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Uptake of L-Ascorbate by Intact Spinach Chloroplasts

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ABSTRACT

Uptake of L-[1-^{14}C]ascorbate by intact ascorbate-free spinach (*Spinacia oleracea* L. cv Vital^r) chloroplasts has been investigated using the technique of silicone oil filtering. Rates greater than 100 micromoles per milligram chlorophyll per hour (external concentration, 10 millimolar) of ascorbate transport were observed. Ascorbate uptake into the sorbitol-impermeable space (stroma) followed the Michaelis-Menten-type characteristic for substrate saturation. A K_m of 18 to 40 millimolar was determined. Transport of ascorbate across the chloroplast envelope resulted in an equilibrium of the ascorbate concentrations between stroma and medium. A pH optimum of 7.0 to 7.5 and the lack of alkalization of the medium upon ascorbate uptake suggest that only the monovalent ascorbate anion is able to cross the chloroplast envelope. The activation energy of ascorbate uptake was determined to be 65.8 kilojoules (16 kilocalories) per mole (8 to 20°C). Interference of ascorbate transport with substrates of the phosphate or dicarboxylate translocator could not be detected, but didehydroascorbate was a competitive inhibitor. Preloading of chloroplasts with didehydroascorbate resulted in an increase of V_{max} but did not change the K_m for ascorbate. Millimolar concentrations of the sulfhydryl reagent *p*-chloromercuriphenyl sulfonate inhibited ascorbate uptake. The data are interpreted in terms of ascorbate uptake into chloroplasts by the mechanism of facilitated diffusion mediated by a specific translocator.

Considerable amounts of L-ascorbic acid are found in algae (10, 17, 33) and in fruits, storage organs, and leaves of higher plants (26). Inasmuch as 30 to 40% of the ascorbate content of spinach leaf protoplasts was found to be associated with the chloroplast fraction (8), a considerable portion of the ascorbate of green leaves appears to be localized within the chloroplasts. On this basis, a stromal concentration of 15 mM can be calculated but concentrations up to 50 mM have been reported (10). Walker (35), when citing earlier literature, ascribed a Chl to ascorbate ratio of 1:1 (w/w) to chloroplasts of field-grown summer leaves which corresponds to a concentration of even 75 mM. Chloroplastic ascorbate may be involved in a variety of physiological processes (for reviews, see 9, 26), the most important of which appears to be the detoxification of photosynthetically reduced oxygen or of its dismutation product H_2O_2 (13). H_2O_2 at a concentration of only 10^{-5} M already inhibits photosynthetic CO_2 fixation by 50% (22) due to an interaction with the light activation system of Calvin cycle enzymes (23). Although the rates of H_2O_2 formation by CO_2-fixing chloroplasts usually do not exceed 1 μmol·mg $Chl^{-1}\cdot h^{-1}$ (31, 34), such a rate results in the attainment of the above-mentioned concentration of 10^{-5} M within 1 s (on the basis of a stroma volume of 30 μl/mg Chl [15]). Therefore, a powerful system to scavenge H_2O_2 at the site of its origin is a prerequisite of photosynthesis. Ascorbate-specific peroxidases and a peroxidative factor have been revealed in chloroplasts (11, 18, 27). The product of the peroxidase reaction, didehydroascorbate, may be re-reduced from NADPH either directly (24) or via NADPH-dependent glutathione reductase and glutathione-dependent dehydroascorbate reductase (13). The former enzyme has been demonstrated in chloroplasts (6), whereas the latter may be localized predominantly in the cytoplasm (7). However, GSH- and light-dependent reduction of dehydroascorbate has also been observed with chloroplasts (27). With respect to the K_m for ascorbate of the various peroxidative systems mentioned above (0.12–3 mM), the high chloroplastic concentrations of ascorbate having been found are sufficient for the reduction of H_2O_2. However, the provenance of ascorbate in the chloroplast is completely unclear. Although the biosynthesis of L-ascorbic acid in plants has not yet been completely elucidated, it is well established that it originates from D-glucose. In contrast to the biosynthetic route in animals or algae (17), ascorbate biosynthesis in higher plants proceeds via oxidation at C-1, epimerization at C-5, and a second oxidation at C-2 or C-3 (12, 16; for earlier references, see 9). The enzymes catalyzing this reaction sequence have not yet been localized in the cell. However, in that ripening strawberries (for reference, see 9) and potato tubers (21) are good systems for the study of ascorbate biosynthesis, this process should not occur in chloroplasts. If ascorbic acid originates outside the chloroplast, this organelle must be capable of reasonable rates of uptake of exogenous ascorbate. Inasmuch as ascorbic acid is present as a monovalent anion at physiological pH, uptake into the stroma solely by diffusion is unlikely. Evidence for a catalyzed ascorbate uptake by intact chloroplasts is shown in the present study.

MATERIALS AND METHODS

Preparation of Chloroplasts. Spinach (*Spinacia oleracea* L. cv Vital^r) leaves were homogenized with an Ultraturrax for 8 s in $Na_4P_2O_7$ buffer (10 mM, pH 6.6) containing 330 mM sorbitol, 5 mM $MgCl_2$ (4), and 4 mM sodium ascorbate. The brei was filtered through eight layers of gauze and one layer of Miracloth and the chloroplasts were spun down at 2000*g* (1 min). The pellet was suspended in buffer B described by Jensen and Bassham (19), however without ascorbate. Cells and cell debris were removed by centrifugation at 25*g* (two times, each 1 min). From the supernatant, the chloroplasts were spun down at 500*g*, resuspended in the same buffer, and washed once. All steps were performed at 0°C. The chloroplasts used were more than 80% intact (14) and did not contain detectable amounts of ascorbate as examined with the ascorbate oxidase assay. Chl was estimated according to Arnon (1). The volume of the packed chloroplasts was determined in hematocrit capillaries with a Heraeus-Christ Mikro-Hematocrit centrifuge. Chloroplasts of a volume of 25 to 70 μl (uncorrected for sorbitol-permeable space) per mg Chl were used.

L-Ascorbate Uptake. To study the uptake of ascorbate, the silicone oil-filtering centrifugation technique was employed. Unless otherwise noted, the experiments were performed in the dark. The ascorbate-free chloroplasts were transferred to a layer of 400 μl of ascorbate free Hepes buffer (pH 7.6, as described by

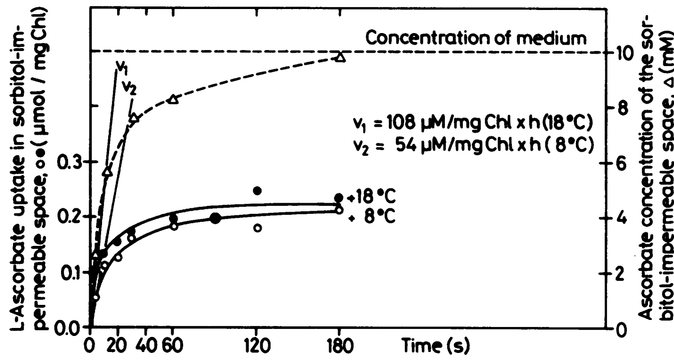

FIG. 1. Uptake of L-ascorbate (10 mM external concentration) into the sorbitol-impermeable space of intact chloroplasts at two different temperatures in the dark. The ascorbate concentration of the stroma was calculated from the experiment performed at 18°C.

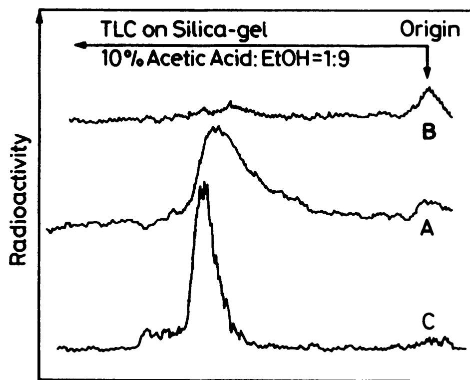

FIG. 2. Radioautograms of chloroplast extracts after 1 min (A) and 25 min (B) uptake of L-[1-^{14}C]ascorbate into isolated intact chloroplasts in the light. (C), Authentic L-[1-^{14}C]ascorbate.

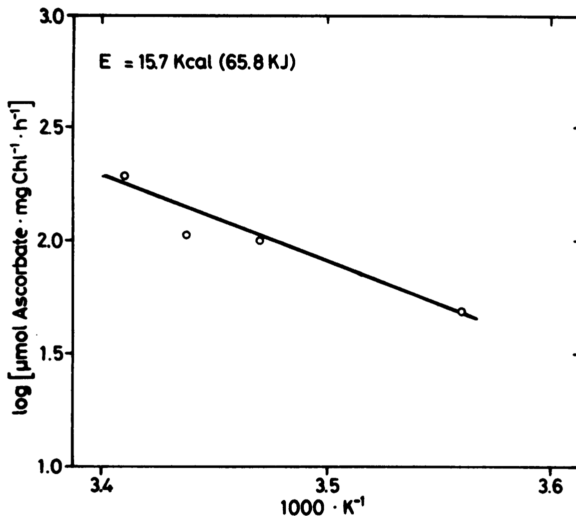

FIG. 3. Temperature dependence of the rates of ascorbate uptake into the sorbitol-impermeable space of intact chloroplasts. The external concentration was 10 mM.

Jensen and Bassham [19]), containing 7 mM $NaHCO_3$ on top of 1 ml silicone oil in a 1.5-ml polypropylene microtube (Eppendorf, Hamburg). The silicone oil was a 1:1 (v/v) mixture of AR 100 and AR 200 (Wacker Chemie Munich). Prior to the addition of ascorbate or other substrates, the chloroplasts were preincubated for 5 min to achieve temperature and pH equilibration. Uptake of ascorbate was started by adding a small volume of the

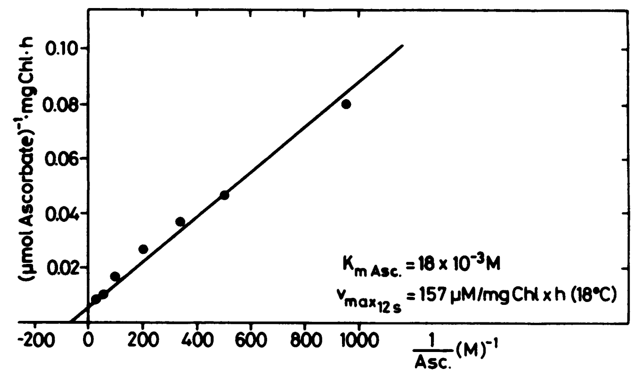

FIG. 4. Concentration dependence of ascorbate transport into the chloroplast stroma. The period of uptake was 12 s (18°C).

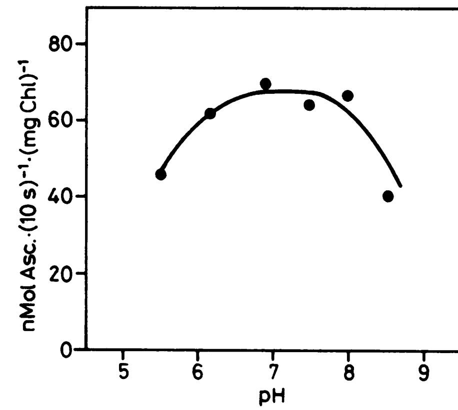

FIG. 5. pH dependence of ascorbate uptake into the chloroplast stroma (10 mM external concentration; 18°C).

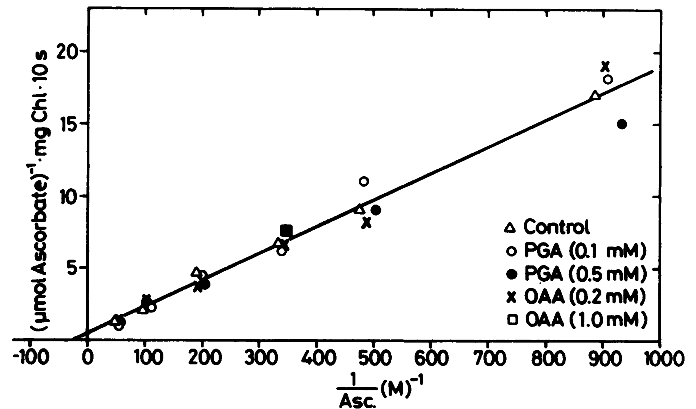

FIG. 6. Concentration dependence of ascorbate uptake into the chloroplast stroma in the presence of 3-phosphoglycerate and oxaloacetate at different concentrations (18°C).

same buffer containing L-[1-^{14}C]ascorbate (Amersham) to the chloroplast suspension which was gently stirred with an air stream. The experiment was terminated by rapid centrifugation in a swing-out bucket rotor (Runne centrifuge model 65) by means of which the chloroplasts were separated from the medium and concentrated as packed organelles in the tip of the microtube. Subsequent to careful removal of the supernatant (from which the specific radioactivity of the substrate was determined) and of the silicone oil, the packed chloroplasts were ruptured by the addition of 130 μl of ice-cold twice-distilled H_2O. From the radioactivity of the resulting suspension, the amount of ascorbate in the chloroplast fraction was determined. From another ali-

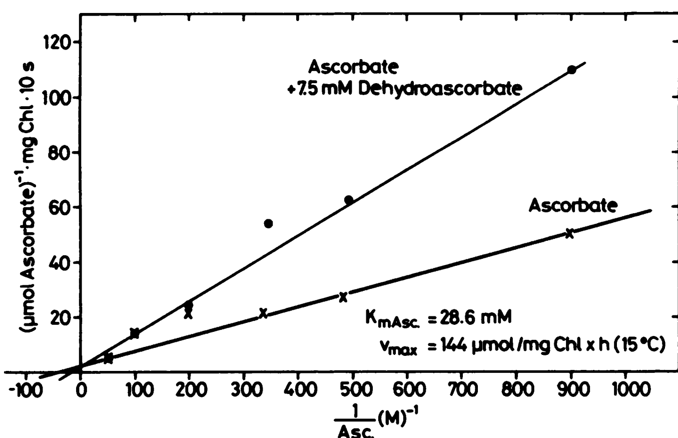

FIG. 7. Concentration dependence of ascorbate transport and inhibition by simultaneously administered didehydroascorbate (15°C).

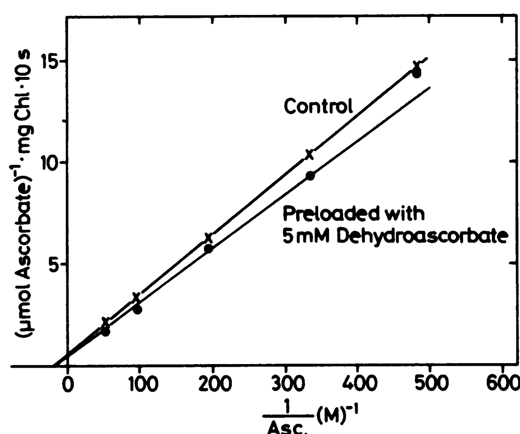

FIG. 8. The effect of preloading of intact chloroplasts with 5 mM didehydroascorbate on ascorbate uptake. Didehydroascorbate was added immediately after preparation of the chloroplasts. Prior to the addition of [^{14}C]ascorbate, chloroplasts were adapted to 15°C for 5 min.

quot, Chl was extracted with methanol and measured as described above. Finally the proportion of the sorbitol-permeable space was calculated (for each sample) from the sorbitol content which was determined with sorbitol dehydrogenase. Uptake of ascorbate into the sorbitol-impermeable space of the chloroplasts was calculated as follows.

$$\begin{aligned}\text{Uptake} = {} & \text{ascorbate } (\mu\text{mol}) \text{ in chloroplasts} \\ & - (\text{vol of sorbitol-permeable space } [\mu l] \\ & \times \text{concn. of ascorbate in the medium})/\text{Chl } (\mu g)\end{aligned} \quad (1)$$

Enzyme Assays. *Ascorbate.* For controls, ascorbate was assayed using ascorbate oxidase (Boehringer Mannheim) and measuring the O_2 consumption with an O_2 electrode. The measurements were conducted at 25°C in 1.5 ml 0.1 M phosphate buffer (pH 5.6) containing 5 units ascorbate oxidase.

Sorbitol. Sorbitol dehydrogenase (Boehringer Mannheim) was diluted with a 1% BSA solution to yield a concentration of 0.4 unit/ml. The assay mixture contained in a total volume of 1.2 ml triethanolamine buffer (pH 7.6, 0.1 M), NAD (15 mM), and an aliquot of the sample. The reaction was started with 50 milliunits of sorbitol dehydrogenase. The rates of NAD reduction were followed at 340 nm and the initial rates over the first 10 min were used as a measure of sorbitol. The calibration curve was linear up to 120 nmol sorbitol.

TLC of Ascorbate and Dehydroascorbate. An aliquot volume of the chloroplast extract was routinely subjected to TLC on silica gel $60F_{254}$ (Merck, Darmstadt) using 10% acetic acid:ethanol, 1:9 (v/v) as a solvent (3). After spraying with *O*-phenylenediamine reagent (3), both ascorbate and dehydroascorbate could be detected with UV light.

RESULTS

Time Course and Temperature Dependency of L-Ascorbate Uptake. Figure 1 shows that L-ascorbate is rapidly taken up into the sorbitol-impermeable space of ascorbate-free chloroplasts (which should correspond to the space surrounded by the inner envelope membrane [36]) from a medium containing 10 mM L-ascorbate. Similar time courses were observed with smaller and higher external concentrations of the ion (results not shown). After an uptake period of 1 to 2 min, the compound inside the plastids was shown by TLC to be unchanged L-ascorbate (Fig. 2A). Ascorbate uptake into the sorbitol-impermeable space is temperature dependent. The Arrhenius plot of the data (Fig. 3) suggests linearity between 8 and 20°C and indicates an activation energy of about 16 kcal/mol (65.8 kJ/mol) corresponding to a Q_{10} of 2.6. Ascorbate was not accumulated in the sorbitol-impermeable space. When the amount of transported ascorbate was related to the volume of the stroma only (which is 7/8 of the sorbitol-impermeable space [15]), a concentration of ascorbate similar to that of the medium was achieved after 3 min (Fig. 1). At that time, the rate of net uptake was zero. No difference in the rate of uptake of L-ascorbate could be detected when the chloroplasts were illuminated or kept in darkness (data not shown). However, after a prolonged incubation in the light, the ascorbate in the chloroplast fraction was found to have been subjected to oxidation to dehydroascorbate which further polymerized to compounds of low mobility in TLC (Fig. 2B). Because such products are not in an equilibrium with ascorbate, radioactivity continuously accumulated in the chloroplasts during illumination.

Kinetic Parameters and pH Dependency. Uptake of ascorbate into the sorbitol-impermeable space followed a Michaelis-Menten saturation kinetics. This is evidenced by the straight line in the Lineweaver-Burk plot (Fig. 4) which furthermore suggests a negligible contribution of free diffusion to the uptake process. A K_m of 18 to 40 mM was calculated from a series of eight experiments and a V_{max} of 160 μmol·mg Chl^{-1} was determined from a Lineweaver-Burk plot of 12-s uptake experiments at 18°C. The rate of ascorbate uptake was decreased at a pH lower than 6 and higher than 8 (Fig. 5). The lower values under acidic conditions indicate that ascorbic acid is transported as an anion, the proportion of which is already remarkably reduced at a pH of 5 (87% ascorbate anion; 13% undissociated ascorbic acid according to a pK_1 of 4.17). No net proton uptake could be observed upon addition of 1 to 20 mM ascorbate (pH 7.8) to a suspension of intact chloroplasts. This observation precludes the possibility that ascorbate is taken up in the protonated, *i.e.* uncharged form. The inhibitory effect of a high pH, on the other hand, can be explained by the rapid oxidation of ascorbate combined with a second deprotonation step of the en-diol (pK_2 = 11.57) in an alkaline milieu.

Interaction of Ascorbate Uptake with Other Translocators of the Chloroplast Envelope. A competitive inhibition by Pi, PGA[1], or a dicarboxylic acid is to be expected if ascorbate uptake is accomplished by the known P-translocator (5) or the dicarboxylic translocator (25). Inasmuch as the recently described glycerate transporter (30) is also accessible to PGA, an inhibitory effect of this compound on ascorbate uptake would not allow discrimination between an interaction with the P- or the glycerate translocator. But ascorbate uptake was not at all affected by high or low concentrations of PGA or of oxaloacetate (Fig. 6). The same lack of interference was noted when the chloroplasts were pre-

[1] Abbreviations: PGA, 3-phosphoglycerate; OAA, oxaloacetate.

loaded with PGA and OAA (data not shown). An increase in the velocity of ascorbate uptake by counterexchange with these compounds must be expected if ascorbate is transported by the known phosphate or dicarboxylate translocators. In addition, the independence of the ascorbate uptake of light provides another argument against the involvement of the glycerate transporter (30).

Inhibition of L-Ascorbate Uptake. Uptake of L-ascorbate by intact chloroplasts was competitively inhibited by its oxidation product didehydroascorbate (Fig. 7). This observation suggests that the uptake is accomplished by a translocator which recognizes both compounds as substrates. This conclusion was corroborated by an experiment in which chloroplasts were preloaded with didehydroascorbate (5 mM) before L-[1-^{14}C]ascorbate was administered. In this case, preloading resulted in an increase of the velocity of ascorbate uptake (Fig. 8), suggesting a counterexchange of both compounds by the same translocator. The rate of L-ascorbate uptake decreased considerably when chloroplasts were stored at room temperature. After 10 and 15 min at 20°C, the rates were 66 and 27%, respectively, of the initial rate found after adaption of the chloroplasts to room temperature. However, this inhibition may be a matter of discussion because the sorbitol- and sucrose-permeable spaces of the packed chloroplasts appeared to increase during storage of the chloroplast preparation at room temperature whereas the permeability of the chloroplast envelope for ferricyanide did not change significantly. If the increase of the extra stromal (sorbitol-permeable) space is only apparent and caused by a slow penetration of sorbitol or sucrose into the stroma, Equation 1 cannot be applied and in turn an inhibition of ascorbate uptake would not be substantiated. For that reason, inhibitory chemicals which need a prolonged preincubation at room temperature, *e.g.* *N*-ethylmaleimide, could not be applied. Inhibition of ascorbate uptake with the sulfhydryl reagent *p*-chloromercuriphenyl sulfonate was significant only at relative high concentrations: an inhibitor concentration of 10 mM resulted in a decrease of 40% and 30 mM was necessary to reduce ascorbate uptake by 60 to 65% (15°C; external ascorbate concentration, 10 mM).

DISCUSSION

The saturation kinetics (Fig. 4) and attainment of high rates of ascorbate uptake by intact chloroplasts together with the competitive inhibition by didehydroascorbate (Fig. 7), the pH optimum (Fig. 5), and the inhibition by the SH-reagent *p*-chloromercuriphenyl sulfonate indicate an uptake mechanism of facilitated diffusion, *i.e.* by a translocator system. This is in contrast to ascorbate transport into liver mitochondria which takes place at a rate of 0.02 μmol·mg protein^{-1}·h^{-1} and is accomplished by pure diffusion (20). Since ascorbate is not accumulated in the chloroplast, uptake does not require additional energy and therefore is independent of illumination or darkening. Like the glucose transporter (32), the ascorbate translocator mediates only the concentration equilibrium of ascorbate between both sides of the chloroplast envelope. This observation also renders evidence that ascorbate had really entered the chloroplast and was not merely trapped by electrostatic interactions with the membrane. The pH optimum as well as the lack of an alkalization of the medium upon ascorbate uptake by the chloroplasts indicate that this compound is transported strictly as an anion. The lack of interference with substrates of the known chloroplastic translocators (Fig. 6) for anionic metabolites (5, 25, 30) and the counterexchange with didehydroascorbate (Fig. 8) suggest specificity of the ascorbate-transporting system. The range of the K_m for ascorbate (18–40 mM) and the activation energy (65.8 kJ) of the system between 8 and 20°C is similar to those for glucose transporters of chloroplasts (32) and liver cells (2). From the observation that ascorbate was readily oxidized by H_2O_2 with ruptured, but not at all with intact, chloroplasts, Nakano and Asada (27) concluded that this compound cannot cross the chloroplast envelope. This finding is in contrast to what other authors observed either directly (34) or via a stimulatory effect of ascorbate on CO_2 fixation (28) by intact chloroplasts. The physiological role of the chloroplastic ascorbate translocator appears to consist of the maintenance of a sufficient ascorbate concentration in the stroma to allow for immediate detoxification of photosynthetically reduced oxygen and its following products. Its function, *in vivo*, may be of particular importance during the induction phase of photosynthesis where O_2 is reduced at high rates (29) and re-reduction of the peroxidatively generated dehydroascorbate cannot keep pace with its formation.

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