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CHANGES IN THYLAKOID STRUCTURE ASSOCIATED WITH THE DIFFERENTIATION OF HETEROCYSTS IN THE CYANOBACTERIUM, *ANABAENA CYLINDRICA*

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Summary

The thylakoids of vegetative cells of the filamentous cyanobacterium, *Anabaena cylindrica*, are capable of oxygen-evolving photosynthesis and contain both Photosystems I and II (PSI and PSII). The heterocysts, cells specialized for nitrogen fixation, do not produce oxygen and lack Photosystem II activity, the major accessory pigments, and perhaps the chlorophyll *a* associated with PSII. Freeze-fracture replicas of vegetative cells and of heterocysts reveal differences in the structure of the thylakoids. A histogram of particle sizes on the exoplasmic fracture face (E-face, EF) of vegetative cell thylakoids has two major peaks, at 75 and 100 Å. The corresponding histogram for heterocyst thylakoids lacks the 100 Å size class, but has a very large peak at about 55 Å with a shoulder at 75 Å. Histograms of protoplasmic fracture face (P-face, PF) particle diameters show single broad peaks, the mean diameter being 71 Å for vegetative cells and 64 Å for heterocysts. The thylakoids of both cell types have about 5600 particles/μm^2 on the P-face. On the E-face, the density drops from 939 particles/μm^2 on vegetative cell thylakoids to 715 particles/μm^2 on heterocyst thylakoids. The data suggest that the 100 Å E-face particle of vegetative cell thylakoids is a PSII complex. The 55 Å EF particle of heterocysts may be part of the nitrogenase complex or a remnant of the PSII complex. The role of the 75 Å EF particle is unknown. Other functions localized on cyanobacterial thylakoids, such as respiration and hydrogenase activity, must be considered when interpreting the structure of these complex thylakoids.

Introduction

The filamentous cyanobacterium, *Anabaena cylindrica*, represents a useful system for the correlation of thylakoid functions with structural features. The thylakoids of the vegetative cells of this and other cyanobacteria are capable of oxygen-evolving photosynthesis. Reactions and spectra attributable to both photosystems are localized there [1—7]. The accessory pigments are complexed with special proteins, the phycobiliproteins, which in turn form larger aggregates, the phycobilisomes, that are bound to the thylakoid surface [8]. *A. cylindrica* is one of several cyanobacteria that produce two types of cells, normal vegetative cells and heterocysts. The latter develop from vegetative cells and function as the sites of nitrogen fixation [9—15]. The differentiation process involves the development of a thickened, lipid-rich cell wall [16,17], changes in the composition of the plasma membrane [18], and, of particular interest to us, a loss of Photosystem II (PSII) activity in heterocysts [12,15, 19,20]. Many of these subjects are discussed in a recent review on heterocysts [21]. By using the freeze-fracture technique to compare the thylakoids of vegetative cells with those of heterocysts, we sought to determine whether a particular morphological difference could be correlated to the absence of PSII activity in heterocysts. Several authors have previously described the ultrastructural changes associated with heterocyst differentiation as revealed by electron microscopy of thin sections [22—27].

Since introduction of the freeze-fracture technique in the early sixties, a number of micrographs of freeze-fractured cyanobacterial thylakoids have appeared in the literature. Jost [28] produced micrographs which showed that particles of different sizes were present on the E-face on the thylakoids of *Oscillatoria rubescens*. Lefort-Tran et al. [29] examined the thylakoids of *Aphanocapsa* sp. 6308. The EF particles ranged from 100 to 140 Å in diameter and occurred in rows. The PF particles had diameters of 60—140 Å. The authors presented a model suggesting that rows of phycobilisomes are aligned with rows of EF particles. Lichtlé and Thomas [30] studied several species of *Oscillatoria* in which both phycobilisomes EF particles occurred in rows. The spacing of the rows of phycobilisomes was found to be the same as the spacing of rows of EF particles. This evidence, taken with the results of Tel-Or and Malkin [31] showing that most light energy absorbed by the phycobiliproteins is transferred to PSII, suggests that certain EF particles make contact with phycobilisomes and represent PSII complexes. Our results provide strong support for this hypothesis and suggest new approaches for further experiments designed to identify the functions of the structural components of cyanobacterial thylakoids.

Methods

Anabaena cylindrica Lemm. was obtained from the Indiana University Culture Collection of Algae (now located at the University of Texas). The strain was maintained on agar plates prepared according to Arnon et al. [5]. Batch liquid cultures were grown in the nitrogen-free medium of Allen and Arnon [32] modified slightly as described previously [18].

Heterocysts were isolated following digestion of the walls of vegetative cells with lysozyme, as described by Fay and Lang [24]. The filaments were harvested by centrifugation at 2000 × *g* for 10 min, washed twice in distilled water and resuspended in a 1% (v/v) solution of glusalase (Endo Labs, Garden City, NY) to aid in the removal of mucilage. Filaments were washed once more in distilled water, then in buffer containing 0.5 M mannitol, 20 mM Na_2 EDTA and 1 mM Tris at pH 7.6. Filaments were then resuspended in buffer containing 0.1% (w/v) lysozyme (Sigma) and incubated at 37°C for 1 h with gentle shaking. The partially lysed filaments were then centrifuged as above and resuspended in cold buffer. The suspension was passed twice through a French press at 1500 lb/inch^2. DNAase was added to a concentration of 33 μg/ml after the first pass. Heterocysts were spun down at 380 × *g* for 5 min. Repeated washes in buffer followed by centrifugation at 380 × *g* were required to obtain a preparation free of vegetative cell fragments.

Samples were prepared for freeze-fracturing by slowly adding an equal volume of 70% glycerol to a suspension of filaments or isolated heterocysts over 1 h with continuous stirring. The glycerinated cells were centrifuged at 20 000 × *g* for 30 min at 4°C. Samples of the pellets were placed on copper support discs, frozen in liquid Freon 12, and stored under liquid nitrogen. Replicas were prepared on a Balzers BA 360 freeze-etch unit and examined in either a Jeol EM 100 or a Phillips EM 200. The micrographs are mounted so that the direction of shadow is from bottom to top. The terminology of Branton et al. [33] is used for designating the fracture faces. Biological membranes consist of a protoplasmic (P) leaflet and an exoplasmic (E) leaflet. The process of freeze-fracturing splits membranes along their hydrophobic interior revealing two complementary fracture faces designated PF for P-face and EF for E-face. Each fracture face has a population of particles which represent intramembrane proteins or protein complexes. Particle size and density determinations were made as described by Staehelin [34].

Results

All cultures of *A. cylindrica* used in this study were grown in the absence of fixed nitrogen. Freeze-fracture replicas of samples composed of intact filaments therefore revealed both vegetative cells and heterocysts. The latter are easily recognized by their thickened, multilayered cell wall and small septa (compare Figs. 1 and 4). In addition, material which has a granular appearance is usually present in the cytoplasm near the poles. This material probably corresponds to the osmiophillic plug material observed in thin sections of heterocysts [25].

The arrangement of the thylakoids was quite variable in both cell types. Figs. 1–3 show thylakoids in vegetative cells. The more frequently observed, highly convoluted arrangement of the thylakoids is shown in Fig. 1. In some cells, the thylakoids occur in parallel concentric sheets as shown in Fig. 2. Similar variability was observed in the arrangement of the thylakoids in heterocysts (Figs. 4 and 5), with parallel thylakoids sometimes occurring near the poles (Fig. 5). Since only about 5% of the cells were heterocysts, and most of the thylakoid fracture faces in these cells were small, it was necessary to isolate

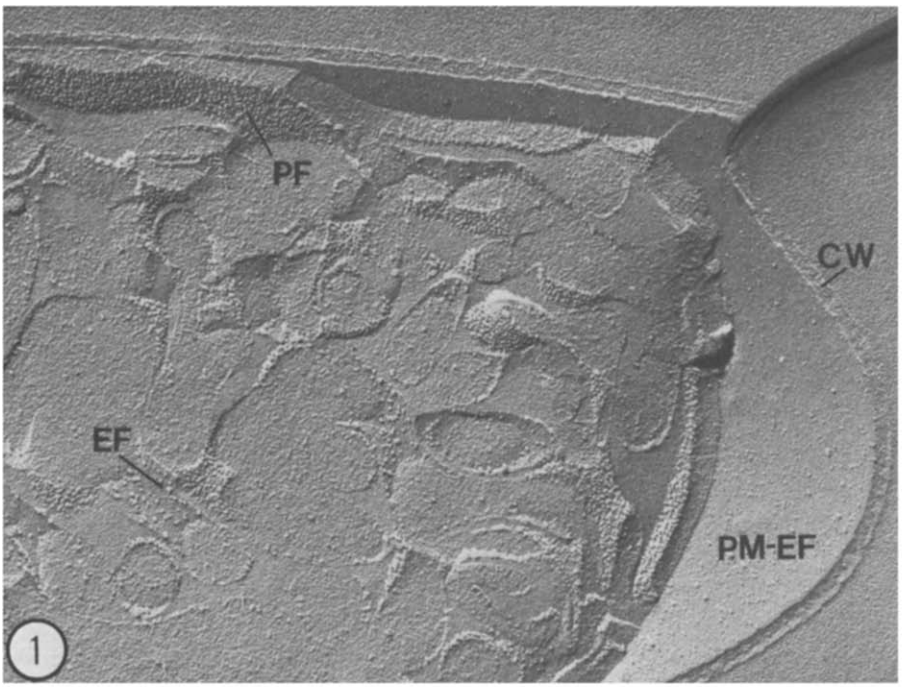

Fig. 1. A freeze-fracture micrograph of a vegetative cell of *A. cylindrica*. Examples of the protoplasmic fracture face (PF) and the exoplasmic fracture face (EF) of the thylakoids are indicated. The cell wall (CW) and the E-face of the plasma membrane (PM-EF) are also shown. Magnification 40 000×.

Fig. 2. Parallel thylakoids in a vegetative cell. The E- and P-faces of the thylakoids and two small areas of the E-face of the plasma membrane (PM), are shown. The width of the white ridge (I) between the E- and P-faces indicates that the intrathylakoid space is extremely narrow. Magnification 100 000×.

Fig. 3. Thylakoids in a vegetative cell showing an unusually extensive alignment of the EF particles for *A. cylindrica*. Magnification 100 000×.

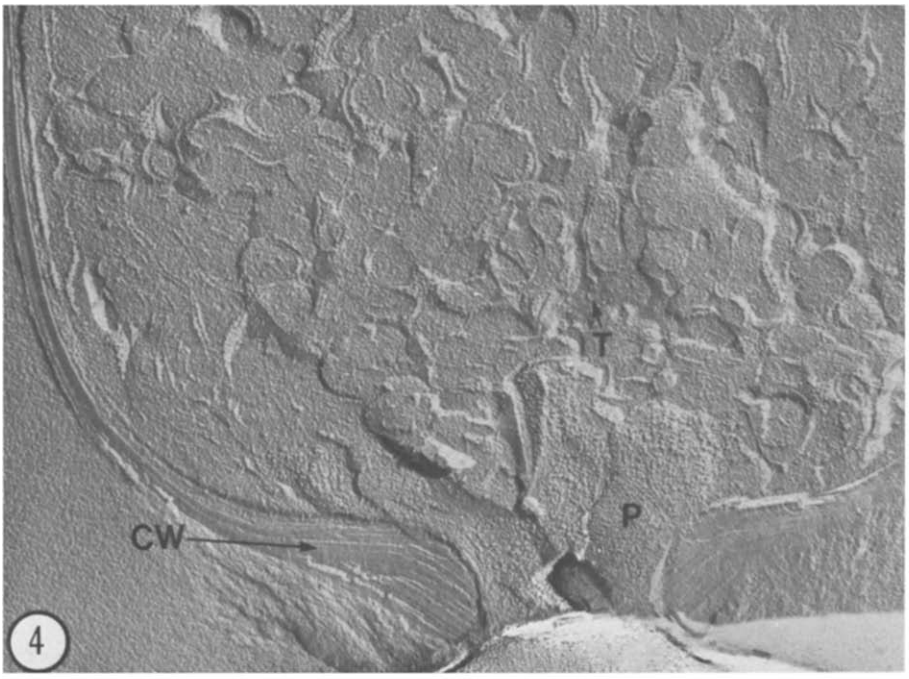

Fig. 4. A heterocyst in an intact filament. The laminated layer of the specialized heterocyst cell wall (CW) is especially evident at the pole. The plug material (P) has a granular appearance. The highly convoluted thylakoids (T) yield very small fracture faces. Magnification 40 000×.

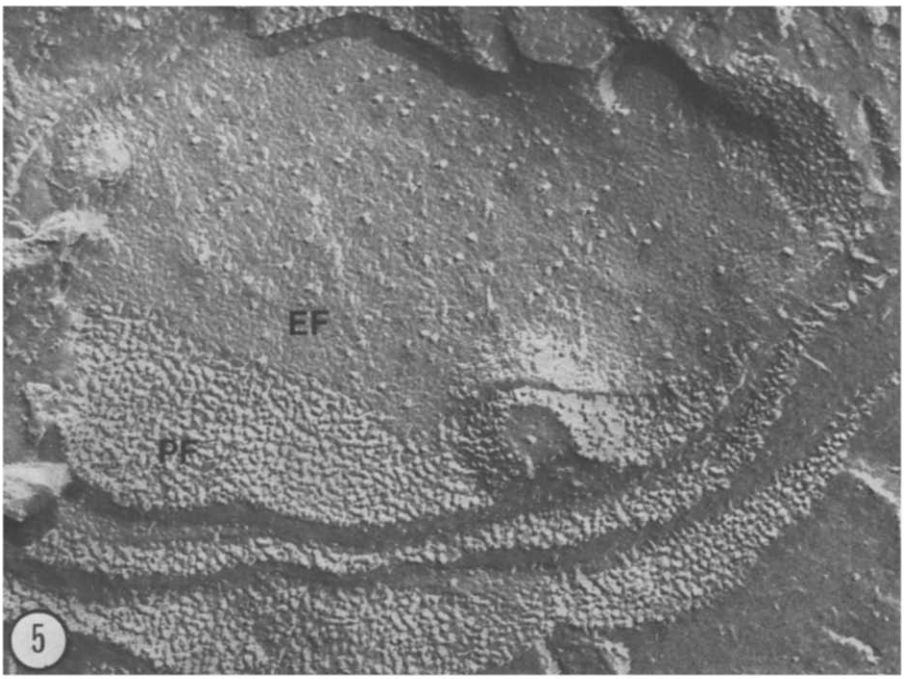

Fig. 5. Thylakoids in an isolated heterocyst. Large fracture faces such as these were rarely observed. Some of our particle density measurements were made on such extensive membrane faces. The intrathylakoid space is virtually undetectable. Magnification, X100 000.

the heterocysts in order to obtain a sufficient quantity of suitable micrographs. The isolation (described in Methods) did not appear to disturb the cytoplasm or thylakoids significantly, although the septa were sometimes broken.

Our analyses of the size and density of particles on the E- and P-faces of thylakoids show that the differentiation of heterocysts from vegetative cells is accompanied by changes in the architecture of the thylakoids. Examples of these fracture faces are shown at high magnification in Figs. 2, 3 and 5. (The freeze-fracture nomenclature is described in Methods.) The results of the particle density determinations are shown in Table I. No difference in particle density was observed on the P-face, both heterocysts and vegetative cells having approx. 5600 particles/μm^2. On the E-face, vegetative cell thylakoids had a mean density of 939 particles/μm^2, while heterocyst thylakoids had 715/μm^2.

TABLE I

NUMBER OF PARTICLES PER μm^2 OF THYLAKOID FRACTURE FACE

Results are expressed as mean ± S.E.

Cell type	E-face	P-face
Vegetative cell	939 ± 57	5643 ± 50
Heterocysts	715 ± 67	5660 ± 52

The difference was statistically significant at the 0.001 level of the Student's *t*-test.

The results of our particle size determinations are shown in Fig. 6. The histograms of thylakoid PF particle diameters show a significant but not a dramatic drop in the mean particle size from 71 Å for vegetative cells to 64 Å for heterocysts. This shift seems to result mainly from the presence of a larger number of particles of the 55–65 Å size classes. The difference between heterocyst and vegetative cell thylakoids is more distinct on the E-face. Here, the mean diameter drops from 95 Å in vegetative cells to 66 Å in heterocysts. The histograms (Fig. 6) show that this difference is primarily due to the absence of particles with diameters of about 100 Å from heterocyst thylakoids. In addition, a very large new category of particles with diameters of about 55 Å is detected on heterocyst thylakoid E-faces. The number of EF particles with diameters of 75 Å is similar in thylakoids of both cell types. Very short rows of EF particles were often observed on vegetative cell thylakoids (Fig. 2), larger rows such as those seen in Fig. 3 only occasionally. Thirty of these aligned EF particles were measured and all appeared to belong to the 100 Å particle size class.

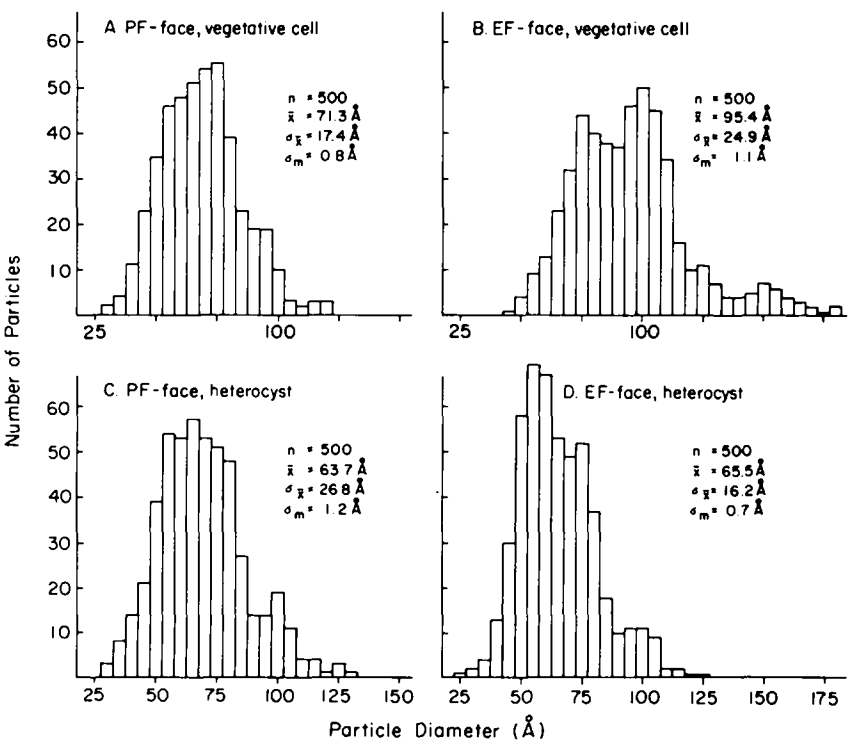

Fig. 6. Particle size histograms of P- and E-faces of heterocyst and vegetative cell thylakoids. n, number of particles measured; $\bar{x}$, mean; $\sigma_{\bar{x}}$, standard deviation; σ_m, standard error of the mean.

Discussion

A considerable body of evidence suggests that heterocysts lack an active PSII complex. Thomas [19] reported that heterocysts generally lack phycocyanin and a chlorophyll absorbance peak considered characteristic of PSII. Donze et al. [20] and Bradley and Carr [12] found that heterocysts do not evolve oxygen and lack the chlorophyll fluorescence properties of PSII. Donze et al. [20] also demonstrated that heterocysts could not photoreduce dichlorophenolindophenol, a reaction catalyzed by PSII reaction centers. Tel-Or and Stewart [14,15] demonstrated that virtually all of the electron transport chain after PSII is present in heterocysts. They produced evidence that a lack of Mn^{2+} at the PSII reaction center could be the critical lesion which prevents oxygen evolution, along with a deficiency of PSII accessory pigments. The results of Flemming and Haselkorn [10], Klein et al. [35], and Sallal and Codd [36] indicate that several peptides present in the thylakoids of vegetative cells are absent from heterocysts. As yet, these peptides have not been identified.

Our results with *A. cylindrica* indicate that changes in freeze-fracture morphology accompany the loss of PSII function from the thylakoids. Since the 100 Å particle size class on the E-face of vegetative cell thylakoids is virtually absent from heterocyst thylakoids, we postulate that it corresponds to the PSII complex. The results of Lichtlé and Thomas [30] suggesting that phycobilisomes are aligned on top of the large EF particles support the same conclusion. It is interesting to note that in our experiments all of the EF particles in rows seemed to be of the 100 Å size class. Thus, it is probable that these particles, but not those of the 75 Å class, are associated with phycobilisomes. Further experimentation is required to confirm this point. The finding that PSII complexes are located on the E-face of freeze-fractured cyanobacterial thylakoids is not surprising in view of the similarity between these and chloroplast thylakoids. In the latter case, considerable evidence suggests that EF particles represent PSII complexes [34,37–43]. Cyanobacterial thylakoids lack the larger EF particle size categories found in higher plant chloroplasts, which have been shown to result from the addition of the chlorophyll *a/b* light harvesting pigment-protein to PSII cores [44]. Like cyanobacteria, red algae lack this chlorophyll *a/b* protein, and have EF particles of about 100 Å diameter [45]. Furthermore, Wollman [46] has recently shown that in *Cyanidium caldarium* the number of 100 Å EF particles corresponds to the number of Photosystem II units in the chloroplast thylakoids.

Our results show that there is a large population of particles on the E-face of heterocyst thylakoids with diameters of about 55 Å. This size class is not evident on the vegetative cell thylakoids. We would like to believe that it represents an enzyme complex which is localized on the heterocyst thylakoids, but absent from vegetative cell thylakoids. As such, components of the nitrogenase system would be the most likely candidates (see Flemming and Haselkorn [10]). Alternatively, it could be a remnant of the PSII complex.

No specific function can yet be attributed to the particles in the 75 Å class which are present in the thylakoids of both cell types. There is evidence, however, that functions other than photosynthesis are localized on cyanobacterial thylakoids. Neushul [47] suggested that the freeze-fracture morphology of

cyanobacterial thylakoids may differ from that of chloroplast thylakoids due to the presence of respiratory enzymes. In this regard, it is interesting to note that the chloroplast thylakoids of red algae lack and EF particle size class at 75 Å, having only one at 100 Å [45]. Bisalputra et al. [48] produced histochemical evidence that respiratory activity is localized on the thylakoids in *Nostoc sphaericum*. Leach and Carr [49] showed that much of the NADPH oxidase activity of *Anabaena variabilis* sediments with a thylakoid fraction following a gentle lysis of the cells but was released to the soluble fraction when cells were lysed by sonication. Biggins [50] did not recover this activity from *Phormidium luridum* after any of several lysis techniques. Glycolate dehydrogenase also appears to be associated with the thylakoids, although it is quite labile [51—53].

Hydrogenase activity has been shown to be at least partially bound to the thylakoids [54]. It is generally agreed that the physiological role of hydrogenase is to recycle H_2 released by the nitrogenase [54—56]. Tel-Or et al. [54] show that hydrogenase activity per mg chlorophyll is several-fold higher in heterocysts than in vegetative cells. The 75 Å particles do not seem to follow this distribution. Further experiments will be undertaken in an effort to correlate a specific freeze-fracture or freeze-etch particle with hydrogenase activity.

The decrease in the mean size of PF particles from 71 Å for vegetative cell thylakoids to 64 Å for heterocyst thylakoids is statistically significant at the 0.001 level of Student's *t*-test. This decrease in size occurs without a net change in the number of particles per μm^2. Among the mechanisms that could contribute to a lower mean particle size are the insertion of more particles of smaller diameter, deletion of some of the larger particles or a decrease in size of some or all of the existing particles. Any of the known differences between the thylakoids of heterocysts and vegetative cells, such as the presence of nitrogenase and hydrogenase, or the reported decrease in PSI unit size [57] in heterocysts, could account for the observed dissimilarity in particle sizes in the P-faces.

Finally, it should be noted that a comparison of the architecture of the thylakoids with that of the plasma membrane [18] reveals significant differences between the two membrane systems in both the size and the densities of the intramembrane particles. In particular, while a large percentage of EF particles on the thylakoids of vegetative cells belongs to the ~100 Å size category, which we have correlated with PSII complexes, the E-face of the plasma membrane of such cells contains only very few particles in this size range. The histograms of EF particle sizes on the thylakoids and the plasma membrane of heterocysts are also clearly dissimilar. These structural differences together with our inability to find direct physical links between thylakoids and plasma membranes in either vegetative cells or heterocysts suggest that thylakoids are not normally derived from the plasma membrane in cyanobacteria as has been proposed by various authors (see discussion in ref. 58).

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