes all plant enzymes that are known to scavenge reactive oxygen intermediates.

**MATERIALS AND METHODS**

**Precultivation Conditions**

* Nicotiana plumbaginifolia L. and Nicotiana tabacum L. cv PBD6 were cultivated in pollutant-free air in controlled environment cabinets (Heraeus-Vötisch, Balingen, Germany) with a 14-h light (150 μmol m⁻² s⁻¹, 6 AM to 8 PM, 25°C/10-h dark cycle (20°C) cycle and RH between 60 and 70%.

Plants were fertilized twice per week with a 10:4:7 N:P:K mixture (Substral, Bamangen, Germany), and pots were watered daily to saturation with deionized water. Two-month-old plants were transferred into walk-in chambers of the phytotron EPOKA (Payer et al., 1994) at the Forschungszentrum für Umwelt und Gesundheit (Munich, Germany) and were acclimated for 7 days in pollutant-free air to higher light intensities without UV-B radiation. To overcome leaf boundary layer resistance, an air current of 0.6 to 1.3 m s⁻¹ was administrated. The light system of the phytotron was designed to simulate the solar spectrum and consisted of metal halide, quartz halogen, and blue fluorescent lamps (238 in total) mounted at the ceiling of a separate lamp cabinet on top of the experimental chamber (for details, see Payer et al., 1994). UV-B and UV-C radiations were excluded during precultivation by a layer of normal float glass between the lamp cabinet and the experimental chamber. Plants received PAR (400-700 nm) was determined by a quantum sensor (Li-Cor, Lincoln, NE) and was about 1120 μmol m⁻² s⁻¹ at the canopy level. PAR increased between 6 and 10 AM and decreased between 4 and 8 PM.

**Exposure to O₃, SO₂, and UV-B**

Plants were exposed to O₃ or SO₂ for 14 h/d (between 10 AM and midnight), with linear increases between 10 AM and noon and decreases between 10 PM and midnight. When not fumigated, plants were exposed to filtered air (Langebartels et al., 1991). O₃ was generated by electrical discharge in pure O₂. Daily mean O₃ levels were 59 ± 2 nL L⁻¹, whereas maximum concentrations were between 94 and 128 nL L⁻¹. The mean SO₂ concentration was 63 ± 2 nL L⁻¹ with daily maxima between 95 and 133 nL L⁻¹. Both pollutants were administered through mass flow controllers into the air stream entering the growth chamber. The chamber atmosphere was sampled using Teflon lines at eight locations per chamber at the canopy level. Concentrations of O₃, SO₂, NO, NO₂, CO₂, and hydrocarbons were recorded 24 h/d; O₃ concentration was measured with a UV-type O₃ analyzer, and SO₂ concentration was measured with a sulfur analyzer (CSI 3100 and CSI SA 285; Columbia Scientific Instruments, Austin, TX).

Supplemental UV-B radiation was provided between 10 AM and 8 PM by UV-B fluorescent lamps (TL 12; Philips, Eindhoven, The Netherlands) mounted at the walls of the lamp cabinet. Two different radiative levels were applied, using 124 lamps during the first and last 2 h of treatment and 198 lamps between noon and 6 PM. UV-C was excluded by two layers of borosilicate glass (Tempax and Pyran; Schott, Mainz, Germany) between the lamp and plant cabinets. The spectral irradiance was measured in the UV region with a modified Bentham double-monochromator spectroradiometer (Seckmeyer and Payer, 1993). The UV-B₈ₑ₉ irradiance was calculated using the generalized plant action spectrum, normalized to 300 nm (Caldwell, 1971), and was approximately 55 mW m⁻².

**Harvest of Plant Material**

For each of the included genes, the changes in steady-state mRNA levels had been analyzed in advance during development as well as during a diurnal cycle. Only the chl Cu/ZnSOD showed dramatic changes in mRNA abundance with leaf age, whereas a diurnal cycle was only observed for Cat1 (Willekens et al., 1994a; J. Kurepa, unpublished results). This was taken into account when selecting times and plant material for analysis. In the case of N. plumbaginifolia, four rosette leaves that are indistinguishable with respect to antioxidant gene expression were harvested from different mature preflowering plants and pooled for RNA extractions. In the case of N. tabacum cv PBD6, plants with six leaves (at the onset of the experiment) to eight leaves (after 1 week of exposure) larger than 8 cm were used for analysis. Leaves 4 and 6 (leaf 1 being the first leaf larger than 8 cm) were harvested from four different plants and pooled for RNA preparation.

**RNA Extraction and Northern Analysis**

RNA from frozen leaf tissue was extracted as described by Logemann et al. (1987). Total RNA was quantified by gel electrophoresis and ethidium bromide staining as well as by spectroscopy. Total RNA (15 μg) of each sample was separated on agarose gels containing 6% (v/v) formaldehyde (Maniatis et al., 1982) and transferred to nylon Hybond-N (Amersham International, Aylesbury, UK) by capillary blotting. Filters were hybridized at 68°C in 50% formamide, 5× SSC (1× SSC is 0.15 μ NaCl, 0.015 μ sodium citrate), 0.5%
SDS, 10% dextran sulfate, and 0.1 mg mL⁻¹ denatured salmon sperm DNA with highly specific ³²P-labeled cDNA fragments of Cat1 (EcoRI-BamHI fragment of pCAT1A; Willekens et al., 1994b), Cat2 (BamHI fragment of pCat2A; Willekens et al., 1994b), Cat3 (BamHI fragment of pCat3A; Willekens et al. 1994b), APx (EcoRI-HindIII fragment of a clone corresponding to APx isolated from Arabidopsis thaliana; S. Kushnir, unpublished results), GPx (EcoRI fragment of 6P229 subcloned in Bluescript vector (Stratagene, La Jolla, CA; Criqui et al., 1992), and chl Cu/ZnSOD (AccI-HindIII fragment of a clone corresponding to pSOD4 isolated from N. plumbaginifolia; J. Kurepa, unpublished results). To obtain specific probes for cyt Cu/ZnSOD, FeSOD, and MnSOD, internal fragments were recloned into pGem2 (Promega, Madison, WI) as described by Tsang et al. (1991). The EcoRI-HindIII fragment of cyt Cu/ZnSOD and the Psfl-HindIII fragments of FeSOD and MnSOD were used in hybridizations. Stringent washing was carried out in 0.1x SSC containing 0.5% (w/v) SDS at 68°C. The relative amounts of mRNA in different samples were determined by densitometric scanning of autoradiographs using an ultrascan laser densitometer (LKB model 2202; LKB, Bromma, Sweden).
The effect of O₃ on mRNA accumulation of SODs and cyt APx was also investigated in the O₃-sensitive N. tabacum PBD6. Plants were exposed to O₃ as described above. After 4 d of treatment, plants started to display spot-like necrotic lesions on the leaves, which are characteristic of O₃ damage. Leaf damage appeared first on the oldest leaves. After 5 d, necrotic spots covered 5 to 10% of the leaf surface of leaves 6 to 8 (leaf 1 being the first leaf from the top that is larger than 8 cm). Leaves 1 to 4 remained symptomless throughout the study.

None of the SODs showed any major alteration in mRNA levels before the onset of visible damage (Fig. 4, t = 3 d). This is in accordance with the results in N. plumbaginifolia. However, after 5 d of O₃ treatment, FeSOD mRNA levels decreased dramatically in the damaged leaf (leaf 6) but not in the undamaged leaf (leaf 4), indicating that the effects on FeSOD mRNA abundance may be closely associated with cellular damage. The decrease in the chl Cu/ZnSOD was not consistently observed and, therefore, should be viewed with caution. In N. tabacum cv PBD6, mRNA and activity levels of the chl Cu/ZnSOD in leaves were highest in young leaves and almost absent in mature leaves (J. Kurepa, unpublished).

Figure 3. RNA gel blot analysis showing the effect of exposure to O₃ on different antioxidant defense genes. Four leaves of different N. plumbaginifolia plants were harvested and pooled for RNA extraction 0, 3, 9, and 33 h after the onset of exposure and additionally after 2 and 5 d.

Figure 2. RNA gel blot analysis showing the effect of exposure to SO₂ on different antioxidant defense genes. Four leaves of different N. plumbaginifolia plants were harvested and pooled for RNA extractions 0, 3, 9, and 33 h after the onset of exposure and additionally after 2 and 5 d.

5 d of SO₂ treatment, Cat3 mRNA levels did not show any increase.

Effect of Exposure to Elevated O₃

N. plumbaginifolia plants of the same age and size as used for UV-B and SO₂ were exposed to daily cycles of O₃ (120 nL L⁻¹, 14 h d⁻¹). As described previously, the exposure regime closely resembled an O₃ episode monitored in July 1990 in Switzerland (Van Camp et al., 1994b). N. plumbaginifolia did not display any visible symptoms of O₃ damage. Therefore, the effect of O₃ could be compared with UV-B or SO₂ without interference of necrosis-induced effects. The antioxidant response to O₃ was again highly similar to the responses to UV-B and SO₂ (Fig. 3). Differences from the previous stress treatments were observed only in the time course of induction or repression. Cat1 mRNA levels started to decrease after 3 h of O₃ exposure, which was the same as with SO₂ but faster than with UV-B. Induction of Cat2 and Cat3 by O₃ was comparable to that of UV-B, but GPx transcript levels increased more slowly during O₃ treatment. FeSOD mRNA levels again showed a continuous decrease, whereas other SODs and cyt APx showed a transient induction.
Response of Antioxidant Genes to \( \text{O}_3 \), \( \text{SO}_2 \), and \( \text{UV-B} \)

DISCUSSION

\textit{N. plumbaginifolia} contains three different Cats, which are very similar in sequence but which nevertheless are thought to be involved in distinct cellular processes (Willekens et al., 1994a). Because their phylogenetic and functional relationship with Cats of maize is not clear (Willekens et al., 1994a), they are denoted arbitrarily \textit{Cat1}, \textit{Cat2}, and \textit{Cat3}. \textit{Cat1} of \textit{N. plumbaginifolia} is highly expressed in photosynthesizing cells, where it may scavenge the \( \text{H}_2\text{O}_2 \) that is produced during photorespiration. \textit{Cat3} of \textit{N. plumbaginifolia} is most abundant in seeds and, therefore, likely to be associated with glyoxysomal functions, whereas \textit{Cat2} is quite uniformly distributed within the plant, although with a preference for vascular tissues. The metabolic processes with which \textit{Cat2} is associated are currently unknown.

This divergence in function is reflected in the expression profile of Cats during \( \text{UV-B} \), \( \text{SO}_2 \), and \( \text{O}_3 \). Results). The position of this gradient along the stem was quite variable during development and between different plants, which might account for the observed inconsistencies. \textit{MnSOD} transcript levels were not altered during the \( \text{O}_3 \) exposure, but the cyt \textit{Cu/ZnSOD} was induced 4- to 5-fold after 5 d, which was concurrent with the appearance of visible leaf necrosis. Comparison of leaves 4 and 6 at that time showed that the induction was confined to the damaged leaves. The cyt \textit{APx}, believed to scavenge the \( \text{H}_2\text{O}_2 \) that is produced by the cyt \textit{SOD}, showed an induction profile similar to that of the cyt \textit{Cu/ZnSOD}, suggesting that both genes might be co-regulated during environmental stress in \textit{N. tabacum}. Cats and GPX displayed a similar expression profile as in \textit{N. plumbaginifolia} (data not shown).

Photosynthetic activity decreases in response to \( \text{UV-B} \), \( \text{SO}_2 \), and \( \text{O}_3 \). In each case, PSI seems to be the major target site (Schreiber et al., 1978; Covello et al., 1989; Renger et al., 1989; Strid et al., 1990). As a consequence, plant metabolic processes involved in fatty acid degradation may be activated for energy supply. In plants, \( \beta \)-oxidation of fatty acids takes place mainly in seed glyoxysomes, but in some recent studies (e.g., Graham et al., 1992) peroxisomes of senescing leaves were reported to adopt glyoxysomal functions. The induction of \textit{Cat3} mRNA levels during \( \text{O}_3 \) and \( \text{UV-B} \) exposure suggests that the formation of these leaf glyoxysomes may also occur during stress conditions, which provoke early senescence. \( \text{O}_3 \) stress and senescence in leaves show analogies with regard to changes in membrane properties (Pauls and Thompson, 1980), production of ethylene (reviewed by Heath, 1994), and protective effects of senescence antagonists, such as cytokinins (Pauls and Thompson, 1982), \( N-[2-(2-\text{oxy}-1-\text{imidazolidinyl})\text{ethyl}]^-\text{-N}'^-\text{phenylurea} \) (Lee and Chen, 1982), and polyamines (Bors et al., 1989), against \( \text{O}_3 \) damage.

The biochemical/physiological processes that \textit{Cat2} is involved in are currently unknown, but the rapid increase in \textit{Cat2} mRNA levels, particularly in response to \( \text{UV-B} \), suggests that \textit{Cat2} plays an important role in the scavenging of \( \text{H}_2\text{O}_2 \) during various stress conditions. In maize, \textit{Cat3} mRNA levels are rapidly induced upon cold treatment, and this increase in \textit{Cat3} is thought to be essential for acclimation to chilling conditions (Prasad et al., 1994). Whether \textit{Cat2} of \textit{N. plumbaginifolia} functionally corresponds to \textit{Cat3} of maize remains to be elucidated, but the fact that both genes have a similar spatial expression, showing highest transcript levels in stems and vascular tissues of leaves, is indicative of a similar role within the plant (Scandalios, 1994; Willekens et al., 1994a).

The dramatic effects of \( \text{O}_3 \), \( \text{SO}_2 \), and \( \text{UV-B} \) on the mRNA levels of different Cats suggest that peroxisomes are very sensitive to environmental adversity. It is tempting to speculate that, if impairment and activation of specific peroxisomal functions are among the first manifestations of incipient stress, peroxisomes could be more crucial for the defense of plants against environmental stress. Analyzing the specific role of various peroxisomal functions in the defense against various stresses could be very rewarding.
the individual Cats and marker enzymes for different peroxisomal processes at the level of enzyme activity should clarify whether these marked changes in mRNA abundance correlate with alterations in peroxisomal processes.

Similarly to Cat2, GPx transcript levels increase rapidly upon stress treatment. GPx has rarely been described in plants (Drotar et al., 1985), and its function has yet to be established. The GPx sequence from *N. sylvestris* used in our analysis as a probe and from citrus (Holland et al., 1993) are most closely related to a phospholipid hydroperoxide GPx from pig (H. Willekens, unpublished results). This enzyme is thought to repair phospholipid hydroperoxides at the expense of GSH (Schuckelt et al., 1991). To date, it is the only protein identified that is actively involved in the repair of lipid-derived peroxides. Its functional importance has not yet been demonstrated, but it is strongly suggested by the fact that in both animals and plants lipid peroxidation is a general consequence of stress. For example, in bean, lipid peroxidation by O₃ has been observed only in an O₃-sensitive variety (McKersie et al., 1982), and interestingly, phospholipids were shown to be particularly affected (Pauls and Thompson, 1980). From the present study we have now identified GPx induction as an early defense response of plants against O₃ and UV-B and, thus, provide experimental evidence for its importance.

In contrast, the amounts of mRNA coding for cyt Cu/ZnSOD and APx increase only with the onset of visible O₃ damage. Previously, a similar correlation was observed at the level of enzyme activity for the cyt Cu/ZnSOD of bean (Pitcher et al., 1992) and tobacco (Van Camp et al., 1994b). The lag period before induction of cyt SOD and APx is, therefore, expected to depend on the O₃ concentration that is applied. Accordingly, exposure of *N. tabacum* PBD6 to high doses of O₃ increased the transcript levels of the cyt Cu/ZnSOD after 5 h (Hérouart et al., 1993). Notwithstanding that O₃ exposure results in increased O₂ radical production in the chloroplasts (Sakaki et al., 1983; Mehlihorn et al., 1990), mRNA levels of FeSOD did not show any positive response to O₃ in *N. plumaginifolia* or in *N. tabacum* PBD6. The observed reduction in FeSOD mRNA levels is also manifested at the level of enzyme activity (Van Camp et al., 1994b) and suggests that the endogenous SOD response to O₃ is ineffective in the case of tobacco chloroplasts. Because this decrease is observed in both the tolerant and the sensitive tobacco species, a simple correlation between FeSOD levels and tolerance does not seem to exist. SO₃ and UV-B, which caused no apparent damage in our experimental conditions, did not induce SODs. In bean, exposure to UV-B led to a marked decrease in mRNA levels of the chl Cu/ZnSOD (Strid, 1993). In contrast, GSSG reductase, which, together with SOD and APx forms part of an enzymic chain for removal of O₂ and H₂O₂, is induced by UV-B in bean. Despite this induction, GSSG was reported to accumulate during UV-B exposure to levels 9-fold higher than in nontreated plants (Strid, 1993).

Taken together, our data suggest that the different stresses O₃, SO₃ and UV-B generate a remarkably similar antioxidant response. The time course of induction or repression is often slightly different for each stress. This shows that the observed changes are not due just to a diurnal cycle because in that case time courses should have been similar for all three experiments. Essentially, an early and a late antioxidant response can be discriminated. The early response includes induction of a specific Cat and GPx but not of SOD and APx. Although indicative of an important role of Cat and GPx, these results do not necessarily imply that SOD or APx are not required during stress protection. As discussed above, cyt Cu/ZnSOD and APx are induced at a later point (late response), whereas the importance of chl SOD was evidenced in a recent study in which a correlation between SOD overproduction in the chloroplasts and O₃ tolerance was shown (Van Camp et al., 1994b). In addition, increased SOD activity has been associated with tolerance to O₃ in tobacco (Shaaltiel et al., 1988; Tanaka et al., 1988) and to SO₃ in *Lolium* (Shaaltiel et al., 1988), poplar (Tanaka and Sugahara, 1980), Chlorella (Rabinowitch and Fridovich, 1983), and bean (Madamanchi and Alscher, 1991).

This general antioxidant response may account for the phenomenon of cross-tolerance (i.e. plants selected for a specific tolerance display cross-tolerance to another unrelated stress) that has been observed in several plants with enhanced antioxidative properties. Also, the pathogenesis-related response was shown to be induced by different stress conditions, which, in addition to various pathogens, include O₃, SO₃, and UV radiation. Enhanced synthesis of phytoalexins by O₃ and SO₃ (Rubin et al., 1983), of polyamines by O₃ (Langebartels et al., 1991) and SO₃ (Priebe et al., 1978), of β-1,3-glucanase and chitinase by O₃ (Schraudner et al., 1992), and of pathogenesis-related proteins 1a and 1b by UV-C (Brederode et al., 1991) has been reported. These data indicate that pathogen, O₃, SO₃, and UV-B activate similar defense genes within the plant, possibly by using the same signal transduction pathway.

Although each gene may be activated similarly by diverse stress conditions, we think that several pathways are involved in inducing different antioxidant genes. Despite the overall homology, some differences regarding the time course of repression or induction were noticed between the studied stress responses. These lag periods do not change in a consistent pattern for different antioxidant genes, which suggests that the regulation of these genes entails several pathways. The nature of these signaling molecules has not yet been elucidated, but ethylene, ABA, and salicylic acid are possibly involved, since they are known to induce antioxidant genes (Bowler et al., 1989; Williamson and Scandalios, 1992). Salicylic acid has recently been shown to increase the intracellular H₂O₂ concentration, probably by inhibiting Cat activity (Chen et al., 1993). This finding not only provides a link between pathogens and oxidative stress but also advocates the importance of H₂O₂ and Cat in plant defense responses. In this respect, the rapid transcriptional activation of Cat2, a Cat of unknown biological function, that was observed in this study is most intriguing. We will investigate further the putative role of this Cat2 in plant defense response.

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