J Am Oil Chem Soc (2010) 87:1475–1480
DOI 10.1007/s11746-010-1632-8

ORIGINAL PAPER

Antioxidative Properties of Ascorbic Acid and Acyl Ascorbates in ML/W Emulsion

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Received: 10 September 2009 / Revised: 27 May 2010 / Accepted: 14 June 2010 / Published online: 30 June 2010

Abstract Octanoyl, dodecanoyl and hexadecanoyl ascorbates were synthesized by the condensation of ascorbic acid and the corresponding fatty acids in acetone using an immobilized lipase from *Candida antarctica*. The oxidation process of methyl linoleate (ML) as an oil droplet in the ML/W emulsion with ascorbic acid or acyl ascorbate was measured at 40 °C, and their antioxidative properties were examined. Hydrophilic proxidant, AAPH, or lipophilic proxidant, AMVN, was added to the water or oil phase to investigate the properties, and the kinetic parameters for the oxidation expressed by the Weibull equation were evaluated. It was suggested that most of the ascorbic acid molecules in the emulsion would be present in the water phase due to its high hydrophilicity and suppress the AAPH-induced oxidation on the interface between the water and oil phases. Dodecanoyl and hexadecanoyl ascorbates would be dissolved in the oil phase and contribute to the suppression of the oxidation in the oil phase rather than on the interface. Octanoyl ascorbate with a HLB number of 11.8 would be in both phases. Regardless of the presence and type of the ascorbate, the rate constant, k, of the Weibull equation decreased as the pH of the water phase increased.

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Keywords Acyl ascorbate · Ascorbic acid · ML/W emulsion · Oxidation · Rate constant

Introduction

L-Ascorbic acid, known as vitamin C, is a water-soluble vitamin, and is widely used as an additive in foods and cosmetics due to its strong reducing ability. The lipase-catalyzed synthesis of 6-*O*-acyl ascorbates through the condensation of ascorbic and fatty acids in a water-soluble organic solvent has been reported [1–3]. Generally, the enzymatic synthesis of the acyl ascorbates is more advantageous than a chemical method due to the simplicity of its reaction process and its high regioselectivity. Acyl ascorbate is an amphiphilic antioxidant, because it consists of ascorbic and fatty acids as the hydrophilic antioxidant and lipophilic group, respectively. It has been reported that the ascorbates, such as 6-*O*-palmitoyl ascorbate, have an anti-tumor activity and metastasis-inhibitory effects [4, 5]. Therefore, acyl ascorbate would be a promising food additive. We have also synthesized acyl ascorbates using an immobilized lipase in a batch or continuous reactor [6, 7], evaluated their surfactant properties [8] and their antioxidative ability in bulky fatty acid [9], and used them for the microencapsulation of lipids [10]. However, there has been no report about the antioxidative property of acyl ascorbate in the O/W type emulsion, in spite of it being an amphiphilic antioxidant. Knowledge of its behavior in the emulsion is indispensable for its effective use.

In this study, the octanoyl, dodecanoyl and hexadecanoyl ascorbates were synthesized by the condensation of ascorbic and the corresponding fatty acids in acetone using an immobilized lipase from *Candida antarctica*. The oxidation process of methyl linoleate (ML) as an oil droplet in

the ML/W emulsion with ascorbic acid or acyl ascorbate was measured at various pHs, and their antioxidative properties were examined. The hydrophilic proxidant, AAPH, or lipophilic proxidant, AMVN, was added to the water or oil phase to elucidate the antioxidative behaviors of the ascorbates. The oxidation process of ML was expressed by the probabilistic Weibull equation and the kinetic parameters were evaluated.

Experimental Procedures

Materials

The immobilized lipase from *Candida antarctica*, Chirazyme® L-2 cf. C2, was obtained from Roche Molecular Biochemicals, Mannheim, Germany. L-Ascorbic acid was purchased from Nacalai Tesque, Kyoto, Japan. Methyl linoleate (ML; purity >95%) was purchased from Tokyo Chemical Industry, Tokyo, Japan. The hydrophilic surfactant, SY-Glyster® ML-750 (decaglycerol monolaurate), was supplied from Sakamoto Yakuhin Kogyo, Osaka, Japan. The octanoic, dodecanoic, and hexadecanoic acids, 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) as a hydrophilic proxidant, 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN) as a lipophilic proxidant, methyl myristate as an internal standard for the GC analysis, and all other chemicals of analytical grade were purchased from Wako Pure Chemical Industries, Osaka, or Yoneyama Yakuhin Kogyo, Osaka. The disposable membrane filter cartridge, 25CS080AN, which held the cellulose-acetate membrane with a pore diameter of 0.8 μm was purchased from Advantec Toyo, Tokyo.

The acyl ascorbates were synthesized by the condensation of ascorbic acid and octanoic, dodecanoic, or hexadecanoic acid in acetone using Chirazyme® L-2 cf. C2, and purified according to previous methods [3]. The hydrophilic-lipophilic balance (HLB) numbers of octanoyl, dodecanoyl and hexadecanoyl ascorbates, which were evaluated according to the Davies' equation [11], are 11.8, 9.90 and 8.00, respectively.

Preparation of ML/W Emulsion and Measurement of the Extent of ML Oxidation in the Emulsion

Five milliliters of a 1.0% (w/v) SY-Glyster® ML-750 aqueous solution as the water phase and the same volume of ML as the oil phase were added to a tube. For each proxidative experiment, AAPH and AMVN dissolved in the water and oil phases at each concentration of 1% (w/v) were employed, respectively, and then transferred to the tube to produce the emulsion. Ascorbic acid or octanoyl ascorbate was added to distilled water as a water phase at a specific concentration, whereas the dodecanoyl or hexadecanoyl ascorbate was added to the oil phase. For the preparation of the emulsion at different pHs, a hydrochloric acid/sodium acetate buffer was used as the water phase. The mixture was emulsified using a rotor/stator homogenizer (Ultra-Turrax T25, IKA® Japan, Nara, Japan) for 5 min at 1×10^4 rpm in the tube immersed in ice water to produce the coarse ML/W emulsion. After the pre-emulsification, the coarse emulsion was circularly passed through a membrane filter using a peristaltic pump at 2.0 mL/min for 30 min to reduce and monodisperse the diameter of the oil droplets. After the emulsification, unoxidized ML remained 95%. The prepared ML/W emulsion was put into an amber glass vial with a screw-cap and stored in the dark at 40 °C with magnetic stirring at 200 rpm.

Measurement of the Particle Size Distribution of Oil Droplets in ML/W Emulsion

Twenty microliters of the emulsion were periodically removed from the vial to measure the particle size distribution of the oil droplets using a centrifugal particle size analyzer (SA-CP3L, Shimadzu, Kyoto). The sample was diluted 100 to 500 times with distilled water prior to the particle size analysis. The particle size distribution in the emulsion was measured in duplicate, and the mean value was calculated. The ratio of the median diameter of the oil droplet at time, r, to the initial diameter during the preparation of the emulsion, r_0, was estimated as the index of the emulsion stability.

GC Analysis

For measurement of the oxidation process, 300 μL of a mixture of methanol/chloroform (1:2 by vol.) was added to 100 μL of the sample from the emulsion and well mixed by a mixer. The mixture was centrifuged at 1.5×10^4 rpm for 5 min using a centrifugal separator (MX-100, Tomy Seiko, Tokyo), and 50 μL of oil phase was sampled. After evaporation of the methanol and chloroform under reduced pressure in a desiccator, a methyl myristate solution in hexane was added to the sample. The amount of unoxidized ML was determined by a gas chromatograph (G-3500, Hitachi, Tokyo) equipped with a hydrogen flame ionization detector and a capillary column, the dimensions of which were 0.32 mm in diameter and 15 m in length, with polyethylene glycol (Rtx-2330, Restek Japan, Tokyo). The injection or detection temperature was 200 °C, and the column temperature was 180 °C. Nitrogen gas was used as the carrier gas, and the flow rate was 1.8 mL/min. The fraction of unoxidized ML was calculated from the ratio of the peak area of ML to that of methyl myristate. A part of

the amount of unoxidized ML in the emulsion was measured multiple times, and the mean value was calculated.

Statistical Analysis

The effect of the concentration of acyl ascorbates or pH on the rate constant for the oxidation of ML in the emulsion was determined by ANOVA. Significant differences were determined by t tests at $p < 0.05$.

Results and Discussion

Effects of the Concentration and Acyl Chain Length of Acyl Ascorbate on the ML Oxidation in the ML/W Emulsion

A hydrophilic proxidant, AAPH, or lipophilic proxidant, AMVN, was added to the water or oil phase to examine the antioxidative properties of ascorbic acid and acyl ascorbates in the ML/W emulsion. Figures 1, 2 and 3 show the oxidation processes of ML as an oil droplet at 40 °C in an AAPH- or AMVN-containing ML/W emulsion with 1, 10 and 100 μmol/L acyl ascorbates or ascorbic acid, respectively. The median diameter of the oil droplet in each emulsion was constant at about 4.5 μm during the oxidation measurement, indicating that the oxidation processes were evaluated for the stable emulsion.

The oxidation kinetics of ML in the emulsion was empirically expressed by the following Weibull equation,

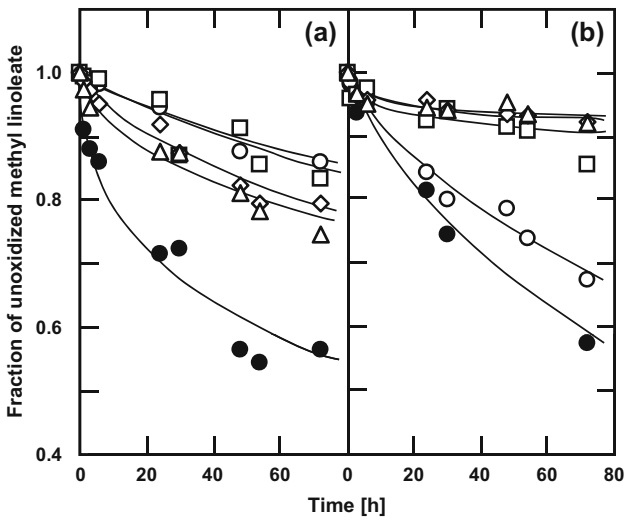

Fig. 1 Oxidative stability of ML as an oil droplet at 40 °C in **a** AAPH- or **b** AMVN-containing emulsion with (*open circles*) ascorbic acid and (*open squares*) octanoyl, (*open diamonds*) dodecanoyl, and (*open triangles*) hexadecanoyl ascorbates at 1 μmol/L, and (*filled circles*) without any additives. The *solid curves* were calculated using the estimated kinetic parameters of the Weibull model

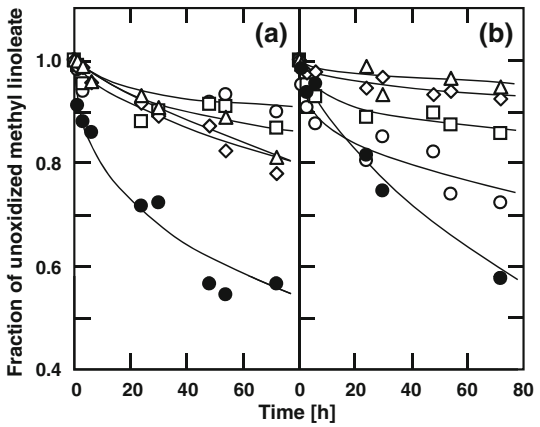

Fig. 2 Oxidative stability of ML as an oil droplet at 40 °C in **a** AAPH- or **b** AMVN-containing emulsion with (*open circles*) ascorbic acid and (*open squares*) octanoyl, (*open diamonds*) dodecanoyl, and (*open triangles*) hexadecanoyl ascorbates at 10 μmol/L, and (*filled circles*) without any additives. The *solid curves* were calculated using the estimated kinetic parameters of the Weibull model

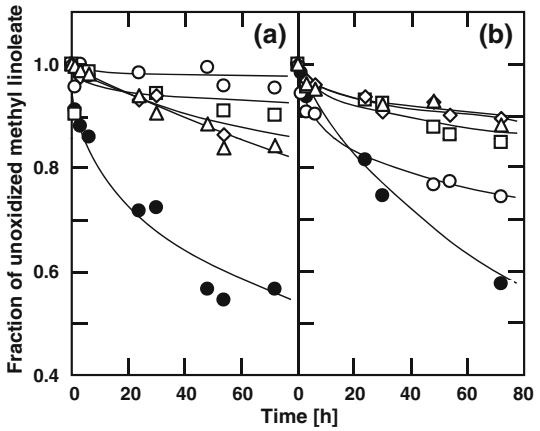

Fig. 3 Oxidative stability of ML as an oil droplet at 40 °C in **a** AAPH- or **b** AMVN-containing emulsion with (*open circles*) ascorbic acid and (*open squares*) octanoyl, (*open diamonds*) dodecanoyl, and (*open triangles*) hexadecanoyl ascorbates at 100 μmol/L, and (*filled circles*) without any additives. The *solid curves* were calculated using the estimated kinetic parameters of the Weibull model

which is flexible and has a potential for describing many deterioration kinetics [12]:

$$Y = \exp[-(kt)^n] \quad (1)$$

where Y is the fraction of unoxidized ML at time t, k is the rate constant, the reverse of which is called the scale parameter, and n is the shape constant. The kinetic parameters, k and n, were calculated by best fitting the experimental results of Solver in Microsoft® Excel 2003, and 95% confidence intervals were determined by t tests. The curves in Figs. 1, 2, 3 were drawn based on the equation using the estimated parameters. Figure 4 shows the relationship between the rate constant of the Weibull model for the oxidation of ML at 40 °C in the AAPH- or AMVN-containing ML/W emulsions

with octanoyl, dodecanoyl and hexadecanoyl ascorbates, and ascorbic acid, and the concentration of the ascorbates. Statistical analysis showed that all the k values for the oxidation with the ascorbates were lower than those without any ascorbates in both the AAPH- and AMVN-containing ML/W emulsions. This suggested that the oxidation in both emulsion systems was suppressed by the ascorbates. In the AAPH-containing O/W emulsion, the k value with ascorbic acid sharply decreased as the concentration of ascorbic acid increased. The k value with octanoyl ascorbate was also in inverse proportion to the concentration of the octanoyl ascorbate, but the extent was lower than that with ascorbic acid. The k value with the dodecanoyl or hexadecanoyl ascorbate was independent of the concentration of the corresponding ascorbate, and all the values were similar to that without any ascorbates. We have reported that there was no difference in the radical scavenging activities of ascorbic acid and acyl ascorbates [7]. Therefore, these results indicate that the highly hydrophilic ascorbates exhibited a high antioxidative activity in the AAPH-containing ML/W emulsion, whereas the ascorbates with a low hydrophilicity only contributed slightly to the suppression of the oxidation. On the other hand, the k value with ascorbic acid in the AMVN-containing emulsion slightly depended on the concentration, though the values at 10 and 100 μmol/L ascorbic acid were nearly equal. The k values for acyl ascorbates in the emulsion increased with the increasing concentrations in contrast to those in the AAPH-containing emulsion. For the AMVN-containing emulsion, the k values were high in the order ascorbic acid > octanoyl > dodecanoyl > hexadecanoyl ascorbate at any concentration. The most hydrophilic ascorbic acid slightly suppressed the oxidation in the AMVN-containing emulsion, and acyl ascorbate with a longer acyl chain showed a higher suppressive effect on the oxidation. The effect of acyl ascorbate, however, decreased as the concentration increased. Niki et al. [13] and Yi et al. [14] reported that ascorbic acid was effective on scavenging radicals in emulsion prepared using phosphatidylcholine. Based on these results, most of the ascorbic acid molecules in the emulsion would be present in the water phase due to its high hydrophilicity. Therefore, ascorbic acid seemed to suppress the oxidation induced by AAPH on the interface between the oil and water phases and slightly acted on the AMVN-induced oxidation in the oil phase. Frankel et al. [15] explained the differences in the efficiency of ascorbic acid and hexadecanoyl ascorbate in preventing lipid oxidation in bulk and O/W emulsion systems by their differences in the affinity toward the air-oil interfaces in bulk oil and the oil–water interfaces in emulsion. Thus, the dodecanoyl and hexadecanoyl ascorbates would be dissolved in the oil phase and contribute to the oxidation suppression in the oil phase rather than on the interface. It is known that an antioxidant exhibits a proxidative activity at the substrate high concentration in the oxidation [16]. The oxidation of ML seemed to be partially accelerated by many acyl ascorbate molecules dissolved in the oil phase, resulting in the proxidative effect. Octanoyl ascorbate having hydrophilic-lipophilic balance of 11.8 would exist in both phases and show the anti- and proxidative abilities.

In the AAPH-containing emulsion, the n values for the oxidation were 0.47 for no ascorbates, and 0.75, 0.28 and 0.17 for the 1, 10 and 100 μmol/L ascorbic acids, respectively. The values with acyl ascorbates were 0.84, 0.50 and 0.25 for octanoyl ascorbate, 0.67, 0.67 and 0.66 for dodecanoyl ascorbate, and 0.53, 0.89 and 0.87 for hexadecanoyl ascorbate at 1, 10 and 100 μmol/L, respectively. In the AMVN-containing emulsion, the n values were 0.78 for no ascorbates, and 0.78, 0.38 and 0.38 for 1, 10 and 100 μmol/L ascorbic acid. The values with the acyl ascorbates were 0.32, 0.31 and 0.40 for octanoyl ascorbate, 0.23, 0.37 and 0.39 for dodecanoyl ascorbate, and 0.21, 0.41 and 0.31 for hexadecanoyl ascorbate at 1, 10 and 100 μmol/L, respectively. No relationship between the n values was found, but all the values were less than unity. This indicated that the oxidation of ML in each emulsion system with ascorbic acid or the acyl ascorbates progressed without an induction period.

Comparison of the Antioxidative Property Between Ascorbic Acid and Acyl Ascorbate in the ML/W Emulsion at Various pHs

Figure 5 shows the oxidation processes of ML in the AAPH-containing O/W emulsion at 40 °C and pH 3, 4 or 5 with 100 μmol/L ascorbic acid and hexadecanoyl ascorbate,

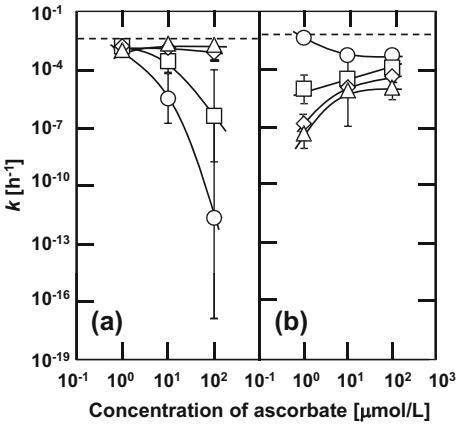

Fig. 4 Relationship between the rate constant, k, of the Weibull model for the ML oxidation at 40 °C in **a** AAPH- or **b** AMVN-containing emulsion with (*open circles*) ascorbic acid and (*open squares*) octanoyl, (*open diamonds*) dodecanoyl, and (*open triangles*) hexadecanoyl ascorbates, and the concentration of the ascorbates. The *dashed lines* represent the k values for the oxidation without any ascorbates. The *bars* show 95% confidence intervals. The *solid curves* were empirically drawn

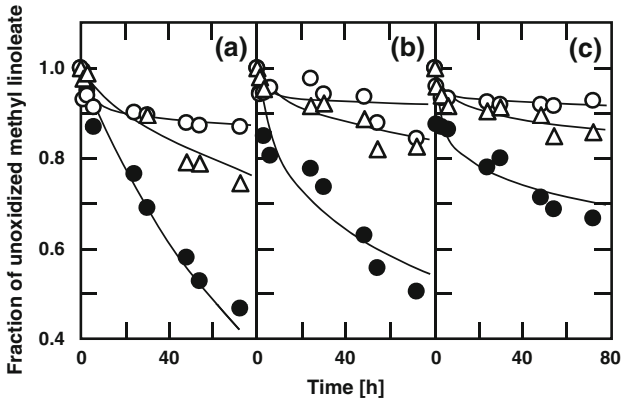

Fig. 5 Oxidation processes of ML in AAPH-containing emulsion at 40 °C and **a** pH 3, **b** pH 4, and **c** pH 5 with (*open circles*) ascorbic acid and (*open triangles*) hexadecanoyl ascorbate at 100 μmol/L, and (*filled circles*) without any additives. The *solid curves* were calculated using the estimated kinetic parameters of the Weibull model

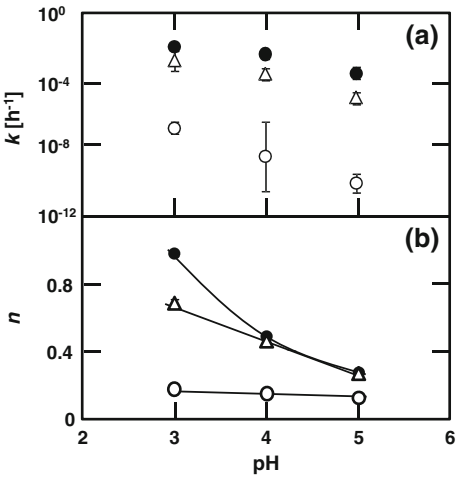

Fig. 6 The pH-dependencies of **a** the rate constant, *k*, and **b** the shape constant, *n*, for the oxidation of ML in AAPH-containing emulsion with (*open circles*) ascorbic acid and (*open triangles*) hexadecanoyl ascorbate at 100 μmol/L, and (*filled circles*) without any additives. The *bars* represent 95% confidence intervals. The *solid curves* were empirically drawn

and without any ascorbates. The oxidation kinetics was expressed by the Weibull equation, and the kinetic parameters, *k* and *n*, were calculated. The curves in Fig. 5 were drawn based on the equation using the estimated parameters. The pH-dependencies of the estimated kinetic parameters are shown in Fig. 6. In every case, the *k* values decreased as the pH of the water phase increased. Furthermore, the AVOVA confirmed that the *k* value for the oxidation in the presence of ascorbic acid was the lowest while that in the absence of any ascorbates was the highest at each pH. The oxidation induced by AAPH in the water phase at the tested pHs was strongly suppressed by ascorbic acid, while hexadecanoyl ascorbate exhibited a low antioxidative

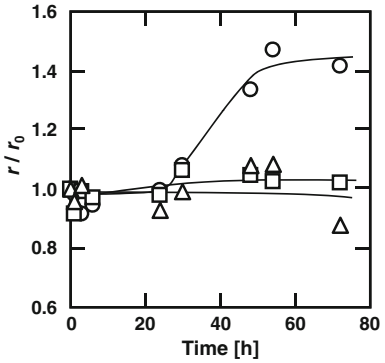

Fig. 7 Stability of AAPH-containing ML/W emulsion without any ascorbates at 40 °C and (*open circles*) pH 3, (*open squares*) pH 4, and (*open triangles*) pH 5. The *r* and r_0 represent the median diameter of the oil droplet at specific times and that at the preparation of the emulsion, respectively. The *solid curves* were empirically drawn

activity. These results would imply the behaviors of the ascorbates as mentioned above, that is, most of the molecules of ascorbic acid and hexadecanoyl ascorbate would be present in the water and oil phases, respectively. Figure 7 shows the stability of the AAPH-containing O/W emulsion without any ascorbates at 40 °C and pHs 3, 4 and 5. The emulsions at pHs 4 and 5 were relatively stable, whereas the median diameter of the oil droplet at pH 3 significantly increased with time. Thus, the pH-dependency of the oxidative stability of ML in the emulsion without any ascorbates seemed to be due to the low stability of the emulsion through flocculation and coalescence of the oil droplets at low pH [17]. A similar tendency was also observed for the oxidation with the ascorbates. However, the *k* value with ascorbic acid more sharply decreased at a high pH than those with hexadecanoyl ascorbate and without any ascorbates. The enhancement of the electron-donating ability of ascorbic acid in the water phase through the progress of deprotonation of the endiol group in the acid molecules due to increasing the pH may affect the oxidation [18]. Hexadecanoyl ascorbate in the oil phase would not show the same behavior. The *n* values with hexadecanoyl ascorbate and without any ascorbates strongly depended on the pH. In contrast, the *n* value with ascorbic acid was almost constant, indicating that the influence of pH was relatively low because of the highly suppressive effect on the oxidation.

Acknowledgments This work was supported by KAKENHI 19780106 for a Grant-in-Aid for Young Scientists (B), MEXT, Japan, and a grant from the Cosmetology Research Foundation, Japan.

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