The Greening Process in Cress Seedlings. III. Age-Dependent Changes in the Capacity of the Tetrapyrrole Pathway

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Dedicated to Professor Werner Wehrmeyer on the occasion of his 60th birthday

Lepidium sativum L., Magnesium Protoporphyrin Monomethylester, Protochlorophyllide, Protoporphyrin

Accumulation of protochlorophyllide (Pchlide) was determined after incubation of etiolated seedlings of garden cress (*Lepidium sativum* L.) with exogenous 5-aminolevulinate for 24 h. Young seedlings (start of incubation 0–12 h after imbibition) accumulated only moderate amounts (< 0.2 nmol per seedling) of Pchlide. Maximum capacity of Pchlide accumulation (*ca.* 14 nmol per seedling) was reached at 30–36 h after imbibition. At 60–72 h after imbibition, the capacity of Pchlide formation from exogenous ALA was only 0.2–0.3 nmol per seedling. Uptake of ALA *via* roots was high in young and old seedlings. The carotenoid content and pattern did not change by ALA incubation. Precursors of Pchlide were also accumulated by ALA treatment. Predominant products were protoporphyrin and magnesium protoporphyrin (MgP) in 30 h old seedlings but magnesium protoporphyrin monomethylester (MgPMe) in 72 h old seedlings. The previously described interaction of Pchlide precursors with cab gene expression cannot be correlated with a single porphyrin. A possible correlation with the ratio MgPMe : MgP is discussed.

Introduction

The specific tetrapyrrole precursor 5-aminolevulinate (ALA) has often been used for studies on chlorophyll biosynthesis. The first short report was that of Granick [1] who found accumulation of large amounts of Pchlide and protoporphyrin in etiolated seedlings after incubation with ALA. Subsequently, several authors concentrated upon elimination of the lag-phase of chlorophyll accumulation after incubation with ALA and upon photoconversion of Pchlide which was produced by incubation with ALA [2–7]. A lag-phase of chlorophyll accumulation the duration of which varies with the age of seedlings is normally observed after transfer of etiolated seedlings to continuous white light. The lag-phase can be shortened or eliminated not only by preincubation with ALA but also by a suitable light pretreatment which operates *via* phytochrome (summarized by Kasemir [8]). The main effect of phytochrome is stimulation of ALA biosynthesis under these conditions [8].

Granick [1, 9] had described not only the accumulation of Pchlide and protoporphyrin after incubation of seedlings or leaves with ALA but also accumulation of small amounts of magnesium protoporphyrin (MgP) and magnesium protoporphyrin monoethylester (MgPMe) especially after additional incubation with 2,2′-dipyridyl. Most authors did not pay attention to these additional chlorophyll precursors. In a systematic study, Ryberg and Sundqvist [6] did not find MgPMe after incubation of leaves of etiolated wheat seedlings with ALA alone but considerable amounts of MgPMe after additional incubation with 8-hydroxyquinoline.

We found a differential effect of ALA in etiolated cress seedlings depending on the time of application: ALA given 48 h after begin of germination led to an increase, ALA given 72–96 h after germination led to a decrease of phytochrome-dependent accumulation of cab-mRNA [10]. Such differential effect of ALA depending on the age of seedlings had not yet been described. In order to evaluate whether different metabolites of ALA might be responsible for this differential effect we investigated the pigment content in etiolated cress

Abbreviations: ALA, 5-aminolevulinate; HPLC, high performance liquid chromatography; MgP, magnesium protoporphyrin; MgPMe, magnesium protoporphyrin monomethylester; $MgPMe_2$, magnesium protoporphyrin dimethylester; Pchlide, protochlorophyllide.

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seedlings incubated with ALA at various times after begin of germination. We included also incubation with the ALA precursor glutamate in order to obtain some information on age-dependent changes in the capacity of early and late steps of the tetrapyrrole biosynthetic pathway in cress seedlings.

Materials and Methods

Plant seeds of garden cress (*Lepidium sativum* L., *cv.* Armada) were germinated in petri dishes (diameter 4.5 cm) on filter paper with 1.25 ml dist. water in the dark at 25 °C. After various times of imbibition the seedlings were treated under dim-green safelight either with 5 mM ALA, 5 mM glutamate, 5 mM ascorbate or 2.5 mM glycine and incubated in the dark for further 24 h.

PChl analysis

At the end of the incubation period, 20 cotyledons were harvested under dim-green safelight and extracted with 3 ml dimethylformamide. The Pchl concentration was calculated from the absorption spectrum as reported by Moran [11].

HPLC analysis

Porphyrins

60 cress seedlings were extracted with 2 ml 70% acetone. After centrifugation the residue was reextracted twice with 70% acetone. The porphyrins were separated by extraction from the acetone-phase into CH_2Cl_2. The solvent was removed by distillation under reduced pressure. The residue which was soluble in acetone was analyzed by reversed-phase high performance liquid chromatography. Column (Rosil C 18, 5 µm, 25 cm) operating on a gradient 60% acetone-/40% H_2O pH 3.5 to 100% acetone in 20 min (flow rate 1.5 ml/min).The pigments were detected by fluorescence with various wavelengths (Pchlide excitation: 435, emission: 625 nm; protoporphyrin excitation: 405, emission: 625 nm and MgP, MgPMe excitation: 415, emission: 585 nm). The following retention times were found: MgP 9.1 min, Pchlide 10.2 min, MgPMe 11.4 min, $MgPMe_2$ 13.1 min, protoporphyrin 14.3 min, protoporphyrin monomethylester 16.7 min.

Carotenoids

15 cress seedlings were extracted with 3 ml dimethylformamide for 16 h. The carotenoids separated from the DMF-solution with 8 ml *n*-hexane. The hexane-phase was used to quantify the carotenoids by HPLC (column Nucleosil 100, 5 µm, 12.5 cm) with petrolether 40–60 °C/acetone 65:35. The flowrate was 1.0 ml/min. Detection was performed by absorbance measurement at 440 nm. Authentic carotenoids were prepared and quantitated after Hager and Meyer-Bertenrath [12]. The isolated carotenoids were used for calibration of the HPLC detection system. The following retention times were found: lutein 3.2 min, violaxanthin 4.3 min.

Determination of ALA

After 30 and 72 h of germination the seedlings were treated with either 5 mM glutamate, 5 mM levulinate, 5 mM glutamate plus 5 mM levulinate, or 5 mM ALA plus 5 mM levulinate. 24 h later one part of the seedlings was illuminated with white light for 10 min and then left for 120 min in the dark. For each test 15 cotyledons were extracted with 1 ml 0.1 N trichloroacetic acid. After centrifugation, 500 µl the supernatant was mixed with 50 µl 1 M sodium acetate and 17 µl methylacetoacetate. The mixture was boiled for 10 min. 500 µl of the cooled mixture was given to 500 µl Ehrlich's reagent. The quantity of ALA was determined photometrically at 550 nm. The method was calibrated with authentic ALA.

Synthesis of magnesium protoporphyrin (MgP), its mono- and dimethylester

10 mg of protoporphyrin were dissolved in *ca.* 20 ml of dimethyl sulfoxide and refluxed under N_2. To the heated solution magnesium acetate was added and refluxed until the spectrum shows formation of the magnesium complex. After cooling the solution was shaken with diethylether. Water was added to the mixture until phase separation. The diethylether phase was washed with water, dried with Na_2SO_4, then the diethylether was removed by distillation under reduced pressure. The residue contains MgP with 98% purity. The dimethylester ($MgPMe_2$) was prepared with excess diazomethane, the monomethylester (MgPMe) with equimolar amounts of diazomethane. In both

cases, the solution of diazomethane in diethylether was added to solid MgP. When the evolution of N_2 ceased, the solvent was removed under reduced pressure. The residue was dissolved in acetone and used for HPLC. With equimolar amounts of diazomethane, we obtained a mixture of MgP : MgPMe : $MgPMe_2$ (relative amounts = 1 : 10 : 20).

Synthesis of protoporphyrin monomethylester

1 g protoporphyrin (disodium salt) was dissolved in 5% methanol/H_2SO_4 and left for 24 h at −20 °C. To the mixture *n*-butanol and water were added until the phases separated. The butanol phase was evaporated under reduced pressure until dryness. The residue was dissolved in acetone (6 l) and 400 g silicagel 60 were added. After standing over night the suspension was filtered through a Büchner funnel. Only the monomethylester was adsorbed at the silica gel whereas the dimethylester remained in the acetone solution. The silica gel was washed with acetone. Protoporphyrin monomethylester was recovered from the silica gel by elution with MeOH.

Results

Cress seedlings accumulate increasing amounts of Pchlide in the dark during the first 72 h of germination; the Pchlide amount remains then constant (about 19 ± 2 nmol/100 seedlings) during the next 24 h (Fig. 1, water control). The limiting factor for Pchlide formation is ALA biosynthesis only during the early stage of development (about 12−48 h after imbibition); incubation with ALA leads to a drastic increase in the amount of Pchlide during this period. Relative changes in the capacity of the biosynthetic chain between ALA and Pchlide can be deduced from the data of Fig. 1: Incubation with ALA was started at various times of germination; Pchlide amounts which accumulated within 24 h of incubation with ALA are presented. The capacity for Pchlide formation has a maximum in 30−36 h old seedlings (for beginning of ALA incubation). The amount of Pchlide at the maximum is more than 100-fold (about 1.100%) higher after incubation with ALA than in the water controls. The capacity declines then rapidly. At 66−72 h, ALA incubation leads only to 20% increase of the Pchlide content over the value of the water control.

The effect of ALA is not due to unspecific feeding of the plants with a nitrogen or carbon source: Incubation with glycine or ascorbate yields Pchlide values identical with the values of the water control, incubation with glycine or ascorbate together with ALA yields identical values with those of application of ALA alone (Table I).

The capacity of ALA biosynthesis from glutamate is very small in dark-grown cress seedlings: Incubation with 5 mM glutamate instead of ALA does not lead to an increase of the Pchlide values over the water control levels (Table II). A very small increase was found in the ALA content of young etiolated seedlings after incubation with glutamate (Table III). The ALA level is increased when further metabolization is inhibited by levulinate (Table III, values ± glutamate). The capacity for ALA formation from endogenous precursors or from exogenous glutamate was increased in 30 h old but not in 72 h old seedlings by preirradiation as expected (Table III). But even this increase in capacity did not lead to the same ALA level as the level which was reached by direct incubation

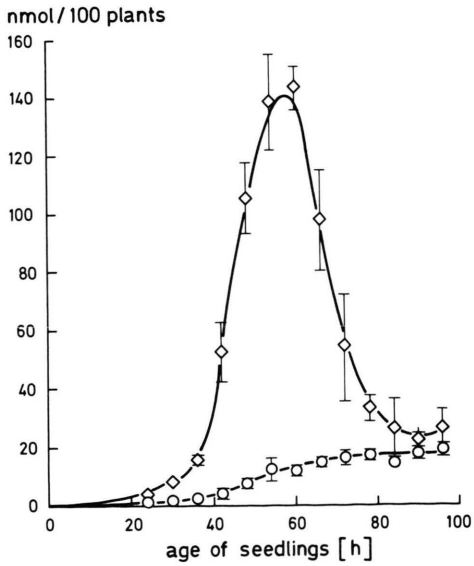

Fig. 1. Accumulation of Pchlide in etiolated cress seedlings. Cress seedlings were grown in the dark for the indicated time (= "age of seedlings"). During the last 24 h of growth, the seedlings were either incubated with 5 mM ALA ( ) or with water ( ). Pigments were extracted from batches of 20 seedlings with dimethylformamide. The Pchlide content was determined by spectroscopy. Means of 5 determinations and standard deviation are given.

Table I. Comparison of Pchlide accumulation with and without glycine or ascorbate. Pchlide was determined at various times according to Fig. 1. Values presented here are percentages based on the corresponding water controls over the whole time range of determinations.

Compound	Percent Pchlide	Number of determinations	Based on (= 100%)
Glycine (2.5 mM)	102.0 ± 13.8	10	water
Ascorbate (5 mM)	95.2 ± 11.9	10	water
Glycine (2.5 mM) + ALA (5 mM)	93.9 ± 17.2	7	ALA
Ascorbate (5 mM) + ALA (5 mM)	91.7 ± 36.5	10	ALA

Table II. Increase of Pchlide in the dark after incubation of cress seedlings with potential precursors. Seedlings were germinated in the dark in water, incubated with the precursor compound in the dark for 24 h and then analyzed for Pchlide.

Start of incubation [h after imbibition]	Precursor compound	Pchlide content [nmol per 100 plants]
18	H_2O	3.5 ± 0.2
	ALA	52.4 ± 9.9
	Glutamate	3.6 ± 0.7
30	H_2O	10.2 ± 1.6
	ALA	138.4 ± 16.4
	Glutamate	9.7 ± 0.9
72	H_2O	16.5 ± 1.2
	ALA	25.7 ± 6.8
	Glutamate	16.9 ± 1.8

with exogenous ALA (Table III). The data show that uptake of ALA through the roots is very effective in etiolated cress seedlings.

Incubation with ALA led to a decrease of lutein (15%) and more drastically of violaxanthin (45%) in etiolated cress seedlings (Table IV). The ratio lutein/violaxanthin is changed consequently from 1.95 (water control) to 2.93 (ALA treated plants).

Besides Pchlide, small amounts of other tetrapyrroles can always be detected in etiolated cress seedlings. For quantitative determination of these compounds, separation by HPLC and detection by fluorescence had at first to be optimized. The system of Witkowski and Halling [13] in which a gradient of ammonium phosphate and methanol is used gave a good separation of porphyrins. However, we experienced problems of corrosion with this system probably due to formation of salt crystals in the presence of the organic solvent. In a salt-free system, a gradient with acetone/water (see Materials and Methods) proved to be more effective than a methanol/water gradient.

Table III. ALA content of cress seedlings determined after incubation with various compounds. Either 30 h or 72 h after start of imbibition, etiolated cress seedlings were incubated with the indicated compound for 24 h. For the test of light induction, plants were irradiated with white light for 5–10 min at 1 h after start of imbibition and then incubated in the dark before analysis.

Start of incubation [h after imbibition]	Compound for incubation	ALA content [nmol per plant] (% of water control) Dark	Light	Factor light/dark
30	Water control	0.80 (100)	1.06 (100)	1.33
	Glutamate	0.96 (120)	1.06 (100)	1.10
	Levulinate	1.41 (176)	2.78 (262)	1.97
	glutamate + levulinate	1.11 (139)	3.72 (350)	3.35
	ALA + levulinate	52.6 (6580)		
72	Water control	0.68 (100)	0.78 (100)	1.14
	Glutamate	0.66 (97)	0.73 (94)	1.11
	Levulinate	0.71 (104)	0.71 (91)	1.00
	glutamate + levulinate	0.72 (106)	0.81 (103)	1.13
	ALA + levulinate	36.4 (5350)		

Table IV. Influence of ALA on the level of the main carotenoids in etiolated cress seedlings. Seedlings were germinated in the dark for 30 h and then incubated with either 5 mM ALA or water for another 24 h. Carotenoids were then extracted from the cotyledons with dimethylformamide, transferred into *n*-hexane and analyzed by HPLC. All values are mean values of 16 analyses in nmol/100 plants.

Compound	Water control	ALA treated
Lutein	19.3 ± 0.8	16.4 ± 2.0
Violaxanthin	9.9 ± 1.8	5.6 ± 1.2
Ratio lutein/ violaxanthin	1.95	2.93

The main tetrapyrrole compound besides Pchlide in 54 h old etiolated cress seedlings is MgP (about 2.4% of Pchlide). This compound cannot be detected in 96 h old seedlings. Its methylester (MgPMe) is the main compound instead (about 0.4% of Pchlide) in 96 h old seedlings. MgPMe occurs also in 54 h old seedlings (1.1% of Pchlide) besides very small amounts of protoporphyrin and protoporphyrin methylester (Table V). Incubation with ALA leads to an increase in the amount of all tetrapyrroles. The increase factor is however different for the various tetrapyrroles and furthermore depends on the age of seedlings for each single tetrapyrrole (Fig. 2). The increase is highest for protoporphyrin (+ 15.800%) and protoporphyrin mono-methylester (+ 5.800%) in young seedlings. The increase of MgP (+ 560%) is similar to that of MgPME (+ 580%) in young seedlings. In 72 h old seedlings, the highest increase induced by incubation with ALA is found for MgPMe (+ 700%) followed by protoporphyrin monomethylester (+ 450%) and protoporphyrin (+ 330%); MgP cannot be detected even after incubation with ALA. Although MgPMe is present in younger and older seedlings in comparable amounts, it is accompagnied by considerable amounts of other tetrapyrroles only in younger seedlings (Fig. 2).

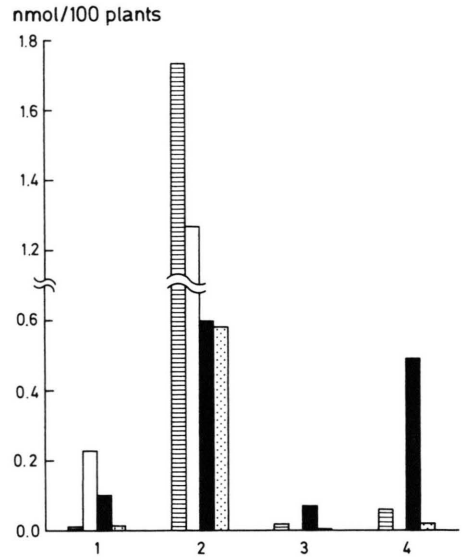

Fig. 2. Accumulation of chlorophyll precursors in etiolated cress seedlings after incubation with ALA. Seedlings were incubated at the age of either 30 h (1, 2) or 72 h (3, 4) with either 5 mM ALA (2, 4) or with water (1, 3). 24 h later, the 54 or 96 h old seedlings were harvested and extracted. The following porphyrins were quantitatively determined in the extracts by HPLC: protoporphyrin □, MgP □, MgPMe ■, and protoporphyrin monomethyl ester □.

Table V. Tetrapyrroles determined in cress seedlings grown in the dark either in water or after incubation with 5 mM ALA for 24 h. Seedlings were then extracted and analyzed for tetrapyrroles by HPLC. All values are percent of Pchlide determined after the same treatment.

Start of incubation	30 h		72 h	
Age of seedlings	54 h		96 h	
	H_2O	ALA	H_2O	ALA
Protoporphyrin	0.1	1.3	0.1	0.2
MgP	2.4	0.9	0	0
MgPMe	1.1	0.4	0.4	1.9
Protoporphyrin mono-methyl ester	0.1	0.4	0.02	0.07
Pchlide	100	100	100	100

Discussion

The capacity of the tetrapyrrole pathway changes dramatically in etiolated cress seedlings in the dark with the age of seedlings. Whereas the capacity of ALA formation from glutamate remains relatively small (and therefore rate limiting) in the dark, the capacity of Pchlide formation from ALA increases to a maximum and decreases again in the dark (see Fig. 1). The time course for this change of capacity corresponds exactly to the time course of the capacity of chlorophyll(ide) accumulation in dark-grown mustard seedlings [14]. Although it is tempting to assume that the same changes in capacity have been measured in both cases one has to

be aware of basic differences between the 2 investigations. Gehring *et al.* [14] did not use any exogenous precursors. Rate-limiting step for chlorophyll formation under those conditions is normally ALA formation. For determination of chlorophyll accumulation, Gehring *et al.* had to irradiate the seedlings with white light which induced the pathway of ALA formation at the same time to a certain extent. We worked in entire darkness so that we can exclude any light-dependent enzyme induction. Stobart and Ameen-Bukhari [15] described a rapid metabolization of exogenous ALA applied to leaf segments of etiolated barley seedlings. The missing increase of ALA after incubation of 72 h old cress seedlings with glutamate (Table III) is not due to rapid metabolization of newly formed ALA: exogenously applied ALA can be detected in large amounts in younger and older cress seedlings (Table III). Since we used exogenous ALA always in excess, we can exclude age-dependent changes in the capacity of ALA formation as explanation for the observed effect. We did not investigate the question which of the enzymes of Pchlide synthesis is primarily changed in its amount or activity. Schneider [16] found a transient activity increase for ALA dehydrogenase and porphobilinogenase (= hydroxymethylbilan synthase) in dark-grown *Helianthus* seedlings. Other enzymes were not investigated.

Age-dependent changes in the capacity of ALA formation from glutamate might also occur. Determination of ALA shows a moderate (about 20%) increase after incubation with glutamate in younger seedlings (age = 30 h at the beginning of incubation) but no significant increase in older seedlings (age = 72 h at the beginning of incubation). This age-dependent increase in the ALA-forming capacity is however much smaller than the light-dependent increase in this pathway (see Table II). The well-known light-dependent increase is regulated *via* phytochrome [17]. Light-regulated changes were not investigated in more detail in the present paper because we concentrated here upon age-dependent changes which occur in the dark. Synthesis of ALA from glutamate is rate limiting for Pchlide synthesis in 30–36 h old cress seedlings. Since ALA feeding leads only to a marginal increase of the Pchlide content in 0–12 h and 60–72 h old seedlings, later steps of Pchlide synthesis must be rate limiting under those conditions.

Watts and Kekwick [18] had described an about 3.3-fold increase of incorporation of isopentenyl pyrophosphate into chlorophyll after incubation of a leaf homogenate of *Phaseolus vulgaris* with ALA. Since the ALA-induced increase of phytol incorporation into Chl was only about 1.7-fold, the authors discussed the possibility that ALA might also act at a stage of the isoprenoid pathway prior to the esterification. According to subsequent investigations [19–21] this could only be the step of geranylgeranyl pyrophosphate formation from IPP. Geranylgeranyl pyrophosphate is the precursor of several compounds including carotenoids. A possible interaction between light-dependent chlorophyll biosynthesis and accumulation of total carotenoids has also been demonstrated [22]. Such an interaction which has been defined as "fine tuning" [23] has been discussed only for the end-product chlorophyll but not for chlorophyll precursors. We find a differential effect of ALA upon accumulation of the single carotenoids in the dark. The reason for decrease in violaxanthin is not clear. If ALA feeding would induce a local decrease of pH at etioplast membranes, activation of deepoxidase would be expected [24]. But we did not find an increase in antheraxanthin or zeaxanthin corresponding to the decrease in violaxanthin. A physiological consequence of the low content in violaxanthin (and other members of the xanthophyll cycle) can be envisaged: The protective role of zeaxanthin which is normally formed from violaxanthin in the light has been described as radiationless dissipation of excess light energy in mature chloroplasts [25, 26]. If such a protection would be functional already in developing etiochloroplasts, the decrease in xanthophylls would increase the photodynamic damage which we found after preincubation with ALA but which had been explained previously only by photosensitization with accumulated porphyrins [27].

Investigation with synchronized cells of *Chlamydomonas reinhardtii* [28, 29] revealed that light-regulated accumulation of cabl mRNA can specifically be inhibited under various conditions, namely treatment with 2,2′-dipyridyl, with cycloheximide or with nitrogen (anaerobiosis). All of these treatments inhibit chlorophyll accumulation and lead to increased levels of chlorophyll precursors. Compounds which inhibit chlorophyll biosynthesis prior to porphyrin formation did not affect

cabl mRNA accumulation. The authors assume that late intermediates of chlorophyll synthesis (preferentially MgPMe) might either destabilize specifically cab mRNA or inhibit its transcription. The authors did not determine the levels of chlorophyll precursors under the applied conditions. We found that the situation might be similar in *Chlamydomonas* and in older etiolated seedlings of *Lepidium* [10]: The light-induced level of cab mRNA is decreased by pretreatment of 3 day old cress seedlings with 2,2′-dipyridyl or ALA. Whereas 2,2′-dipyridyl leads to a general inhibition of transcription in cress seedlings the effect of ALA treatment is specific for cab mRNA. The present investigation shows that the effect of ALA cannot simply be explained by accumulation of MgPMe: ALA leads to increased levels of this compound in older seedlings (in which inhibition of cab mRNA accumulation occurs) and in younger seedlings (in which no inhibition of cab mRNA accumulation can be observed). The only parameter derived from porphyrin analysis which could be correlated with accumulation of cab mRNA is the ratio of MgPMe to other porphyrins (preferentially MgP, but also protoporphyrin and its methyl ester): This ratio is higher in (untreated) older cress seedlings than in young seedlings (see Fig. 2). This corresponds to a lower level of cab mRNA in older than in younger seedlings (dark water controls [10]). The ratio is even higher in older cress seedlings which were pretreated with ALA, in this case the light-induced increase in the cab mRNA level is reduced compared to the untreated water control [10]. Further experiments will show whether this ratio is directly related to accumulation of cab mRNA or whether the correlation discussed here is only accidental.

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