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Free radical scavenging potential of L-proline: evidence from in vitro assays

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Summary. An assessment of the potential of proline to scavenge free radicals was made in a couple of in vitro assay systems, namely graft co-polymerization and autooxidation of pyrogallol. Both these assays are essentially dependent upon free radical mechanisms. Graft co-polymerization involved a ceric (Ce^{4+}) ion- or γ-radiation-induced grafting of methyl acrylate (MA) onto a cellulose backbone. The degree of grafting, measured gravimetrically, was taken as a measure of free radical generation. The γ-radiation-dependent grafting was far greater than that due to Ce^{4+} ions. Inclusion of proline in the assay, irrespective of the initiator used, led to suppression of grafting in a concentration-dependent manner indicating the ability of proline to scavenge free radicals. The γ-radiation-dependent grafting was also suppressed by hydroquinone and glutathione but not by ascorbate, glycine and spermine. In contrast to graft co-polymerization, proline did not inhibit the autooxidation of pyrogallol, a reaction involving superoxide radical generation. A subset of data constitutes an evidence for the ability of proline to scavenge free radicals in vitro. It is implied by extension that free proline, known to accumulate in plant tissues during abiotic stresses, would contribute to scavenging of surplus free radicals produced under a variety of abiotic stresses.

Keywords: Cellulose – Free radicals – Graft co-polymerization – Methyl acrylate – Proline

Abbreviations: MA, methyl acrylate; CAN, ceric ammonium nitrate

Introduction

Plants, during their life cycle, experience a variety of environmental stresses. Examples are water-deficit, salinity, temperature extremes, toxic metal ion concentrations and UV radiations. All these abiotic stresses restrict the plant growth and productivity to varying degrees depending upon the severity of stress. A common plant response to different stresses is the stimulated generation of free radicals particularly the reactive oxygen species (ROS) e.g., $OH^{\bullet}$, $O_2^{\bullet -}$, H_2O_2 etc. These are capable of causing considerable cellular damage through peroxidation of membrane lipid components and also via direct interaction with various macromolecules. Cells are sufficiently equipped to keep the ROS generated as a consequence of normal cellular metabolic activities within limits. Certain low ROS concentrations, in fact, participate in signal transduction mechanisms (Foyer and Noctor, 2005). Both low molecular weight antioxidant metabolites e.g., glutathione, ascorbic acid, α-tocopherol and antioxidative enzymes e.g., catalase, ascorbate peroxidase, superoxide dismutase contribute to the scavenging of free radicals (Dietz et al., 1999). Under different stress conditions, however, the free radical generation often exceeds the overall cellular antioxidative potential leading to a situation referred to as oxidative stress. The latter substantially contributes to the stress-dependent adverse effects on plant growth.

In response to different abiotic stresses, plants accumulate high concentrations of free proline in their tissues. The phenomenon is known to occur under water-deficit (Hare et al., 1998), salinity (Rhodes et al., 2002; Munns, 2005), low temperature (Naidu et al., 1991), heavy metal exposure (Bassi and Sharma, 1993a, b; Schat et al., 1997; Sharma and Dietz, 2006), UV radiation etc. Proline has been ascribed several adaptive functions against stresses in plants such as osmoregulation (Kavikishor et al., 1995; Hare and Cress, 1997), N-reserve, heavy metal chelation (Farago and Mullen, 1979; Sharma et al., 1998). Besides, the evidence is emerging implicating proline in antioxidative activities. For example, Mehta and Gaur (1999) demonstrated a reduced heavy metal (Cu, Cr, Ni, Zn) dependent lipid peroxidation and K^+ efflux from *Chlorella vulgaris* pretreated with exogenous proline. Further, the transgenic green microalga *Chlamydomonas reinhardtii*

expressing the mothbean Δ^1-pyrroline-5-carboxylate synthetase (P5CS) was shown to contain 80% higher free proline levels than the wild type cells and exhibit substantially enhanced tolerance to toxic Cd concentrations (Siripornadulsil et al., 2002). P5CS catalyses the first reaction in biosynthesis of proline from glutamate (Hu et al., 1992). Free proline levels correlated well with GSH redox state and malondialdehyde levels in heavy metal treated algae. Free proline was suggested to act as an antioxidant in Cd stressed cells, the resultant increased levels of GSH supporting the synthesis of phytochelatins for Cd complexation. Proline dependent scavenging of ROS such as hydroxyl radicals (Smirnoff and Cumbes, 1989) and singlet oxygen (Alia et al., 2001) has also been reported. In view of these findings together with the stress-induced production of this compatible solute, proline is likely to be an important component of the cellular antioxidative network involved in mitigating the abiotic stress-dependent oxidative stress effects. The aim of the present study was to obtain further evidence for the free radical scavenging potential of proline in a couple of in vitro free radical generation/detection assay systems. The first assay system comprised of a graft co-polymerization reaction wherein a free radical mediated grafting of a monomer onto a polymeric backbone was accomplished; the degree of grafting assessed gravimetrically serving a quantitative measure of free radical production. Another assay system comprised of $O_2^{\bullet -}$ radical generation through autooxidation of pyrogallol and its spectrophotometric detection.

Materials and methods

Graft co-polymerization assays

Methyl acrylate (monomer) was grafted onto a cellulose (polymer) backbone. The grafting is essentially dependent upon free radical generation in the reaction system. Two different initiators were used.

Ceric ion dependent initiation

Ceric ions were employed as redox initiator for grafting of methyl acrylate (MA) onto cellulose in an aqueous medium. Initiation by ceric ammonium nitrate (CAN) was carried out in the presence of nitric acid. In a typical graft co-polymerization procedure, 0.2 g pure oven-dried microcrystalline cellulose powder was dispersed in 10 ml deoxygenated water in a three-necked flask placed in water bath (50 °C). The reaction flask was flushed with purified nitrogen (passed through freshly prepared alkaline pyrogallol solution (>1%) to remove traces of oxygen): nitrogen flow was maintained through out the reaction period. 0.2 g CAN and 1.5 ml of nitric acid (1 N) were added slowly to the reaction flask. Finally, 1.0 ml MA was added drop wise to the reaction mixture from the dropping funnel. The graft co-polymerization reaction was allowed for 3 h under constant stirring with a magnetic stirrer. After 3 h, the reaction mixture was filtered and the homopolymer removed by extraction with acetone. The grafted sample was dried in an oven at 45 °C and the grafting percentage was calculated from the increases in the initial weight of cellulose:

$$\%\ \text{Grafting} = \frac{W_2 - W_1}{W_1} \times 100$$

W_1 = weight of original cellulose powder
W_2 = weight of grafted cellulose after acetone extraction

γ-radiation dependent initiation

The reaction mixture as above without the addition of CAN and HNO_3 and without nitrogen flushing was irradiated in a "Gamma chamber 900" autotimer having cobalt-60 (2000 ci) as source of γ-radiation for 2 h. Thereafter, the samples were processed for removal of homopolymer and grafting percentage determined as above.

Different metabolites/chemicals tested for their free radical scavenging properties assessed in terms of decrease in degree of grafting namely, proline, glycine, spermine, hydroquinone, glutathione and ascorbate were included in the reaction mixture at stated concentrations.

Autooxidation of pyrogallol

The reaction mixture contained 3 ml of 1 mM EDTA (prepared in 50 mM Tris, pH 8.0) and 50 µl of 48 mM pyrogallol (prepared in 10 mM HCl). Pyrogallol in basic solution autooxidizes forming $O_2^{\bullet -}$ and a lemon yellow coloured end product. The latter was quantified spectrophotometrically at 420 nm. The change in absorbance could be taken as an index of free radical generation (Marklund and Marklund, 1974). Incubation of 10 min was allowed. The effect of proline, glycine, spermine and putrescine on the reaction was assessed by including them in the reaction mixture.

Results

Graft co-polymerization assays

Ceric ion initiated reaction

Reaction mechanism: Graft co-polymerization of a vinyl monomer, methyl acrylate (MA), onto cellulose essentially occurs via a free radical mechanism wherein ceric ions (Ce^{4+}) act as redox initiator. Generation of active sites onto the cellulose backbone and initiation of monomer take place simultaneously via formation of respective complexes with Ce^{4+}. In the process, ceric ions concomitantly switch their redox states through 1 e^- transfer. The reaction could be summarised as follows:

Initiation and propagation:

$$Ce^{4+} + RCellOH \leftrightarrow Complex_1 \rightarrow RCellO^{\bullet} + H^+ + Ce^{3+}$$

$$Ce^{4+} + M \leftrightarrow Complex_2 \rightarrow M^{\bullet} + Ce^{3+} \xrightarrow{nM} (M)^{\bullet}_{n+1}$$

$$RCellO^{\bullet} + M \rightarrow RCellOM^{\bullet} \xrightarrow{nM} RCellO(M)^{\bullet}_{n+1}$$

Termination:

$$RCellO^{\bullet} + {}^{\bullet}(M)_{n+1} \rightarrow RCellO(M)_{n+1}$$

$$RCellO(M)^{\bullet}_{n+1} + Ce^{4+} \rightarrow RCellO(M)_{n+1} + Ce^{3+}$$

$$(M)^{\bullet}_{n+1} + Ce^{4+} \rightarrow (M)_{n+1} + Ce^{3+}$$

Reaction optimization: Prior to determining the effect of proline, different graft co-polymerization reaction components/parameters were optimized; the optimized reaction is described in the Materials and methods. For the purpose of optimization, different reaction parameters were tested in the following respective ranges: CAN, 0.05–0.30 g; H_2O, 10–50 ml; HNO_3, 0.5–3.0 ml; temperature, 30–50 °C (data not shown).

Effect of proline on the degree of grafting: Under the described reaction conditions, a 61% grafting of MA on cellulose was observed. Inclusion of proline (0.25–1.00 M) in the reaction mixture resulted in a concentration-dependent suppression of the process. In the presence of 1 M proline only 17% grafting was evident (Fig. 1).

γ-radiation initiated reaction

Reaction mechanism: Upon passing through matter (reaction mixture), the intensity of electromagnetic radiations declines as a consequence of partial absorption. The absorbed radiations in turn activate the grafting reaction via interaction with different reaction components in the following manner:

Initiation:

$$RCellOH \xrightarrow{\text{gamma-rays}} RCellO^{\bullet}$$

$$H_2O \xrightarrow{\text{gamma-rays}} H^{\bullet} + OH^{\bullet}$$

$$M \xrightarrow{\text{gamma-rays}} M^{\bullet}$$

$$RCellOH + OH^{\bullet} \rightarrow RCellO^{\bullet}$$

$$M + {}^{\bullet}OH \rightarrow {}^{\bullet}MOH$$

$$RCellO^{\bullet} \xrightarrow{M} RCellOM^{\bullet}$$

Propagation:

$$M^{\bullet} \xrightarrow{nM} (M)_n - M^{\bullet}$$

$${}^{\bullet}MOH \rightarrow HO - (M)_n - M^{\bullet}$$

$$RCellOM \xrightarrow{nM} RCellO(M)_nM^{\bullet}$$

Termination:

$$RCellO(M)_nM^{\bullet} + {}^{\bullet}MOH \rightarrow RCellO(M)_{n+2}OH \quad (\text{Graft})$$

$$RCellO^{\bullet} + (M)_n - M^{\bullet} \rightarrow RCellO - (M)_{n+1} \quad (\text{Graft})$$

$$(M)_n - M^{\bullet} + {}^{\bullet}M - (M)_n \rightarrow (M)_{2n+2} \quad (\text{Polymer})$$

Reaction optimization and effect of proline and other effectors: Prior to determining the effect of proline and other effectors, the reaction was optimized with respect to the duration of irradiation. The extent of MA grafting on cellulose increased linearly with an increase in irradiation time until 3 h; a more than 400% grafting was yielded in a 3 h irradiation (Fig. 2). The latter, however, was associated with an excessive formation of homopolymer. Therefore, for subsequent studies, a 2 h irradiation at a dose of 1.02 kGy was adopted.

Inclusion of proline in the reaction system resulted in a concentration-dependent decline in the magnitude of grafting. Thus, the grafting was reduced from 310% in control (without proline) to 305, 122 and 22% at 0.25, 0.50 and 1.0 M proline, respectively (Fig. 3). In contrast, glycine (another amino acid) and spermine (a polyamine) did

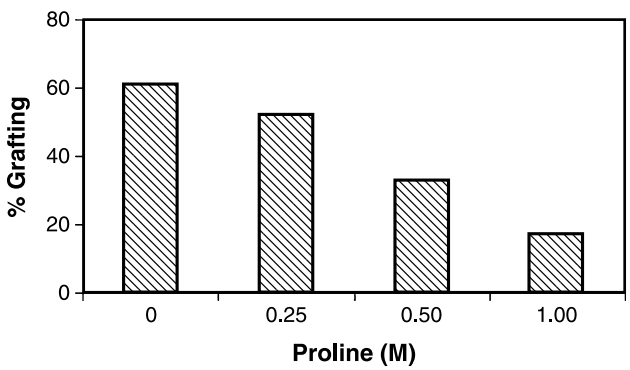


Fig. 1. Effect of proline on ceric ion-dependent methyl acrylate (MA) grafting on cellulose

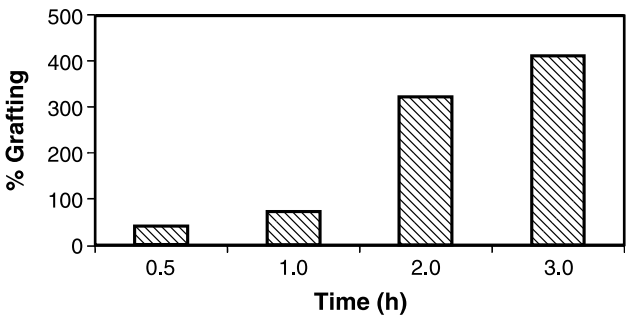


Fig. 2. γ-radiation-induced grafting of methyl acrylate (MA) onto cellulose as a function of time of irradiation

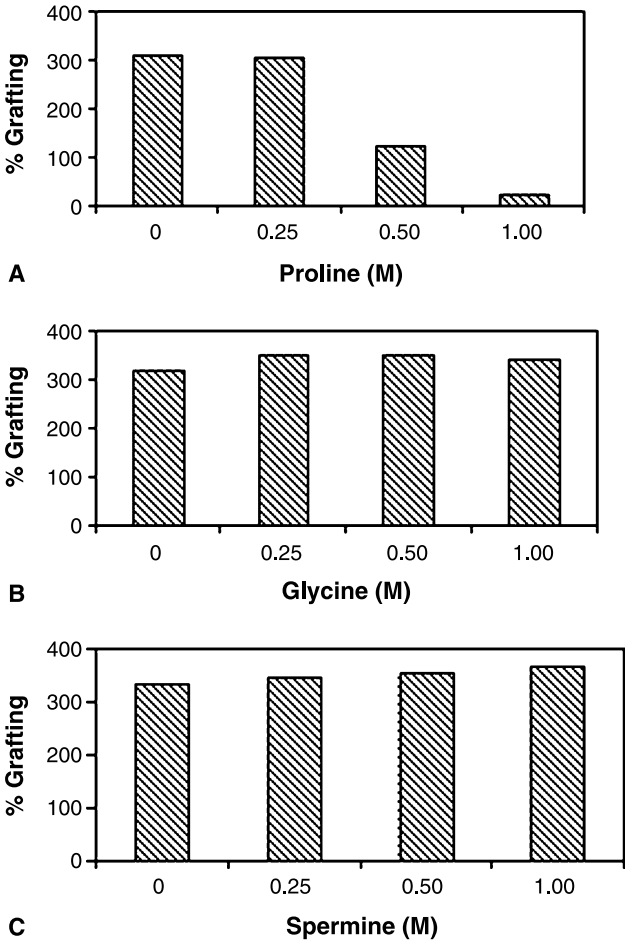


Fig. 3. Effect of proline (**A**), glycine (**B**) and spermine (**C**) on γ-radiation-dependent methyl acrylate (MA) grafting onto cellulose

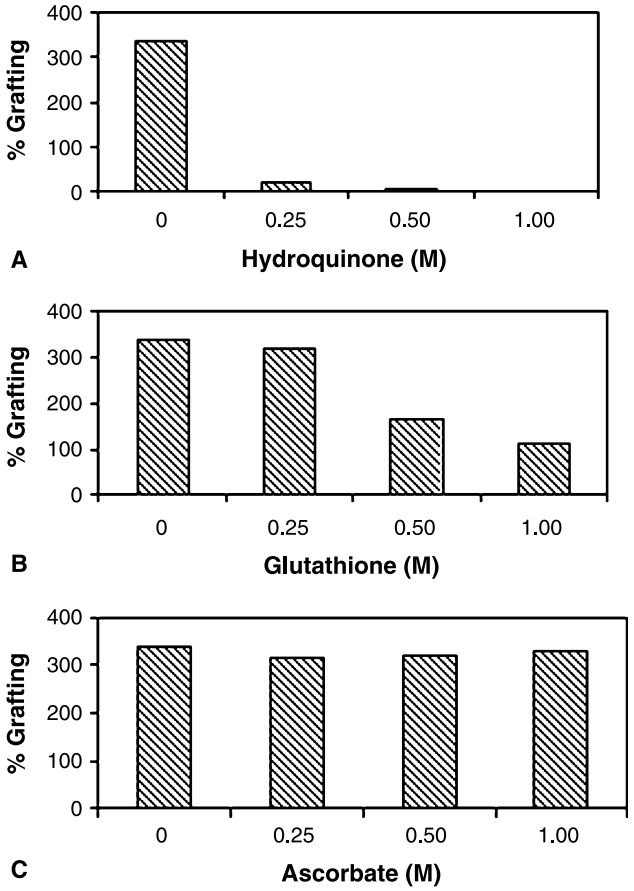


Fig. 4. Effect of hydroquinone (**A**), glutathoine (**B**) and ascorbate (**C**) on γ-radiation-dependent methyl acrylate (MA) grafting onto cellulose

not at all affect the grafting extent (Fig. 3). Of the three antioxidants tested, hydroquinone and glutathione effectively suppressed the grafting of MA onto cellulose. Hydroquinone was most effective causing a total suppression of grafting at 0.50 M (Fig. 4). Glutathione suppressed the process in a concentration-dependent manner leading to a two-thirds suppression of grafting (Fig. 4). In contrast, ascorbic acid did not affect the process (Fig. 4).

Pyrogallol autooxidation assay

Pyrogallol autooxidizes under the assay conditions producing $O_2^{\bullet -}$ and an end product that could be measured spectrophotometrically at 420 nm. Proline, included in the reaction mixture in a range of 5–200 mM, did not inhibit the reaction. There was no marked change in A_{420} until 50 mM proline; at higher concentrations there was, in fact, an increase in A_{420} value (Fig. 5). Glycine, however, marginally suppressed the A_{420} (data not shown).

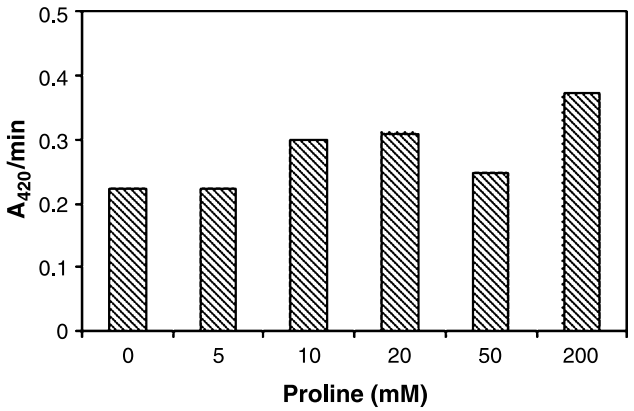


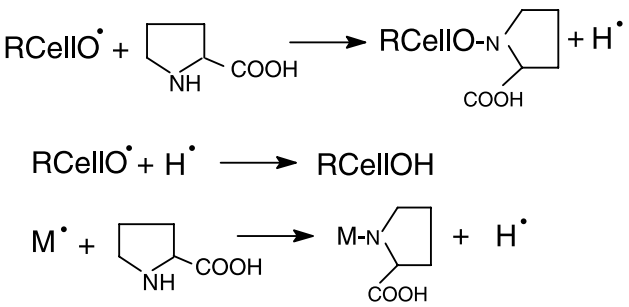
Fig. 5. Effect of proline on autooxidation of pyrogallol (A_{420}/min)

Discussion

The present data provide clear evidence for a strong potential of L-proline as free radical scavenger in an in vitro assay system, namely the graft co-polymerization reaction. The latter involved an essentially free radical mediated grafting of a monomer, methyl acrylate (MA),

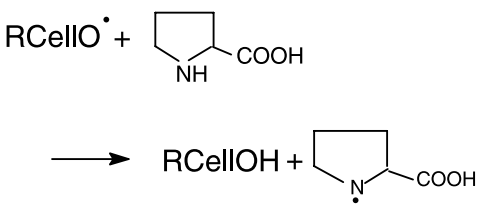
onto the cellulose backbone; the degree of grafting could be taken as a reliable quantitative measure of free radical generation. Ce^{4+} and γ-radiation were employed as reaction initiators which facilitated, via appropriate e^- transfer(s), the transformation of both cellulose and MA into corresponding free radicals and eventually the grafting. In order to enhance the reliability and reproducibility, the assay was optimized with respect to different reactants, reaction conditions and also the post-reaction removal of homopolymer from the graft. A vast difference in the degree of Ce^{4+}- (61%) and γ-radiation- (>300%) induced grafting reflected the relative efficacy of two initiators.

The inclusion of L-proline in the assay, irrespective of reaction initiator, led to a concentration-dependent reduction of the degree of grafting. The reduction of a magnitude of 61 and 93% of γ-radiation-induced grafting by 0.5 and 1.0 M proline respectively is remarkably large and convincing. A possible manner in which proline could interact with the free radicals generated in the reaction killing/scavenging their activity is as follows:



The UV spectral analysis of MA, proline and their mixture with or without γ-irradiation provided a preliminary indication of complex formation between M• and proline (data not shown).

Alternatively, direct abstraction of hydrogen by cellulose or monomeric radical might lead to free radical scavenging as follows:



The proposed mechanisms, in fact, need not be mutually exclusive; they may contribute variably to the overall free radical scavenging that is reflected in the reduced degree of grafting. A similar suppression of Ce^{4+}-induced MA grafting onto cellulose by 5-hydroxytryptophan and 5-hydroxytryptamine has been reported earlier (Misra et al., 1993). Proline has been reported to scavenge a couple of ROS ($OH^•$ and $^1O_2$) in certain in vitro generation/detection systems. Thus, proline was shown to scavenge $OH^•$ radicals generated by ascorbate/H_2O_2 or by xanthine oxidase/hypoxanthine/H_2O_2 and detected either by hydroxylation of salicylate or by denaturation of malate dehydrogenase (Smirnoff and Cumbes, 1989). Proline has been suggested to react with $OH^•$ under H abstraction (Rustgi et al., 1977). Similarly, Alia et al. (2001) demonstrated a proline-dependent quenching of photochemically generated singlet oxygen ($^1O_2$) which could be explained on the basis of the capability of proline to form charge transfer complex due to its low ionization potential.

The present data involving non-ROS free radical species reinforce the free radical scavenging potential of proline; the antioxidative activity of proline, by extension, could be expected in vivo too. In accordance, it is quite likely that the elevated tissue proline levels under stressful growth conditions, among other functions, constitute a component of cellular antioxidative network involved in mitigation of stress effects. Although free radical scavenging in graft co-polymerization assays required rather high proline concentrations (>0.25 M) probably owing to assay conditions employed, in a cellular scenario, similar activity might occur at actual proline concentrations. Proline accumulation of the magnitude of more than 100-times greater than that of normal levels in stressed plants is known (e.g., Aspinall and Paleg, 1981); high millimolar proline concentrations are likely. In fact, an antioxidative role of proline in vivo has been occasionally shown (Smirnoff, 1993; Mehta and Gaur, 1999; Siripornadulsil et al., 2002). However, the precise evaluation of its role in the presence of other cellular antioxidants is awaited. In contrast to the graft co-polymerization assays, proline did not affect the pyrogallol autooxidation that is mediated by $O_2^{•-}$ radicals. Obviously, proline, exhibiting selectivity, did not interact with superoxide radicals possibly due to redox restrictions. Effect of proline on graft co-polymerization was compared with some antioxidants (hydroquinone, glutathione and ascorbate) and other compounds (glycine, spermine). Whereas glutathione and hydroquinone compared well with proline in suppressing the grafting process, ascorbate was altogether ineffective. The reaction also remained unaltered in the presence of glycine and spermine. These observations could be explained in terms of the redox compatibility/restrictions between the free radical species in question and the tested effectors.

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