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PROTON INVOLVEMENT WITH THE LIGHT-INDUCED HINDRANCE OF SPIN LABEL MOTION IN THE LUMEN OF SPINACH THYLAKOIDS

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Summary

The light-induced hindrance of spin label motion increases linearly with light intensity. However, it has not been possible to unambiguously demonstrate light saturation due to the very high rates of spin label reduction at high light intensity. The light-induced hindrance of spin label motion may be mimicked in the dark by subjecting thylakoids to appropriately low pH regimes. Uncouplers such as gramicidin-D and methylamine reduce the light-induced hindrance to dark levels as does ethylenedinitrilotetraacetate (EDTA) treatment. Valinomycin plus KCl which destroys the electric potential is only partially effective in reducing the light-induced hindrance. These results indicate that protons in the aqueous lumen of the thylakoids are closely involved with the observed light-induced hindrance of spin label motion.

Introduction

Electron paramagnetic resonance (EPR) spectroscopy is now being used to measure internal viscosity, volume and pH of membrane-enclosed aqueous regions of cells and organelles [1—10]. We have been principally interested in measuring relative motion of the membrane permeable spin label 2,2,6,6-tetramethylpiperidine-*N*-oxyl-4-amine (Tempamine) while it resides in the aqueous lumen of the spinach thylakoid [7,8,10]. Our earlier studies [7,8] dealt only with the motion of Tempamine in the lumen of thylakoids in the dark. However, we recently described techniques [10] which allow us to observe the mo-

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Abbreviations: Tempamine, 2,2,6,6-tetramethylpiperidine-*N*-oxyl-4-amine; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

tion of spin labels in illuminated thylakoids. These experiments have not been possible until recently, due to the widespread observation that thylakoids rapidly photoreduce nitroxyl radicals [7,8,10—13] to the corresponding diamagnetic hydroxylamines [13]. This chemical reduction leads to temporally disproportionate line heights in the EPR spectra, making motion analysis far more difficult.

In this paper, we confirm the light-induced hindrance of rotational motion of Tempamine in the lumen of the thylakoids [10] and we show that the observation of the light-induced hindrance seems to require that the lumen be highly protonated. Our findings are discussed in terms of the light-induced conformational changes reported by others [14—21] and the internal buffering capacity of the lumen [22,23].

Materials and Methods

Tempamine and tris(oxalato)chromate(III), (chromium oxalate) were obtained as previously described [8]. Gramicidin and valinomycin were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.).

EPR spectra were obtained from illuminated thylakoid suspensions as has been previously described [10]. Actinic light intensity was varied by changing the voltage applied to the projector filament. Rotational correlation times (τ_c) were calculated as has been described previously [7—10]. EPR signals arising from Tempamine located only in the aqueous lumen of the thylakoid were observed using techniques described elsewhere [7,8]. Typical EPR samples were prepared as described in the legend of Fig. 1.

In illuminated samples, τ_c was calculated following the averaging of spectral parameters as we have described previously [10]. These averaging techniques may only be applied if the line heights and widths are changing in linear fashion. To demonstrate linearity, signal height tracings are obtained with the magnetic field constant at 3242.2 G which corresponds to the midfield line (h_0) and at 3259.2 G which corresponds to the highfield line (h_{-1}). In the light, these tracings show (Fig. 1 of Ref. 10) that the decrease in signal amplitude is linear between about 20 s and 4.5 min during sample illumination. When the midfield line width is plotted against time (Fig. 2 of Ref. 10), one observes a light-induced increase followed by a slower decrease in W_0. The decrease is linear after about 30 s of illumination. Since the total time required for a forward/reverse scan of a sample is 4 min, it is possible to average the values obtained for W_0, h_0 and h_{-1} and to calculate τ_c, as long as the scans of the mid- and highfield lines are recorded over the period of linear change. Fig. 1A of this paper shows typical forward/reverse spectra obtained from thylakoids maintained in the dark. The small decrease in signal amplitude with time does not appreciably affect the calculation of τ_c as seen in Table I. Fig. 1B shows that upon illumination, the signal rapidly decreases. Since a scan takes 2 min, the midfield line is scanned after about 1 min of illumination and it is rescanned (in reverse) after about 3 min of illumination. The highfield line is also scanned twice during 1 and 3 min. Thus, both the mid- and highfield lines are scanned during the period of linear reduction and the averaging techniques may be applied. The average values for W_0, h_0, and h_{-1} are given in Table I and are used

to calculate τ_c. The values give a close approximation of the actual τ_c of Tempamine in the illuminated thylakoid.

Thylakoids were isolated from fresh market spinach leaves (*Spinacea oleracea*) as described in [7]. Chlorophyll concentrations were determined by the methods of Arnon [24].

Ethylenedinitrilotetraacetate (EDTA) treated thylakoids were prepared from typical thylakoid preparations by washing the membrane in 0.1 mM EDTA, 200 mM sucrose and 5 mM Tricine/NaOH (pH 8.0) for 5 min, followed by centrifugation at $3000 \times g$. The resulting pellet was resuspended in the same EDTA buffer to a concentration of 0.1 mg chlorophyll per ml and incubated at 20°C until methyl viologen-mediated photophosphorylation ceased (about 30 min). Electron transport and photophosphorylation were performed as described elsewhere [25].

For the pH studies, control thylakoid preparations were washed two times in the following buffer of varying pH: 200 mM sucrose, 30 mM Tricine, 20 mM KH_2PO_4, 20 mM citric acid, 10 mM KCl and 3 mM $MgCl_2$. Each thylakoid sample was then adjusted to 5 mg chlorophyll per ml with the buffer of appropriate pH.

Results

Fig. 1 shows the forward and reverse spectra of Tempamine from thylakoid suspensions under a variety of conditions. In Fig. 1A, the spectra from dark thylakoids can be observed. Calculations of τ_c from both the forward and reverse spectra yield essentially identical results, as shown in Table I. Apparently, the small amount of dark reduction occurring, which is detected as the reduced magnitude of all line heights on the reverse scan, does not signifi-

TABLE I

Summary of parameters measured and calculated from the EPR spectra in Fig. 1. The water sample contained only Tempamine (pH 7.5) at 3 mM. The thylakoid samples are as described in Fig. 1 as are the EPR settings. $\eta = \tau_c(\text{sample})/\tau_c(\text{water})$.

Spectrum	Condition	h_0	h_{-1}	h_0/h_{-1}	W_0 (G)	τ_c(s) X10^{10}	η
1A	dark, forward	7.25	4.45	1.63	2.13	3.82	9.6
	dark, reverse	6.63	4.11	1.61	2.15	3.76	9.4
	Average	6.94	4.28	6.94 ÷ 4.28	2.14	3.80	9.5
1B	low light, forward	5.20	1.86	2.79	2.98	13.0	33
	low light, reverse	1.90	1.53	1.24	2.88	2.13	5.3
	Average	3.55	1.70	3.55 ÷ 1.70	2.93	8.48	21
1C	high light, forward	2.94	0.72	4.08	2.62	17.4	43
	high light, reverse	*	0.30	*	*	*	*
	Average	*	*	*	*	*	*
1D	like 1B + DCMU, forward	7.94	5.09	1.56	2.15	3.48	8.7
	like 1B + DCMU, reverse	7.69	4.83	1.59	2.09	3.55	8.9
	Average	7.82	4.96	7.82 ÷ 4.96	2.12	3.53	8.8
	Tempamine in water	11.6	10.8	1.07	1.70	0.4	1.0

* Not possible to measure or calculate this parameter.

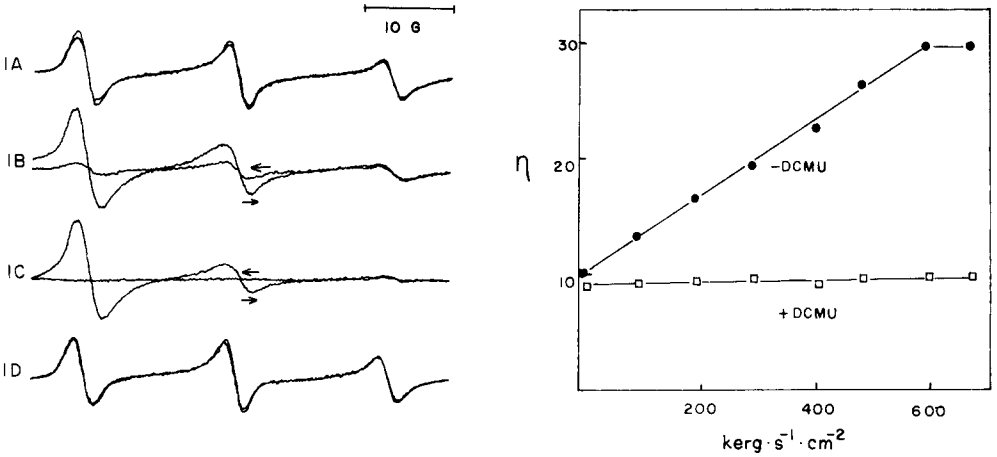

Fig. 1. Forward and reverse EPR spectra from thylakoid preparations under a variety of conditions. Typical EPR samples were prepared as follows: 83 μl of chloroplasts at 5 mg chlorophyll per ml were added to a test tube containing 1 μl of 100 mM ADP, 1 μl 500 mM NaH_2PO_4 and 2 μl of 12.5 mM methyl viologen, all at pH 7.5. Then 3 μl of 100 mM Tempamine and 10 μl of 400 mM chromium oxalate also at pH 7.5 were added. When the inhibition or uncouplers were present, they were added last. Spectra were recorded from 65 μl samples contained in 1 X 100 mm Kimax capillaries in 4 mm quartz NMR sample tubes. Modulation amplitude is 0.125 G, the microwave power is 5 mW and the scan rate is 20 G/min. Higher light intensities are associated with rapid signal loss and measurement of τ_c or η at light intensities above 600 kergs · s^{-1} is essentially impossible. →, forward scan, ←, reverse scan. Thylakoid preparation conditions: A, dark; B, 400 kergs · s^{-1} · cm^{-2}; C, 1200 kergs · s^{-1} · cm^{-2}; D, 400 kergs · s^{-1} · cm^{-2} and 100 μM DCMU.

Fig. 2. Relationship between light intensity and the value of η. Reaction conditions are like those described in Fig. 1. The value of η has not reached clearly saturating levels of light intensity even at 600 kergs · s^{-1} · cm^{-2}. Also, as seen in Fig. 1C, spectral measurements become very difficult at high intensity due to rapid Tempamine reduction.

cantly affect the τ_c calculation. In the light, (Fig. 1B) the nitroxyl reduction is far more rapid as detected by the large differences in the line heights between the forward scan and the reverse scan. This rapid reduction does begin to affect the calculation of τ_c as shown in Table I. The errors are especially large at higher light intensities. Above 600 kergs · s^{-1} · cm^{-2}, the Tempamine signal is essentially gone before the reverse scan has begun and it is impossible to estimate line heights and widths from the reverse scan as can be seen in Fig. 1C. The potent inhibitor of electron transport 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), is able to almost completely inhibit the reduction of spin label in the light. Table I shows that the values for τ_c from the forward and reverse spectra in Fig. 1D remain essentially unchanged.

It is clear from Table I that neither the forward nor the reverse scan of the magnetic field yields a spectrum from which τ_c can be meaningfully calculated when the reduction of the label is rapid. In both scans, the midfield line height and the highfield line heights are temporally disproportionate and so the h_0/h_{-1} ratio is either over or underestimated. In Materials and Methods, we have described techniques which allow the averaging of midfield line heights, highfield line heights, and midfield line widths as long as the rate of spin label reduction is linear during the period of the forward and the reverse scan. These averaged line heights and widths can be used to calculate the τ_c which would be

calculated from an instantaneous spectrum taken at the moment the scan direction changes. Throughout the remainder of this paper, we have chosen conditions where the rates of spin label reduction are linear and where we can apply our averaging techniques to estimate τ_c.

Rotational correlational time (τ_c) is a precisely defined physical property of a rotating molecule wich may be calculated from a first derivative EPR spectrum, but only when all the assumptions incumbant on the equation are realized [26—28]. We do not place emphasis on the actual τ_c under a given set of conditions, but we stress the changes in τ_c of Tempamine in our samples relative to the τ_c of Tempamine in bulk water. We call this relative increase in τ_c the internal microviscosity (η) and define $\eta = \tau_c$ (thylakoid)/τ_c(bulk water).

Fig. 2 shows that in the dark, η is about 9 indicating that the motion of the spin label is hindered about 9 times more than it is in the bulk water. This dark value varies somewhat between thylakoid preparations, but it is usually between 7 to 11. As white light of increasing intensity is applied to the EPR sample, η increases from about 9 to about 30. Unfortunately, the rate of Tempamine reduction also increases. At light intensities beyond 600 kergs $\cdot$ s^{-1} $\cdot$ cm^{-2}, the spectra are not suitable for motion analysis. In subsequent experiments, we have chosen light intensities around 400 kergs $\cdot$ s^{-1} $\cdot$ cm^{-2}. These intensities provide about a 2-fold increase in η over the dark levels and still provide very good spectra for our calculations.

Fig. 3 shows that in the dark, the potent thylakoid uncoupler gramicidin-D, has no effect on η. However, when the thylakoids are illuminated in presence of increasing amounts of gramicidin, the ability of light to induce the higher levels of η is greatly reduced. However, even at very high concentrations of

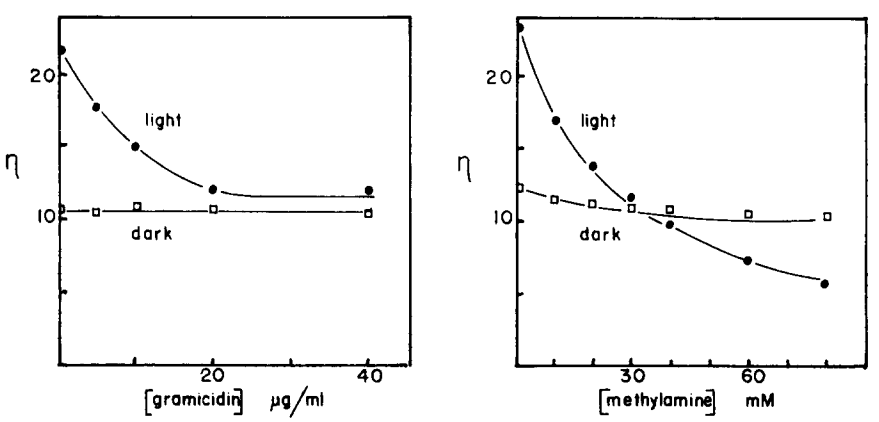

Fig. 3. Loss of the light-induced change in η with the uncoupler gramicidin-D. Maximum reduction in η occurs at about 20 μg/ml gramicidin. This concentration is much higher than what is usually required to uncouple thylakoids (approx. 1 μg/ml) but the chlorophyll concentrations are about 100 times greater than those used for ordinary phosphorylation assays. There is no change in the dark value of η. Gramicidin was added to samples such as those described in FIg. 1.

Fig. 4. Loss of the light-induced change in η with the uncoupler methylamine. The value of η decreases to and even falls below the dark value at 35 mM methylamine. Beyond 35 mM, the thylakoids probably begin to osmotically swell to volumes greater than the normal (dark) thylakoid. There is little change in the dark value of η with increasing methylamine, or in the value of η in the light in the presence of 100 μM DCMU. Methylamine was added to samples like those described in Fig. 1.

TABLE II

Efffect of EDTA treatment on the light-induced change in η. The removal of coupling factor from the thylakoids abolishes the light-induced change in η. DCMU has no further affect on these samples. The right-hand column shows that the EDTA treatment (described in Materials and Methods) totally inhibits any ATP formation. Spin label samples are as described in Fig. 1. Thylakoid preparations were assayed for photophosphorylation as has been described elsewhere [25].

Sample	η(dark)	η(light)	nmol ATP/ml
Normal thylakoids	10.2	22.1	202
Normal +DCMU	10.3	10.3	0
EDTA thylakoids	9.9	10.0	0
EDTA +DCMU	10.1	9.9	0

gramicidin, η in the light does not quite reach the values of η in the dark.

Methylamine is also a potent thylakoid uncoupler, but it uncouples by a different mechanism [29]. Fig. 4 shows that in the dark, η does not respond significantly to increasing concentrations of methylamine. In illuminated thylakoids, however, η decreases with increasing methylamine concentration. In contrast to gramicidin, methylamine reduces η in illuminated thylakoids to values well below those observed in the dark, probably due to methylamine induced swelling of the thylakoids [30]. In the presence of DCMU in illuminated thylakoids, there is no change in η at any of the methylamine concentrations tested.

Since these two different uncouplers (which uncouple by different mechanisms) have profound effects on the observed light-induced η, we were prompted to explore the effects of EDTA treatment on thylakoid preparations. Treatment with EDTA at low ionic strength is known to remove coupling factor from the thylakoid membrane, rendering the membranes completely uncoupled. Table II shows that after EDTA treatment, the thylakoids no longer show a light-induced increase in η. The effectiveness of the EDTA treatment is manifest in the membranes inability to synthesize measurable ATP.

Valinomycin transports potassium ion across the membrane in response to its potential gradient. This potassium transport dissipates the small membrane potential which develops in thylakoids upon illumination. Valinomycin does not effect steady-state photophosphorylation since it does not appreciably effect the pH gradient [31]. Fig. 5 shows that valinomycin does decrease the light-induced microviscosity changes, but not to dark levels as gramicidin, methylamine and EDTA treatment are able to do.

The results described above suggest that a membrane ΔpH may be involved in our observed light induced increase in η. Thus, we wanted to know whether ΔpH was required or whether η was only dependent on the presence of high concentrations of protons in the lumen. Fig. 6 shows that the light-induced microviscosity changes can be mimicked by lowering the pH of the suspending medium in the dark. Between pH 6 through 9, there is no observable change in η. However, as the pH drops to 5.5 through 4.5, η begins to increase. When these same dark samples are illuminated, we see an increase in η to about 21. The light-induced value of η is independent of the medium pH. Unfortunately, at very low pH, the proportion of neutral Tempamine molecules which are available to cross the membrane is decreased dramatically. Therefore, the signal

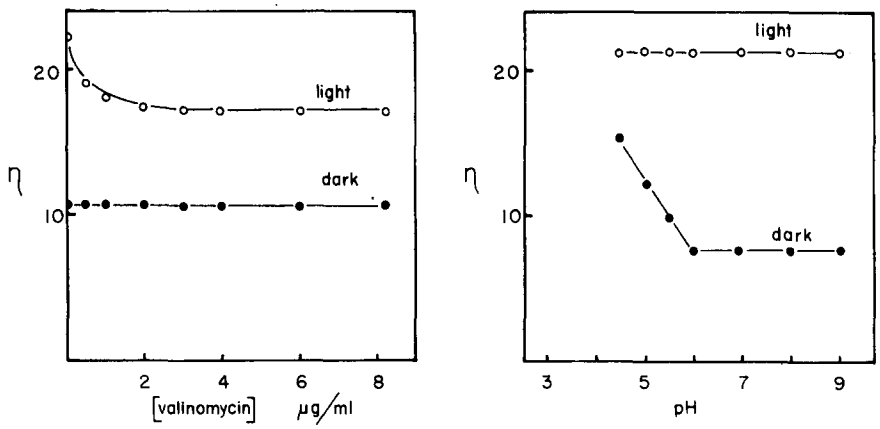

Fig. 5. Decrease in the light-induced change in η with valinomycin. Valinomycin maximally reduces η in the light at about 5 μg/ml, although the total reduction is much less than that observed for gramicidin (Fig. 3). Valinomycin was added to samples like those described in Fig. 1.

Fig. 6. Effect of pH on the value of η in the dark and light. Thylakoids were homogenized normally and then washed three times in buffers of varying pH as described in Materials and Methods. Sample conditions are like those described in Fig. 1. The value of η in the light is independent of the applied pH over the range pH 4.5 to 9.0, but the value in the dark drops between pH 4.5 and 6, and then remains constant through pH 9.0. The spin label will only cross the thylakoid membrane in the unprotonated form. Below pH 4.5, almost all of the label is protonated. Therefore, most of the label remains outside the thylakoid and the internal signal becomes too small to measure.

intensity from the thylakoid interior decreases and below pH 4.5, there is no evidence of Tempamine residing in the lumen.

Discussion

The results in this paper strongly suggest that protons are closely involved with our observed light-induced η. Although we do not have direct evidence at this time, we feel it is likely that the light-induced η also correlates with the decrease in lumen volume and membrane approach observed by others [18,19, 32] in well-coupled thylakoids. Transmission electron microscopy is underway to determine whether uncouplers prevent the light-induced membrane approach.

It also seems clear that the important parameter is the proton concentration in the lumen, not the ΔpH across the membrane. This conclusion is based on the fact that the light-induced η may be mimicked by subjecting the thylakoids to low pH regimes where there is no appreciable ΔpH. Thus, although uncouplers have striking effects on the light-induced change, the effects are not due directly to a disruption of energy coupling mechanisms, rather the effects are due to physical changes of the membrane in response to lumen acidification. These physical changes in the membrane seem to be independent of ΔpH.

We have previously discussed some of the factors which might restrict the motion of Tempamine in the thylakoid lumen and we concluded that the aqueous phase of the lumen was more motionally restrictive than bulk water [7].

When we consider illuminated thylakoids and the observed light-induced changes in η, the interpretation becomes more difficult. In the light, just as we have seen in the dark, there is no evidence of immobilization in our spectra and an intact membrane is absolutely required. But the theoretical considerations must be changed when the membranes come closer together. The first problem is one of interpreting the published micrographs. How far apart are the membranes? Where does the membrane start and the lumen begin? These questions are hard to answer in dark thylakoids when the distances are large, but in the light, when the membranes almost (or perhaps do) touch, the measurements are very difficult to make. The data of Murakami and Packer [18] allow us to estimate that in the dark and in the light the intermembrane distances between opposing thylakoid membranes are about 70 and 30 Å, respectively. If we assume that these are reasonable approximations, then we can consider the possibility of momentary spin label binding to the membrane. Interaction between Tempamine and the thylakoid membrane which may occur during the lifetime of the excited state (10^{-8}s) of the spin label [2] requires that Tempamine be within a certain distance from the thylakoid membrane. This distance (X) can be calculated as we have described elsewhere [7]. For Tempamine in a 1 cP solution, where r = 3 Å and $t = 10^{-8}$s, X is 31 Å. In the dark, if we take into account the observation that Tempamine motion is restricted (by whatever mechanism) by a factor of 10, X is reduced to 14 Å. In the light where motion is restricted by a factor of 20 (at moderate illumination), X is further reduced to 7 Å. This means that in order to account for our results in terms of Tempamine-membrane interactions, only those Tempamine molecules located within 14 or 7 Å of the membrane may interact with the membrane at any given time. If Tempamine is randomly distributed in the thylakoid lumen, then in the dark, about 40% of the intrathylakoid Tempamine is available for interaction with the membrane assuming the thylakoid interior under these conditions is 70 Å across. In the light, the lumen shrinks to 30 Å, but X also decreases, and therefore, the amount of intrathylakoid Tempamine available for interaction is only increased to about 47%. If we further assume that the intrathylakoid space is similar to bulk water, then the remaining 60 or 53%, respectively, of Tempamine would yield an EPR signal characteristic of Tempamine in bulk water. The total signal from the thylakoid under these conditions would consist of a broadened signal from the Tempamine close to the membrane and a sharp signal from the Tempamine in the thylakoid lumen. The signal height of the broadened signal would be greatly reduced compared to the sharp signal due to the relationship between line height and line widths (W^2h = constant). Thus, the sharp signal would predominate in the EPR signal arising from the thylakoid interior and it is from this signal that correlation time and microviscosity are calculated. Therefore, Tempamine interaction with the thylakoid membrane could contribute to the total EPR intrathylakoid signal, and may be reflected in our measurement of η, but we feel that a 7% increase in the probability of Tempamine binding, cannot adequately explain a 2- to 3-fold increase in τ_c.

Thus, we are again left with the conclusion that Tempamine inside the thylakoid is indeed tumbling in a motionally restrictive environment. We have previously suggested [7,8,10] that ordered water could account for our observa-

tions, and that this model was consistent with the proton translocation function of the lumen. We have no new evidence which speaks directly to this question, but the evidence presented in this paper remains consistent with our previous conclusions.

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