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Antioxidant properties of violacein: Possible relation on its biological function

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Abstract—Violacein, a violet pigment produced by *Chromobacterium violaceum*, has attracted much attention in recent literature due to its pharmacological properties. In this work, the antioxidant properties of violacein were investigated. The reactivity with oxygen and nitrogen reactive species and 1,1-diphenyl-2-picryl-hydrazyl (DPPH), a stable free radical, was evaluated. EPR studies were carried out to evaluate the reactivity with the hydroxyl radical. The action of violacein against lipid peroxidation in three models of lipid membranes, including rat liver microsomes, Egg and Soy bean phosphathidylcholine liposomes were also evaluated. The compound reacted with DPPH (IC_{50} = 30 μM), nitric oxide (IC_{50} = 21 μM), superoxide radicals (IC_{50} = 125 μM) and decreased the hydroxyl radical EPR signal. The compound protected the studied membranes against peroxidation induced by reactive species in the micromolar range. The reconstitution of violacein into the membranes increased its antioxidant effect. These results indicate that the compound has strong antioxidant potential. Based on these results we suggest violacein plays an important role with the microorganism membrane in defense against oxidative stress.
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1. Introduction

Chromobacterium violaceum was the first bacterium to have its genome sequenced by the Brazilian Sequencing Network, a project in which our group also participated.[1] It is a Gram-negative proteobacteria found in the soil and water in tropical and sub-tropical environments. Its complete genome sequence revealed widely varying alternative pathways for energy generation, complex and extensive systems for stress adaptation, motility, and widespread utilization of quorum sensing for control of its inducible systems. All these findings demonstrate the organism's versatility and adaptability. Studies on the bacteria metabolism have revealed it is a facultative anaerobe and presents an oxidative or fermentative metabolism, producing organic acids from glucose and some other carbohydrates.[1,2]

One of the main characteristics of this microorganism is the ability to produce a purple pigment, known as violacein 3-[1,2-dihydro-5-(5-hidroxy-1H-indol-3-yl)-2-oxo-3H-pyrrol-3-ilydene]-1,3-dihydro-2H-indol-2-one (Fig. 1), under aerobic conditions. The production of secondary metabolites by bacteria is of great interest for pharmacological applications. Many of these secondary metabolites show antibiotic and cytotoxic activities, and aquatic microorganisms have become important sources of natural products for research.[3]

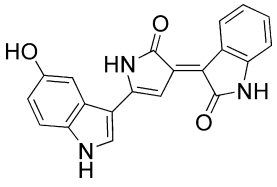

Figure 1. Chemical structure of violacein.

Keywords: Violacein; Free radicals; *Chromobacterium violaceum*; Liposomes.
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Although the physiological function of violacein is not yet clarified, the *C. violaceum* genome sequencing has given some insight into the mechanisms and control of the pigment production.[1,4] Violacein isolation and chemical characterization began in 1958[5] and it was finally spectroscopically analyzed in 1984.[6] Violacein has been shown to have antibiotic,[7] tripanocide,[8] antitumoral,[9–12] antiviral,[13] and genotoxic [14] properties.

Also according to the *C. violaceum* genome content, it has a versatile energy-generating metabolism and is normally exposed to diverse environmental conditions.[15] The bacterium is able to live under anaerobic and aerobic conditions but violacein is produced only in the presence of oxygen. It has been proposed that violacein is involved with protection against radiation.[16] In a recent study the authors showed that violacein is toxic to bacterivorous nanoflagellates suggesting that this activity would be useful for survival since it undermines protozoan grazing and population dynamics.[17]

It is well known that under aerobic conditions reactive oxygen and nitrogen species are produced as part of the normal metabolism of living organisms. However, many pathologies can provoke an imbalance in homeostasis, and the production of reactive oxygen species (ROS) as well as reactive nitrogen species (RNS) particularly the peroxynitrite[18,19] may increase greatly, compromising the affected organism. When a mammal is invaded by an antigen such as a bacterium the neutrophils form the principal front of the cell-mediated immune system. Their natural function is to overcome foreign organisms, inactivating them and destroying the residues through reactive species generation.[20] Antioxidant defenses, enzymatic and/or non-enzymatic, protect the invading organisms against the oxidative stress caused by their invasion.

The principal enzyme that defends against microorganism invasion is myeloperoxidase (MPO). This enzyme transforms H_2O_2 into potent microbicidal oxidants in activated neutrophils. It was also shown that MPO produces the tyrosyl radical, suggesting that there is also a phagocyte pathway for lipid peroxidation and dityrosine cross-linking of proteins.[21] MPO, which consists of azurophilic granules in the phagolysosomes, has a chlorinating activity besides its regular peroxidative activity, unlike other peroxidases.[22,23]

As already mentioned, the principal marker of oxidative stress is the production of ROS and RNS, and biological membranes are one of their important targets. They induce lipid peroxidation (LPO) of membrane phospholipids, unsaturated fatty acids.[24,25] At the cellular level, reactive species have been found to be responsible for alterations in membrane physical properties, inducing changes in its fluidity causing alterations in cell permeability, enzyme activities, and cell death.[26,27] Liposomes, in which phospholipid composition, structure, and dynamics can be fully controlled, are appropriate models for studies involving phenomena related to natural membranes. They are surrounded by a lipid bilayer, structurally similar to the cell membrane lipidic matrix.[24,28] In addition, it is known that individual membranes often exhibit distinct lipid compositions.[29]

In this work we report violacein antioxidant efficiency against oxygen and nitrogen reactive species, as a scavenger of hydroxyl ($^{\bullet}OH$), superoxide ($O^{\bullet-2}$), and nitric oxide ($NO^{\bullet}$) radicals, and also of the DPPH radical; inhibiting lipid peroxidation induced by peroxynitrite ($ONOO^{-}$) and ascorbyl ($Asc^{\bullet}$), and inhibiting MPO activity. Additionally we show that violacein reconstituted into liposomes has its antioxidant potential increased.

2. Results and discussion

To initiate the evaluation of violacein antioxidant properties the DPPH free radical scavenging system was used. As a tool to study the action of molecules as free radical scavengers it has the advantage of being independent of metal or enzymatic activity. This assay characterizes the antioxidants reacting with the stable free radical 1,1-diphenyl-2-picryl-hydrazyl (DPPH) producing a colorless 1,1-diphenyl-2-picryl-hydrazine. When DPPH receives an electron or hydrogen radical to become more stable, its absorption decreases. More details are described in Section 3. The violacein was able to scavenge the free radical completely, and the IC_{50} of 30 μM was obtained, indicating its antioxidant potential (Fig. 2).

The lipid membranes are the principal targets of reactive species and it is well known that such membranes vary in lipid composition and their response to ROS action (for a review, see Lima et al., 2004),[29] we tested the antilipid peroxidation properties of violacein in three lipid-membrane systems including rat liver microsomes, Egg PC liposomes, and Soy bean PC liposomes. Figure 3 shows violacein protection against microsomal membrane oxidation induced by the ascorbyl radical. Violacein was able to inhibit the radical action at 200 μM completely, and the IC_{50} obtained was of 10 μM, a very low concentration compared to the values for quercetin (about 80 μM) results not shown.

The next data are related to the lipid peroxidation of Egg PC and Soy bean PC liposomes induced by the ascorbyl radical and peroxynitrite. Violacein at 80 μM was able to avoid the peroxidation of Egg PC membranes induced by both reactive species almost completely, and the IC_{50}s

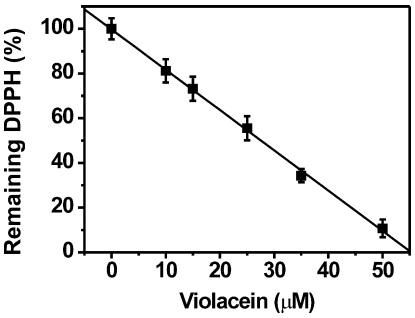

Figure 2. Effect of violacein on DPPH radical.

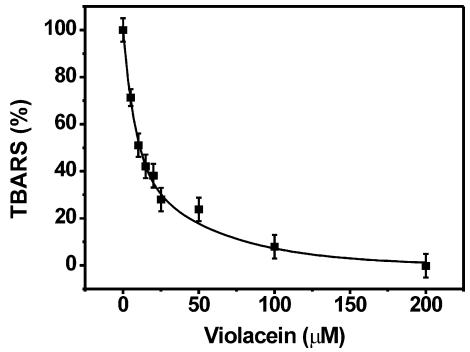

Figure 3. Effect of violacein on liver microsomes peroxidation induced by ascorbyl radical. Results are expressed as percentage of lipid peroxidation. Considering 100% of peroxidation 30.2 ± 1.2 μmol per mg of protein.

obtained were 29 μM (for ascorbyl) and 33 μM (for peroxynitrite). Violacein was less efficient in protecting Soy bean liposomes from peroxidation. It did not reach IC_{50} until a concentration of 0.5 mM for both reactive species tested. A control with the amount of DMSO present in the violacein solution was run in parallel in all experiments.

Considering that violacein has hydrophobic characteristics we assumed that it would be easy to incorporate the molecule into liposomes. Before starting the vesicles formation through dialysis process, violacein was added to the lipid solution as described in Section 3. After violacein incorporation, no traces of the compound were found either in the dialysis buffer or after the liposomes centrifugation. After that the membranes reconstituted with violacein were analyzed and exposed to the reactive species ascorbyl and peroxynitrite. Violacein reconstituted into the membranes increased the lipoperoxidation protection by ~60% against peroxynitrite and ~20% against the ascorbyl radical. It means that violacein when in solution competes with the reactive species in the membrane permeation process, perhaps permitting the ROS to reach the oxidation site first. From these results one can suggest that when the compound is inside the membrane, the reactive species do not easily reach the target in the lipid carbon tail, possibly because the antioxidant is nearer to the oxidation site. This effect is more evident with peroxynitrite. This is interesting because the lipid bilayer seems not to be a barrier for peroxynitrite. Its calculated permeability coefficient is approximately 8.0×10^{-4} cm s^{-1}, comparable with that of water.[30,31] In this case Soy bean PC liposomes were used, of the kind that violacein could not effectively protect against lipid peroxidation when it was in solution (Table 1). A control with 100% peroxidation was run in parallel for each reactive species. The reason why violacein is less efficient permeating the Soy bean PC than Egg PC liposomes is not known, however it is well known that the acyl chain of the fatty acids of PC from distinct natural fonts are completely different. For Egg PC the percentage of palmitic, palmitoleic, estearic, oleico linoleico, linolenics, and araquidonic acid is 34, 2, 11, 32, 18, 0, and 3, respectively, and for Soy PC is 17, 0, 6, 13, 59, 5, and 0, respectively.[32,33] The composition of the lipid membranes define their physicochemical properties such as phase transition temperature, acyl chain mobility, permeability, etc. It is expected that the hydrophobic compounds as well as the ROS would interact in distinct way with different membranes.[29,33]

Table 1. Effect of violacein on Soy bean PC liposomes peroxidation by peroxynitrite and by ascorbyl radical

Reactive specie	Violacein	Lipoperoxidation protection %
Peroxynitrite	In solution	47
	Reconstituted	91
Ascorbyl radical	In solution	44
	Reconstituted	61

Violacein in solution and reconstituted into liposomes. Control, assay without violacein. Violacein concentration was set to 0.5 mM in all cases.

Concerning the RNS, violacein was able to protect against lipid peroxidation induced by peroxynitrite as described above and was also able to scavenge nitric oxide as shown in Figure 4. The compound at 40 μM scavenges the radical almost completely, and the IC_{50} obtained was 21 μM. A standard curve with sodium nitrite, was run in parallel to each experiment, allowing the amount of nitric oxide released by sodium nitroprusside in the reaction medium to be calculated.

Violacein exerts a double protection against the deleterious actions of $NO^{\bullet}$ since it scavenges the molecule and protects against the lipid peroxidation induced by $ONOO^{-}$. The decrease in $NO^{\bullet}$ supply is a positive event when the organism is in an oxidative stress state since an overproduction of this radical triggers the production of peroxynitrite that is not scavenged by physiological antioxidant.[34,35] Additionally violacein was also able to scavenge the superoxide with an IC_{50} of 50 μM (Fig. 5). Considering that the synthesis of $ONOO^{-}$ involves $NO^{\bullet}$ and $O^{\bullet -2}$, the ability of violacein to scavenge both reagents of this reaction forms an important barrier against the host defense, since peroxynitrite is also fundamental in the combat of organism infections.[18,19]

Violacein was also able to scavenge the hydroxyl radical, the most deleterious among the ROS. This was shown through EPR experiments with the violacein

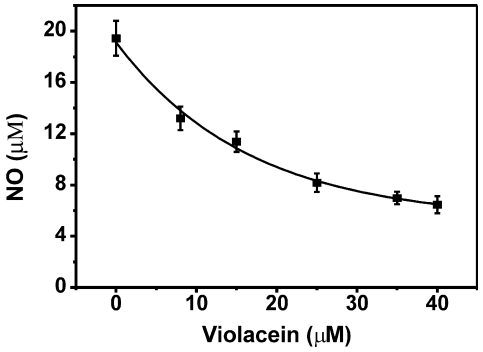

Figure 4. Effect of violacein as scavenger of nitric oxide.

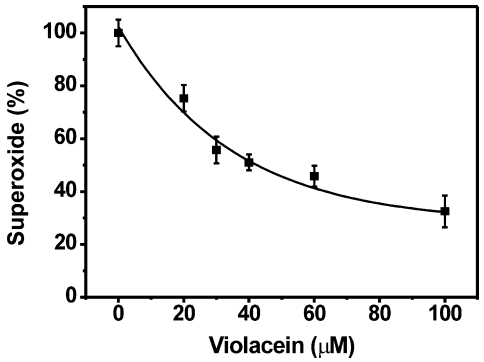

Figure 5. Effect of violacein as scavenger of superoxide radical.

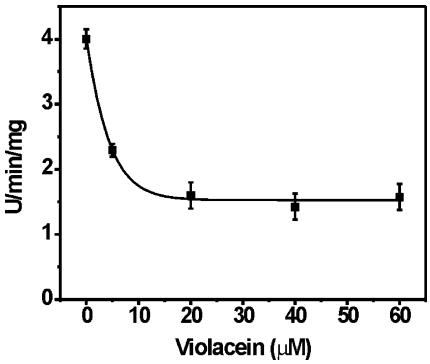

Figure 7. Effect of violacein concentration on activity of myeloperoxidase. Considering 100% of activity 4.0 ± 1.0 U/min/mg.

co-reconstituted into liposomes since the solvent (DMSO) utilized to dissolve the compound reacts with the hydroxyl radical,[36] interfering in the indirect spectrophotometric methods for this reactive specie. The EPR spectra show that violacein decreases the signal of the free radical in both systems, Egg PC and Soy bean PC liposomes (Figs. 6A and B, respectively).

Myeloperoxidase activity was also inhibited by violacein (Fig. 7) and this enzyme plays a central role in infection and inflammation. Its role is to convert hydrogen peroxide and chloride to hypochlorous acid (HOCl) to damage the microorganisms. The violacein was able to inhibit the myeloperoxidase activity. Although the activity did not reach zero, the $K_{0,5}$ obtained was 18 μM (Fig. 7). The enzyme activity was determined by the slope of the absorption curve measured at 450 nm for the first 30 s in each concentration of violacein. For more details, see Section 3.

We can conclude from these results taken together that: (i) violacein incorporates into the lipid bilayer with very high yield; (ii) the protection was higher when violacein was reconstituted into the liposomes than in solution, and it depends on lipid composition; (iii) violacein was able to protect lipid membranes against oxidative stress in all assays carried out; (iv) violacein was able to scavenge free radicals in solution and to inhibit the action of a pro-inflammatory and oxidizing enzyme; (v) EPR measurements showed that violacein also scavenges the hydroxyl radical; (vi) violacein was not present in the supernatant of the *C. violaceum* culture, that is the majority was found in the cellular mass fraction of the bacteria and to extract the violacein it was necessary to destroy the cells.

Although *C. violaceum* is an opportunistic microorganism, that is, not pathogenic, when an infection settles in it is lethal, especially in children.[37] Violacein probably interacts with the bacterium membrane to help in its protection against the oxidative stress generated by the host immune system. Although we proved that violacein has a high affinity for the lipid bilayer we cannot discard the possibility that violacein is also located in the cell wall of the *C. violaceum* since it is a Gram-negative bacterium.

3. Materials and methods

3.1. Materials

Egg and Soy bean PC (phosphatidylcholine), 1,1-cholic acid, deoxycholic acid, 1,1-diphenyl-2-picryl-hydrazyl (DPPH), thiobarbituric acid (TBA), nitro blue tetrazolium (NBT), superoxide dismutase (SOD), hexadecyl trimethyl-ammonium bromide (HTMAB), (*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]) (HEPES), diethoxyphosphoryl-5-methyl-1-pyrroline-*N*-oxide (DEPMPO), MPO, and θ-dianisidine were purchased from Sigma® (St. Louis, MO, USA). All other chemicals obtained were of analytical grade. The experiments were performed with $n = 3$ and in triplicate. The results are represented as means ± standard error.

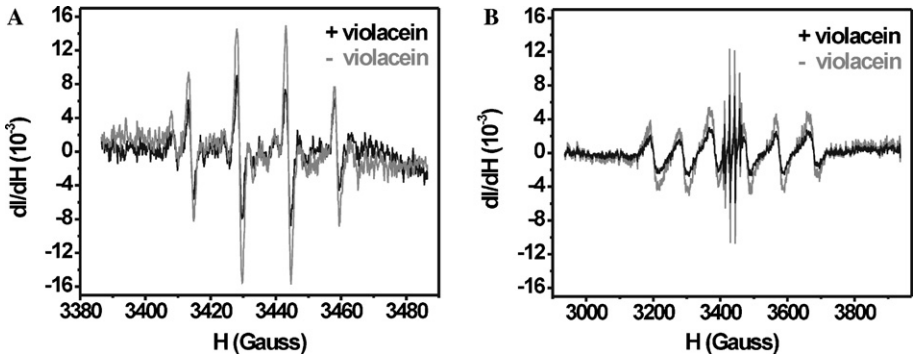

Figure 6. EPR DMPO-Hydroxyl adduct spectra of liposomes containing violacein incorporated. (A) Egg PC and Egg PC-VIO, (B) Soy bean PC and Soy bean PC-VIO.

3.2. Microorganism

The violacein was isolated and purified from *C. violaceum* (ATCC12479) as described previously.[8] To violacein production, *C. violaceum* was cultivated in 500 mL of Terrific Broth, in flasks under agitation (150 rpm), at 37 °C, for 5 days. After cultivation the cells were harvested by centrifugation (5000*g*, for 10 min). The cells were dried end violacein was extracted utilizing methanol as solvent. Additional purification, in order to separate deoxyviolacein and other pigments was carried out through silica gel chromatography. The fraction containing violacein was separated and the methanol evaporated. The resulting violacein powder was dissolved in DMSO and used in the subsequent assays.

3.3. Liposome preparation

Unilamellar liposomes were prepared by cholate dialysis as described previously.[38,39] Briefly, the method consists of dissolving the phospholipids at (50 mg/mL) in a buffer containing Tricine (10 mM), cholic acid (20 g/L), and deoxycholic acid (10 g/L) at pH 8.0, followed by a dialysis procedure at 30 °C for 5 h. The detergents go through the membrane dialysis of ~2 nM pore in favor of a concentration gradient while the vesicles are forming (maximal 1 mL sample against 2 L of dialysis buffer).

3.4. Reconstitution of violacein into liposomes

An aliquot of the lipid dissolved in detergent and an aliquot of violacein dissolved in DMSO were mixed before starting the dialysis process. The obtained mixture was transferred to a dialysis recipient. In parallel, liposomes with the same amount of DMSO, in the absence of violacein, were prepared to be used as control. After the dialysis process, an aliquot of the liposomes was centrifuged at 14,000*g* (10 min), and the violacein content in the supernatant was evaluated spectrophotometrically at 597 nm ($\varepsilon = 17{,}000\ M^{-1}\ cm^{-1}$).[40]

3.5. Microsome preparation

Microsomes were obtained by differential centrifugation with calcium aggregation. The liver was homogenized in Tris (10 mM) / sucrose (250 mM) pH 7.4 (1:6 w/v), and centrifuged at 600*g* for 5 min at 0 °C. The supernatant was centrifuged at 12,000*g* for 10 min. The microsomal fraction resulted from the precipitation of the supernatant with $CaCl_2$ (80 mM) and centrifugation at 25,000*g* for 15 min. The pellet was washed with Tris (10 mM) / KCl (150 mM), pH 7.4 and centrifuged at 25,000*g* for 15 min. After washing, microsomes were resuspended with glycerol (20%) in Na_2HPO_4 (0.1 M).[41] This fraction was immediately placed in liquid nitrogen for the later determination of antioxidant activity.

3.6. Lipid Peroxidation induced by ascorbyl radical and peroxynitrite

Lipid peroxidation was induced by the addition of $FeSO_4$ (25 μM) and ascorbate (500 μM), for the ascorbyl radical or peroxynitrite (2.7 mM) in a reaction medium containing 2 mg microsomal protein/mL or liposomes (lipids at 12.5 mg/mL), and Tris–HCl (0.1 M), pH 7.4. The samples were incubated for 30 min at 37 °C and the extent of lipid peroxidation was determined by the thiobarbituric acid method.[42] The amount of TBARS was calculated using an extinction coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$. The amount of organic solvent in all experiments was kept constant, by adding the same volume of stock solutions of violacein with different concentrations. In all cases, a blank run with the same amount of the organic solvent was made to check for its interference in the assays.

3.7. Radical $NO^{\bullet}$

Nitrogen monoxide was produced by the incubation of sodium nitroprusside (40 mM) solution in a medium containing NaH_2PO_4 (20 mM), at pH 7.2 at room temperature, in the absence or presence of violacein in different concentrations.[43] NO was measured indirectly through nitrite formation. Nitrite was monitored spectrophotometrically at 540 nm by the Griess reagent: *N*-1-naphthylethylenediamine (0.1% w/v) and sulfanilamide (1% w/v) in H_3PO_4 (5% v/v) with the help of a sodium nitrite standard curve.[44]

3.8. Radical DPPH

The radical DPPH is considered a stable radical and has its maximal absorption at 517 nm. The assay is based on the incubation of all reagents for 1 h at 37°C in an ethanolic solution of DPPH (200 μM) and measuring the optical density afterwards at 517 nm. A control run in the absence of the molecule being tested was made in parallel with 100% DPPH.[45]

3.9. Scavenger of superoxide radical

The superoxide radical was generated through adrenaline oxidation. The assay was carried out in a reaction medium containing glycine buffer (50 mM), pH 10.2, adrenaline (0.12 mM), pH 2.0, incubated for 1 min, and then NBT (6 μM) and violacein were added.[46] The assay was monitored following the reduction of NBT during the first 2 min of the reaction, at 480 nm.[47] As a control experiment SOD (100 U/mL) were added to ensure superoxide radical formation.

3.10. Myeloperoxidase activity

Rat lungs were homogenized in an ice-cold phosphate buffer (50 mM) at pH 6.0, containing HTMAB (0.5%) as previously described and freeze-thawed three times.[48] The samples were centrifuged at 12,000*g* at 4 °C for 20 min. The supernatant was assayed in a reaction medium containing phosphate buffer (50 mM), pH 6.0, at 25 °C, θ-dianisidine (0.167 mg/mL), and H_2O_2 (0.0006%). The enzyme activity was determined by the slope of the absorption curve measured at 450 nm for the first 30 s in each violacein concentration. A standard curve of myeloperoxidase activity was obtained previously with a commercial enzyme batch. Sodium azide (250 μM) was used to

inhibit the myeloperoxidase activity as a control of enzyme preparation.[49]

3.11. Electron paramagnetic resonance (EPR)

Different liposomes, free or co-reconstituted with violacein, were submitted to the action of the hydroxyl radical by incubation in a medium containing ascorbate (0.1 mM), $FeCl_3$ (0.025 mM), H_2O_2 (1.4 mM), KH_2PO_4 (pH 7.0), and analyzed by EPR (Bruker, Mod. ESP 300-E). DEPMPO was used as the adduct.[50,51] Control experiments were carried out monitoring the formation of TBARS to verify radical formation both with liposomes and deoxyribosis.[52]

Acknowledgments

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