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STUDIES ON THE NATURE OF THE INHIBITORY EFFECT OF TRYPSIN ON THE PHOTOSYNTHETIC ELECTRON TRANSPORT OF SYSTEM II IN SPINACH CHLOROPLASTS

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SUMMARY

The effect of trypsin on the photosynthetic electron transport of spinach chloroplasts has been investigated by measurements of the flash-induced absorption changes, indicating chlorophyll a_I at 703 nm, chlorophyll a_{II} at 690 nm and at 515 nm via electrochromism the electrical potential gradient across the thylakoid membrane, respectively, and of the fluorescence induction caused by moderate actinic light. It was found:

(1) In the presence of benzyl viologen as electron acceptor and with water as natural electron donor trypsin, incubation leads to a complete suppression of the absorption changes of the electrochromic effect and of chlorophyll a_I and chlorophyll a_{II}.

(2) Addition of System I electron donors (*N*-methylphenazonium sulfate plus ascorbate or 2,6-dichlorophenolindphenol plus ascorbate) fully restores the chlorophyll a_I photoreaction, whereas the initial amplitude of the electrochromic absorption change at 515 nm amounts about 50 % of the control value without trypsin. The chlorophyll a_{II} inhibition remains uneffected by System I electron donors.

(3) System II electron donors (benzohydroquinone plus ascorbate or TPB) are unable to overcome the inhibition of electron transport by trypsin.

(4) The fluorescence induction curve in 3-(3,4-dichlorophenyl)-1,1-dimethylurea-blocked chloroplasts is modified by trypsin. The level of maximal fluorescence is remarkably decreased, whereas the initial fluorescence remains constant. The rise in kinetics is slightly decelerated.

From these results, it is concluded that in the linear electron transport from

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Abbreviations: DCIP, 2,6-dichlorophenolindophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; PMS, *N*-methylphenazonium sulfate; Tricine, tris(hydroxymethyl)-methylglycine.

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water to benzyl viologen, mild trypsin treatment specifically attacks System II at a site very close to the reaction center, either on the oxidizing or on the reducing side. The reaction center of System II itself is relatively stable against trypsin. Arguments are presented which argue in favor of the trypsin attack being primarily directed at the reducing side of System II.

INTRODUCTION

Trypsin, a large water-soluble protease enzyme is known to hydrolize peptide chains at arginine and lysine residues only. Because of its size, it cannot penetrate into lipid membranes and therefore, can be used as a fairly mild specific modifier of the outer phase of energy-transducing membranes. Recently, it has been found that trypsin in chloroplasts uncouples as well as inhibits photosynthetic electron transport [1–4]. On the basis of measurements of DCIP photoreduction with either water or artificial System II electron donors it has been concluded that trypsin inhibits System II electron transport at least at two different sites on the oxidizing side of System II [3], whereas System I remains uneffected [2, 3].

The accessibility of the water-splitting enzyme system Y to tryptic digestion would imply that this enzyme is located at least partly near the outer phase of the thylakoid membrane. This would be in accordance with recent results obtained on diazonium benzene sulfonic acid-incorporated chloroplasts [5, 6], but in contradiction to conclusions drawn on the basis of proton liberation measurements in normal uncoupled chloroplasts [7, 8] and on manganese release in Tris-treated chloroplasts [9]. With respect to the action of trypsin, it is interesting to note that according to recent measurements [4] in contrast to many other inhibitors acting on the oxidizing side of System II, such as Tris [10], hydroxylamine [11] or chaotropic agents [12], trypsin does not change the manganese content of spinach chloroplasts.

Hence, the question arises whether trypsin primarily interfers with the oxidizing side of System II, especially with the water-splitting enzyme system Y.

In order to clarify the effect of trypsin on photosynthetic electron transport measurements of the absorption changes of chlorophyll a_{I} [13, 14] and chlorophyll a_{II} [15, 16] indicating the function of the reaction centers of Systems I and II, respectively, of the field indicating absorption change at 515 nm [17, 18] and of the fluorescence induction [19, 20] have been carried out. The obtained results indicate that trypsin digestion blocks the linear electron flow from water to benzyl viologen by interrupting the System II electron transport rather on the reducing side than on the oxidizing side, whereas System I remains unaffected. Furthermore, trypsin leads to an increase of membrane permeability.

MATERIALS AND METHODS

Stripped spinach chloroplasts have been prepared according to the method of Winget et al. [21] as described in part I of this series [22].

The reaction mixture is given in the legends of the figures. Commercially available trypsin by Boehringer has been added to the chloroplast suspension. After

a definite dark incubation time, the measurement was started without addition of trypsin inhibitor.

The measurements of the absorption changes have been performed with a repetitive flash photometer technique as described in ref. 23. Excitation: saturating red light flashes (filter RG1, 4 mm+2 mm KG 2 from Schott) for 515 nm absorption changes and saturating blue light flashes (filter BG 28, 2 mm+2 mm KG 2 from Schott) for absorption changes at 690 and 703 nm, respectively, flash duration approx. 20 μs. The electrical band width ranged from 0 to 37 kHz. The optical pathlength through the cuvette was 1.2 mm, the band width of the monitoring light (grating monochromator) was 5 nm, the light intensity approx. 50 ergs $\cdot$ cm^{-2} $\cdot$ s^{-1}.

Fluorescence measurements have been carried out with the same apparatus. Chloroplasts were excited by actinic blue light, 430 nm, approx. 2000 ergs $\cdot$ cm^{-2} $\cdot$ s^{-1}. Fluorescence intensity was measured at 685 nm (filter DAL 685).

All measurements were made at room temperature.

RESULTS AND DISCUSSION

Fig. 1 shows the initial amplitudes ΔA_0 of the absorption changes at 515 and 703 nm, respectively, as a function of the incubation time of trypsin. The amplitudes

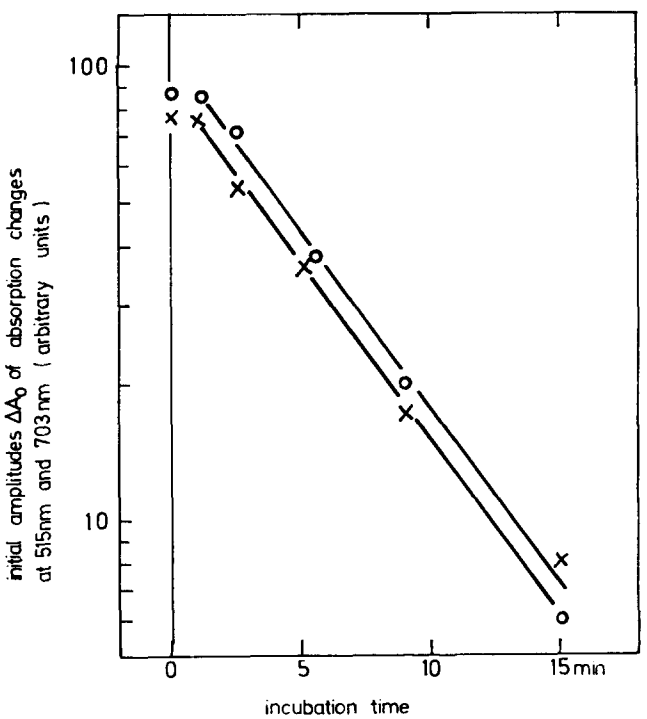


Fig. 1. Initial amplitudes ΔA_0 of the flash-induced absorption changes at 515 nm (×–×) and 703 nm (○–○) as a function of incubation time with trypsin in spinach chloroplasts. Chloroplast suspension: chloroplasts (100 μM chlorophyll), 100 μM benzyl viologen, 20 mM NaCl, 1 mM $MgCl_2$, 20 mM Tricine/NaOH, pH 7.5, 20 μg trypsin/ml suspension. After the dark time indicated on the abscissae, the measurements began. 64 signals were averaged, repetition rate 5 Hz.

ΔA_0 of the 515 nm absorption change that indicate the electrical field across the thylakoid membrane generated by Photosystem I and II, respectively, and of the 703 nm absorption change representing the number of electrons flowing from water to chlorophyll a_I decrease in a parallel fashion and nearly exponentially with incubation time. This indicates that the linear electron transport from water to benzyl viologen is progressively inhibited by trypsin digestion. Addition of tetraphenylboron, known to be a potent System II electron donor [24, 25], and the use of $Na_3[Fe(CN)_6]$ instead of benzyl viologen, do not significantly modify the decrease of the initial amplitudes ΔA_0 of 703 nm absorption change with increasing incubation time of trypsin. This is in contrast to Tris-washed chloroplasts, where tetraphenylboron significantly enhances the amplitude ΔA_0 of 703 nm absorption change [25]. Hence, it can be concluded that the inhibition of the linear electron flow from water to benzyl viologen by trypsin is not mainly caused by a destruction of the water-splitting enzyme system Y, which is the case with Tris washing.

Fig. 2 shows the amplitudes ΔA_0 of the 515 nm absorption change as a function of the incubation time of trypsin in the presence of System I electron donor couple DCIP plus ascorbate. In the presence of trypsin, the initial amplitude ΔA_0 of the 515 nm absorption change decreases with increasing incubation time down to 50–60 % of the control. The decay kinetics are dependent on the trypsin concentration. On the other hand, the amplitudes ΔA_0 of the 703 nm absorption changes remain practically unaffected (not explicitly shown, but see Fig. 3). These results confirm the conclusion that trypsin does not inhibit System I electron transport from

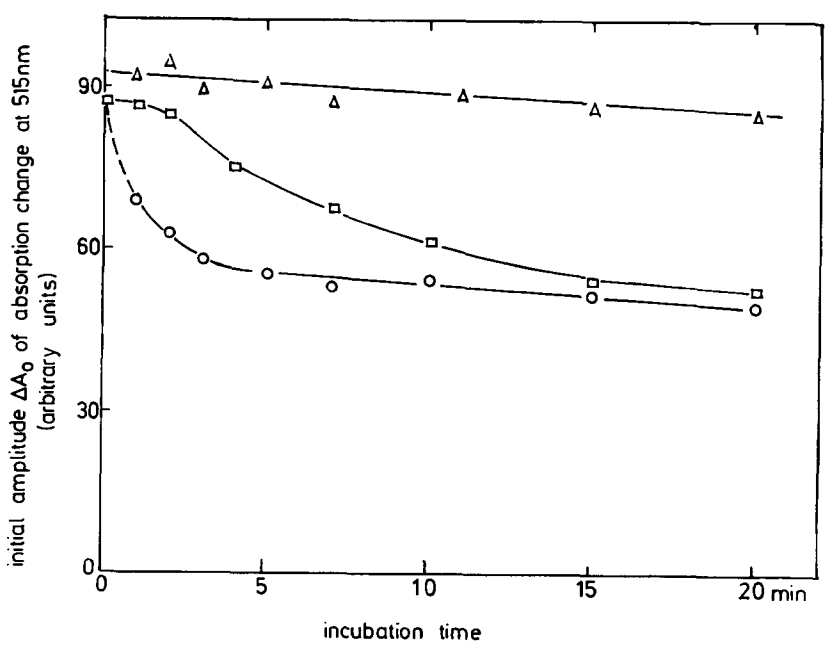
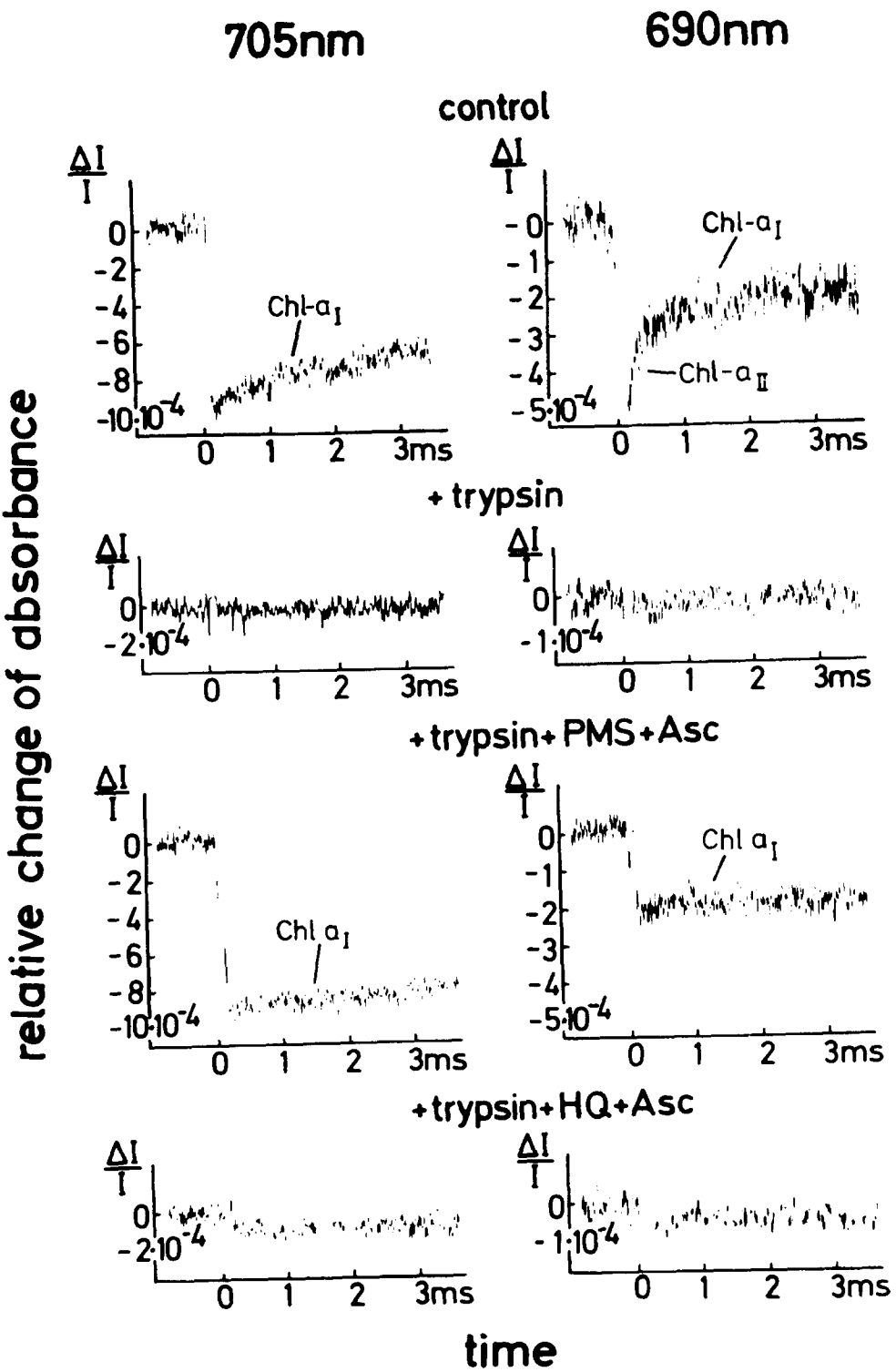


Fig. 2. Initial amplitude ΔA_0 of flash-induced absorption change at 515 nm as a function of incubation time of trypsin in the presence of 50 μM DCIP plus 2 mM ascorbate as System I donor couple in spinach chloroplasts. Trypsin concentration: △–△, no addition; □–□, 20 μg/ml trypsin and ○–○, 200 μg/ml trypsin. Other experimental conditions as in Fig. 1.


705nm
690nm
control
+ trypsin
+ trypsin + PMS + Asc
+ trypsin + HQ + Asc
ΔI/I
Chl-a I
Chl-a II
Chl a I
0
-2
-4
-6
-8
-10·10-4
-5·10-4
-2·10-4
-1·10-4
0 1 2 3ms
relative change of absorbance
time

DCIP plus ascorbate to benzyl viologen [3, 4] and that Photosystems I and II contribute to nearly the same degree to the generation of the electrical field across the thylakoid membrane [17, 18]. Furthermore, it has been found that the decay kinetics of the 515 nm absorption change is progressively accelerated by trypsin incubation (see ref. 37). This indicates that trypsin digestion leads to an unspecific increase of the membrane permeability thereby inducing an uncoupling effect [4].

In Fig. 3, the absorption changes at 690 and 703 nm are compared under different experimental conditions. The 703 nm absorption change ($\tau_{\frac{1}{2}} \approx 15$ ms) reflects the reaction of chlorophyll a_I (for details see ref. 26), whereas at 690 nm the faster components (35 μs, 200 μs) of the absorption change are caused by chlorophyll a_{II} [15, 16] and the slow kinetics (15 ms) indicate chlorophyll a_I (this is explicitly denoted in the top curves of Fig. 3). It is seen that trypsin digestion completely suppresses the absorption changes of both chlorophyll a_I and chlorophyll a_{II}, if benzyl viologen is used as an electron acceptor and water acts as a natural electron donor.* Addition of PMS plus ascorbate known to catalyze System I electron transport [27] completely restores the reactivity of chlorophyll a_I in agreement with the experimental findings of Fig. 2. On the other hand the chlorophyll a_{II} reactions remain blocked. Furthermore, the chlorophyll a_{II} reactivity cannot be restored by addition of benzohydroquinone plus ascorbate, which is known to be a System II electron donor [28]. These results corroborate the conclusion that the interruption of the linear electron transport from water to benzyl viologen by trypsin is not caused by a specific destruction of the water splitting enzyme system Y.

Taking into account all the results presented above there remain three possible sites for the interaction of tyrpsin with the photosynthetic electron transport: (a) trypsin blocks the oxidizing side of System II between chlorophyll a_{II} and the secondary intrinsic donor system which is able to accept electrons from exogeneous artificial System II electron donors or (b) trypsin destroys the reaction center complex of System II, X 320 · chlorophyll a_{II} [22], responsible for the primary photoinduced charge separation or (c) trypsin blocks the reducing side of System II. In order to gain further information about the mode of action of trypsin the fluorescence induction has been measured in DCMU-blocked chloroplasts. Fig. 4 shows the induction curves in the absence and in the presence of trypsin. It is seen that trypsin significantly reduces the variable fluorescence $F_{max}-F_0$ without influence on the initial fluorescence F_0.

Similarly, the area over the fluorescence induction curve is decreased by trypsin ($A_{trypsin}$) to about 40–60 % of the control value, $A_{control}$. Also, the fluorescence rise kinetics are slightly decelerated in the presence of trypsin. As can be anticipated from the failure of System II electron donors to restore the absorption changes of chlorophyll a_I and chlorophyll a_{II}, respectively, in trypsin-incubated chloroplasts these substances do not affect the fluorescence rise even in the presence of DCMU. It has been found that the *Chlamydomonas reinhardi* mutant hfd-49 lacking photochemical

* See Note Added in Proof, p. 285.

Fig. 3. Flash-induced absorption changes at 690 and 703 nm as function of time in spinach chloroplasts. Chloroplast suspension: chloroplasts (100 μM chlorophyll), 100 μM benzyl viologen, 2 mM NH_4CL, 20 mM Tris · HCl, pH 7.2. Other additions as indicated in the figure: 80 μg trypsin/ml suspension, dark incubation 10 min. 20 μM PMS plus 2 mM ascorbate and 30 μM benzohydroquinone (HQ) plus 1 mM ascorbate, respectively. 2048 signals were averaged, repetition rate 10 Hz.

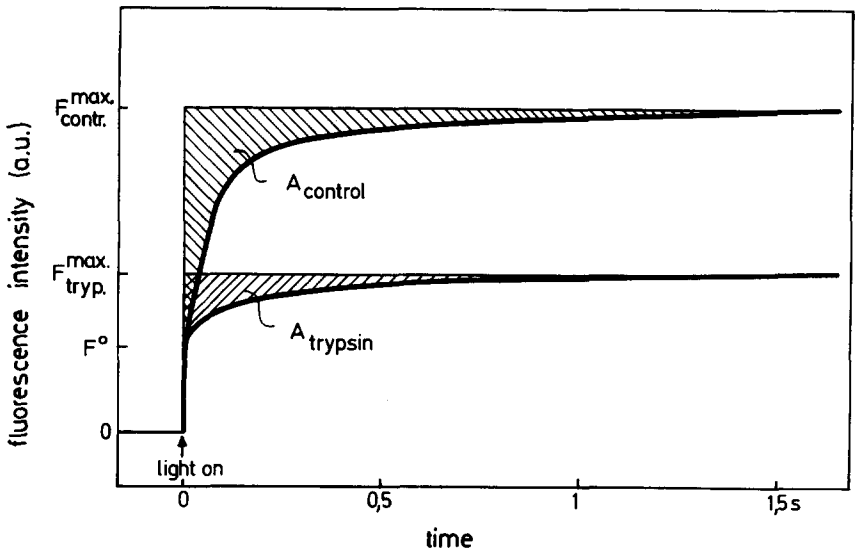


Fig. 4. Fluorescence induction in normal and in trypsin-treated chloroplasts in the presence of 2 μM DCMU. Chloroplast suspension as in Fig. 3, but without benzyl viologen.

activity of System II, does not show any fluorescence induction curve [29]. A nearly complete suppression of variable fluorescence has also been found to occur in chloroplasts incubated with pancreatic lipase [30]. Beyond the modification of cytochrome *b*-559 this treatment concommittantly destroys the *C*-550 component, known to indicate the reaction of the primary electron acceptor of Photosystem II [31]. Hence, the conclusion has been drawn that lipase treatment causes a destruction of the System II reaction center itself. However, contrary to lipase incubation, in trypsinated chloroplasts a significant part of the variable fluorescence remains even under conditions where the electron transport is completely blocked. In this respect it must be emphasized that according to Etienne et al. [32] the area over the induction curve normalized to maximal variable fluorescence, i.e. $(/F_{max}-F_0)^{-1}\int_0^\infty[F_{max}-F(t)]dt$, is linearly correlated to the photochemical quencher capacity even in the presence of external artificial quenchers. Because the normalized area remains nearly invariant to trypsin, it can be inferred that trypsin primarily does not destroy the reaction centers of System II in contrast to lipase.

Hence, the present results indicate that trypsin modifies either the properties of the primary electron acceptor (or acceptors, see. refs. 33 and 34) or that the intrinsic donor system very close to chlorophyll a_{II} is attacked. Recently, it has been found that in chloroplasts damaged on the oxidizing side of System II, a cyclic electron flow around System II occurs [35, 36] so that the chlorophyll a_{II} reaction is not impaired. In the presence of trypsin this effect has not been observed. Furthermore, because it has been inferred that chlorophyll a_{II} is located near the inner phase of the thylakoid membrane [16] it is reasonable to assume that the intrinsic donors that are closest related to chlorophyll a_{II} are also prevented from a fast attack by trypsin added to the outer phase. Hence, it seems to be very probable that trypsin interfers with the functional connection of the primary electron acceptor of Photosystem II with the plastoquinone pool, rather than with the oxidizing side. In a forthcoming paper, a direct proof

will be given for this assumption [37].

In the present study we did not analyze in detail the uncoupling effect of trypsin as is manifested by the increase of membrane permeability. Because trypsin is known also to attack the coupling factor CF_1 [38, 39] the uncoupling effect could be related to a modification of this enzyme system. Recently it has been shown that removal of CF_1 from the thylakoid membrane of spinach chloroplasts significantly enhances the membrane permeability [40, 41].

NOTE ADDED IN PROOF (Received June 3rd, 1976)

Very recently it has been found by Döring [42] that, in the presence of $K_3[Fe(CN)_6^-]$, tyrpsin digestion does not suppress the chlorophyll a_{II} reaction but only that of chlorophyll a_I. This is in agreement with the results reported in ref. 37.

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