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Bicarbonate effects on photo-inhibition

Including an explanation for the sensitivity to photo-inhibition under anaerobic conditions

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Isolated thylakoid membranes were found to be efficiently protected against photo-inhibition by sodium bicarbonate (20 mM $NaHCO_3$) under both anaerobic and aerobic conditions. Furthermore, data are presented which indicate that the pronounced sensitivity to photo-inhibition under anaerobic compared to aerobic conditions is due to the removal of protecting bicarbonate, rather than oxygen, from the medium. A second type of bicarbonate effect on photo-inhibition, in apparent contradiction to the protective effect of added $NaHCO_3$, is that thylakoid membranes that were depleted in their endogenous bicarbonate by treatment with formate were found to be less susceptible to photo-inhibition than thylakoids in the normal non-depleted state.

Photo-inhibition; Bicarbonate; Carbon dioxide; Regulation of photosynthesis; Spinach thylakoid membranes; Mehler reaction

1. INTRODUCTION

This paper is concerned with effects of bicarbonate ions on photo-inhibition. Since carbon dioxide is the ultimate substrate for photosynthesis, bicarbonate ions may well be an important regulating factor, acting on the thylakoid membranes of the chloroplasts in vivo. The background to the investigations presented in this paper, is that in an earlier study [1], isolated thylakoid membranes were slightly illuminated in absence of an electron acceptor in order to evoke phosphorylation of the thylakoid membrane polypeptides. Sometimes we noticed a decrease in the PS2 activity after this illumination (photo-inhibition) and we also discovered that in samples, to which 20 mM $NaHCO_3$ was added before illumination, there was no such decrease in the PS2 activity. We therefore concluded, that this observation indicated a protection against photo-inhibition by added sodium bicarbonate [1,2].

In the present study, I have further investigated the bicarbonate effects on photo-inhibition. Not only do these data confirm that indeed 20 mM sodium bicarbonate ($NaHCO_3$) protects thylakoid membranes against photo-inhibition, under anaerobic as well as aerobic conditions. These data also suggest, that the long known increased sensitivity to photo-inhibition under anaerobic conditions [3] is at least partly explained by a bicarbonate depletion of the medium. Data are also presented which indicate that, in apparent contradiction to the effect of added sodium bicarbonate in protection against photo-inhibition, there is also an other, different type of bicarbonate effect on photo-inhibition, manifested when not only the medium, but also the thylakoid membranes themselves, are depleted in their content of endogenous bicarbonate.

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Abbreviations: LHC2 - light-harvesting chlorophyll *a/b* complex of photosystem 2; PpBQ - phenyl-*p*-benzoquinone; Hepes - *N*-2-hydroxyethylpiperazine-*N*2-ethane sulfonic acid; chl - chlorophyll

2. MATERIALS AND METHODS

2.1. *Preparation of thylakoid membranes*

Thylakoid membranes were prepared from spinach as in [4].

2.2. *Pretreatment of thylakoid membranes*

Before the photo-inhibitory treatment, the thylakoid membranes were pretreated in different ways (details are directly taken from [1], where the effect of bicarbonate on photo-inhibition was first seen). The thylakoid membranes were suspended at a concentration of 600 μg chl/ml in 50 mM Hepes, pH 7.4/5 mM sodium chloride/5 mM magnesium chloride/100 mM sucrose. This suspension was gently N_2-bubbled for 30 min (also before use the medium was bubbled with N_2 gas for 30 min). In the case of photo-inhibition of bicarbonate depleted thylakoids, the N_2-bubbling of the thylakoid membrane suspension described above was performed at pH 5.8 in the presence of 100 mM sodium formate. After the N_2-bubbling the pH was adjusted up to 7.4. In a control experiment the N_2-bubbling was performed at pH 5.8 but without 100 mM sodium formate. After the N_2-bubbling the thylakoid membranes were diluted to 200 μg chl/ml (medium above, N_2-bubbled), and supplied with 0.4 mM ATP and 10 mM NaF and kept in darkness for at least 2 min. Then either 20 mM sodium bicarbonate was added, from a freshly prepared 0.5 M stock

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solution (= sample with added $NaHCO_3$), or the corresponding volume of the N_2-bubbled distilled water that the bicarbonate stock solution was made from (= sample without added $NaHCO_3$). After 2 min the photo-inhibitory treatment was started.

2.3. *Photo-inhibitory treatment*

The thylakoid membranes were illuminated at room temperature, in the absence of any added electron acceptor, with red light (OG 590) of intensity 200 $\mu E/m^2 \cdot s$ or higher. After 10, 20 and 30 min, samples and corresponding dark control samples, were spun down and kept as pellets on ice until activity measurements were performed.

2.4. *Activity measurements*

At least 2 min before measurement of the PS2 activity, the sample pellet was resuspended to 1 mg chl/ml in the assay medium described below, supplied with 20 mM $NaHCO_3$. Determination of PS2 activity was performed in a Clark type of oxygen electrode with 0.4 mM PpBQ as an electron acceptor and saturating red light. The assay medium contained 50 mM Hepes, pH 7.4/10 mM sodium phosphate/5 mM sodium chloride/5 mM magnesium chloride/100 mM sucrose and thylakoid membranes corresponding to 20 μg chl/ml. The PS2 activities were expressed as % of the oxygen evolving capacity for the corresponding dark control samples. The control samples all had activities around 150 $\mu mol\ O_2/mg\ chl \cdot h$.

3. RESULTS

3.1. *Bicarbonate added to the medium protects against photo-inhibition*

In Fig. 1a it is seen that when the thylakoid membranes are assayed for the PS2 activity subsequent to a photo-inhibitory treatment for 30 min, the activity has gone down to 37% of the activity of a corresponding sample kept in darkness. However, if 20 mM sodium bicarbonate ($NaHCO_3$) was added to the thylakoid membrane suspension prior to the photo-inhibitory treatment, nearly 90% of the activity has remained after the same treatment. As was concluded already in [1], this shows that the thylakoid membranes are protected against photo-inhibition by the addition of 20 mM $NaHCO_3$.

The photo-inhibitory treatment resulting in Fig. 1a was applied under anaerobic conditions, obtained by N_2-flushing for 30 min before the illumination. If the photo-inhibitory treatment was performed under aerobic conditions, i.e. without prior N_2-flushing, again the samples supplied with 20 mM $NaHCO_3$ prior to photo-inhibition were less inactivated than the samples without $NaHCO_3$ (Fig. 1b). However, under the aerobic conditions in Fig. 1b the samples without added bicarbonate are less inactivated compared to the corresponding samples under the anaerobic conditions in Fig. 1a, and the effect of $NaHCO_3$-protection therefore appears to be less pronounced.

3.2. *Effect of depletion of bicarbonate from the thylakoid membranes*

Bicarbonate is well-known to bind to one or several sites on the thylakoid membrane (see [5]). Even when 20 mM $NaHCO_3$ is not supplied to the thylakoid membrane suspension as in the experiments described above,

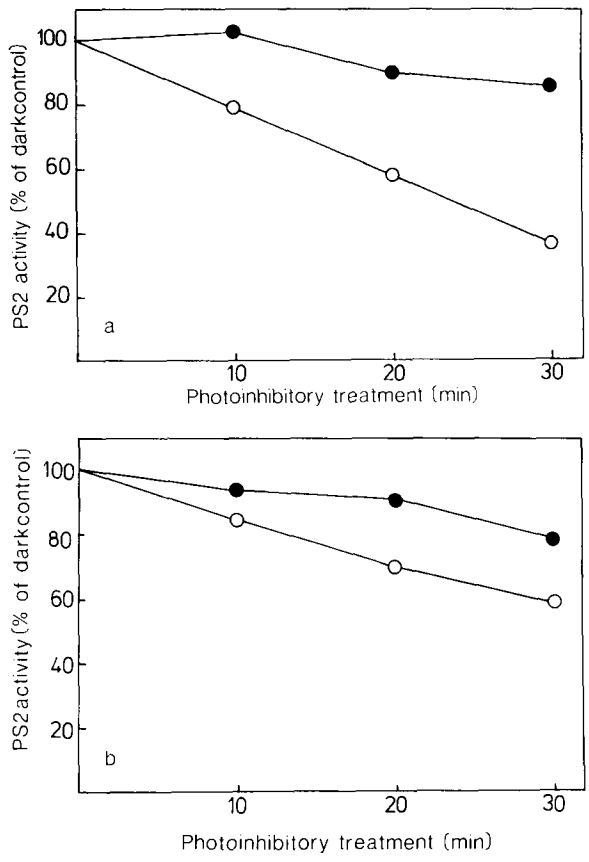

Fig. 1. Photo-inhibition of thylakoid membranes under anaerobic conditions in the presence (●) or absence (○) of added 20 mM sodium bicarbonate ($NaHCO_3$).

the medium in itself still contains some bicarbonate, originating from dissolved carbon dioxide from the air and corresponding to about 0.130 mM $NaHCO_3$ at pH 7.4. Under such 'normal' conditions, certain amounts of bicarbonate ions are bound to high-affinity sites on the thylakoid membranes. Depletion of this endogenous bicarbonate can be obtained by treatment at low pH with excess amounts of sodium formate [6]. In this way formate ions, identical to bicarbonate ions in all respects except for a missing hydroxyl group, replace the bicarbonate ions. To see if it was possible to further increase the $NaHCO_3$ effect in protection against photo-inhibition, seen in Fig. 1, by adding the bicarbonate to thylakoids that were first depleted in their endogenous bicarbonate, the samples were treated by N_2-flushing at pH 5.8 in the presence of 100 mM sodium formate. Thereafter they were subjected to photo-inhibitory treatment in the presence or absence of $NaHCO_3$ (Fig. 2). Surprisingly, this did not lead to the expected increase in bicarbonate protection. Instead it was found that the formate-treated thylakoid membranes that were depleted in endogenous bicarbonate prior to the pre-illumination (Fig. 2a, open symbols) were *less* susceptible to photo-inhibition than were the non-depleted control thylakoid membranes (Fig. 2b,

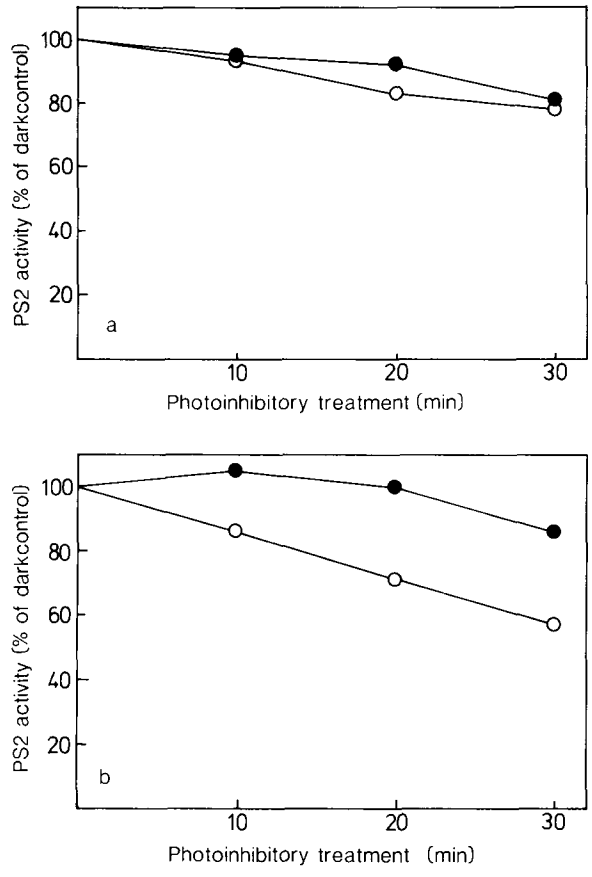

Fig. 2. Photo-inhibition of thylakoid membranes depleted in endogenous bicarbonate by formate treatment prior to photo-inhibititory treatment (a) and non-depleted control thylakoid membranes (b), in the presence (●) or absence (○) of added 20 mM $NaHCO_3$.

open symbols). Addition of 20 mM $NaHCO_3$ before the photo-inhibitory treatment however showed a protective effect again, both to the only slightly inhibited depleted thylakoid membranes (Fig. 2a, closed symbols) and to the non-depleted control membranes (Fig. 2b, closed symbols).

The endogenous bicarbonate ions are required for normal rates of electron transport [5,7]. Depletion of endogenous bicarbonate with formate ions inhibits electron transport and this inhibition is reversed upon readdition of $NaHCO_3$. Separate activity measurements were performed on the depleted (= pH 5.8 and 100 mM formate) and the non-depleted (= pH 5.8 or 7.4 with no formate) thylakoids, which showed that the PS2 activity of the depleted thylakoids were indeed stimulated by bicarbonate while non-depleted control thylakoid membranes were not (Table I). These data also indicate, that even if N_2-flushing to obtain anaerobiosis removes CO_2 and thus bicarbonate from the medium, it does not affect the endogenous bicarbonate ions much.

3.3. *Thylakoid membranes are sensitive to photo-inhibition under anaerobic conditions because of bicarbonate depletion*

It is known since the discovery in 1962 by Trebst [3] that under anaerobic conditions thylakoid membranes become photo-inhibited at much lower light-intensities than under aerobic conditions. The information that addition of bicarbonate protects against photo-inhibition, especially after N_2-bubbling of the sample, suggests that the reason for the sensitivity to photo-inhibition under anaerobic conditions, is not due to anaerobiosis per se but to a depletion of bicarbonate from the medium. To test this idea, thylakoid membranes were N_2-flushed for 30 min to obtain anaerobic conditions. Thereafter, the thylakoid membrane suspension was either directly subjected to the photo-inhibitory treatment (Fig. 3, open circles) or made fully aerobic again by 10 min flushing with air, which was freed from HCO_3^- by passage through a solution of 50% KOH, and thereafter subjected to the photo-inhibitory treatment (Fig. 3, open squares). It is clearly seen, that the readdition of oxygen into fully aerobic conditions did not have any effect at all on the degree of photo-inhibition. Contrastingly, in a sample still anaerobic but supplied with 20 mM $NaHCO_3$ (Fig. 3, closed circles) a pronounced protection against photo-inhibition occurs.

Table I

Measurement of the bicarbonate stimulation of PpBQ-dependent PS2 activity as an estimation of the degree of depletion of the endogenous bicarbonate

	PpBQ-dependent PS2 activity (μmol O_2/mg chl·h)		
N_2-flushing of thylakoids with:	Activity measured with no $NaHCO_3$	Activity measured with $NaHCO_3$	$NaHCO_3$ stimulation of activity
pH 5.8 and formate	53	210	3.8
pH 5.8 (no formate)	177	174	1.0
pH 7.4 (no formate)	173	159	1.1

Analysis of the degree of depletion of endogenous bicarbonate in the thylakoid membranes described in Fig. 2 was done in the following way: After 30 min N_2-flushing, before any $NaHCO_3$ was added and before the photo-inhibitory treatment was started, samples of 33 μl each were withdrawn, directly pipetted into the reaction vessel of the oxygen electrode and their PS2 activity assayed with PpBQ as acceptor. To estimate the degree of bicarbonate depletion obtained in the 3 cases, the activity was determined with and without reactivation of 20 mM $NaHCO_3$ in the assay medium.

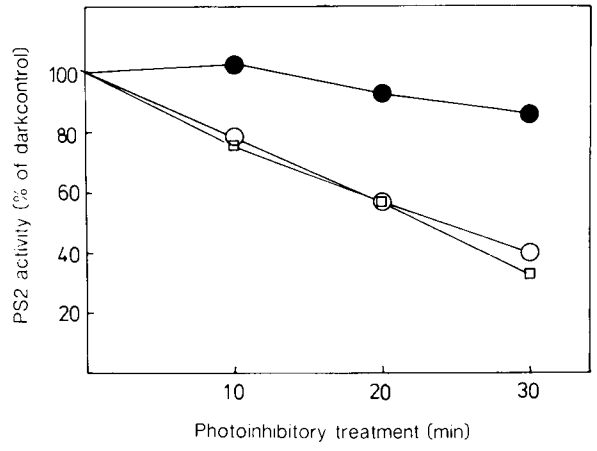

Fig. 3. Photo-inhibition of thylakoid membranes that are N_2-bubbled to obtain anaerobic conditions (○) and of thylakoid membranes that subsequent to the N_2-bubbling but before the photo-inhibitory treatment were flushed with CO_2-free air back into aerobiosis (□) or supplied with 20 mM $NaHCO_3$ (●).

Even if the anaerobic conditions in these experiments were not so called strictly anaerobic using an enzymic oxygen trap [8,9], it is quite clear that the oxygen readded to the anaerobic samples above has not any effect at all in protection against photo-inhibition, thus giving strong support to the idea, that upon flushing with nitrogen or argon gas to obtain anaerobiosis, the resulting sensitivity to photo-inhibition is due to the removal of carbon dioxide (and thus bicarbonate), rather than of oxygen, from the medium.

3.4. *Further characterization of the protective bicarbonate effect on photo-inhibition*

That bicarbonate protects against photo-inhibition was first seen in connection with studies on thylakoid protein phosphorylation [1], thus, in the presence of ATP. Illumination of thylakoid membranes in presence of ATP is known to cause phosphorylation of LHC2, which in turn causes a decrease in the antenna size of PS2 [8–12]. We found that both phosphorylation and antenna size decrease was further accentuated by bicarbonate [1]. This may of course affect the amount of light arriving into PS2 upon illumination. In other words, one possible explanation for a less pronounced photo-inhibition in presence of bicarbonate could be that the antenna size of LHC2 becomes decreased due to phosphorylation and that less light therefore reaches PS2. To test if this is so, the bicarbonate effect on photo-inhibition was also tested using a protocol where no ATP was added prior to the photo-inhibitory treatment (Fig. 4). This result shows clearly that the $NaHCO_3$ protective effect is not dependent on the presence of ATP and therefore the action of $NaHCO_3$ cannot be explained by a decreased LHC2 antenna.

That the $NaHCO_3$ effect is not related to the antenna size is in agreement with the data obtained when the intensity of photo-inhibitory light was increased. In Fig. 5 the photo-inhibitory treatment was applied for 20 min to samples with and without added $NaHCO_3$ and the light intensity of the photo-inhibitory light was varied from 200 up to 1000 $\mu E/m^2 \cdot s$. The degree of photo-inhibition increases with light intensity, both with and without $NaHCO_3$. However, protection by added $NaHCO_3$ is considerable and the difference between samples with and without $NaHCO_3$ is much the same over the whole range of light intensities.

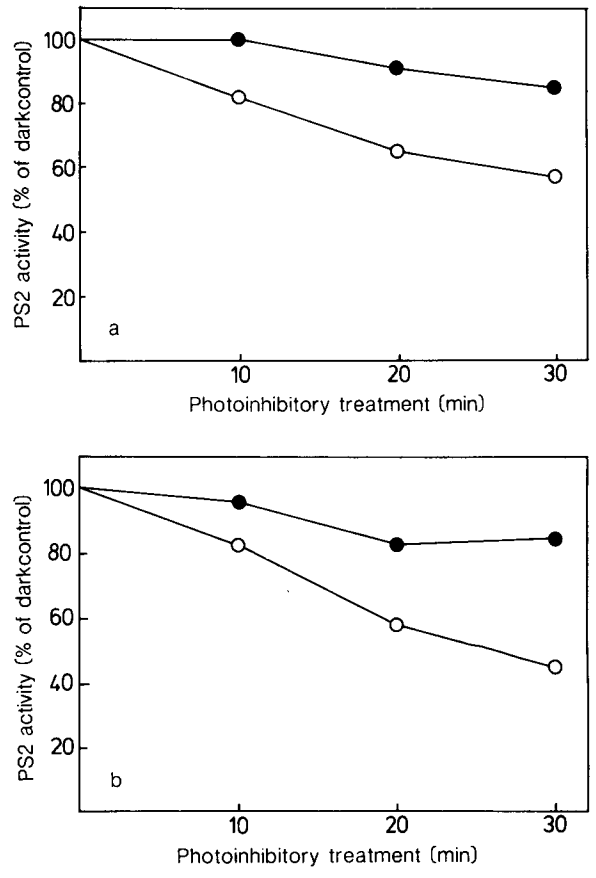

Fig. 4. Photo-inhibition of thylakoid membranes in the presence (a) or absence of ATP (b), and in the presence (●) or absence (○) of added 20 mM $NaHCO_3$.

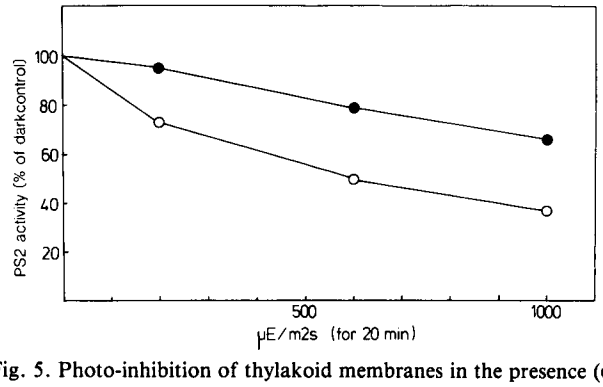

Fig. 5. Photo-inhibition of thylakoid membranes in the presence (●) or absence (○) of added 20 mM $NaHCO_3$ as a function of increasing intensity of the photo-inhibitory light.

It should also be mentioned that the protective effect of bicarbonate seems quite specific in that 20 mM Na_2SO_4 had absolutely no, and 20 mM Na_2HPO_4 no or a very small, protective effect (data not shown).

4. DISCUSSION

There seem to be two different bicarbonate effects on photo-inhibition. One is the effect seen under both anaerobic and aerobic conditions, when 20 mM $NaHCO_3$ is supplied to either normal or depleted thylakoid membranes and clearly counteracts photo-inhibition. In apparent contradiction is the other effect, seen when thylakoid membranes are depleted in their endogenous bicarbonate by formate treatment, leading to a lesser susceptibility to photo-inhibition compared to non-depleted thylakoid membranes. At present it is not obvious how to explain this paradoxal role of bicarbonate in photo-inhibition. It may be related to the recent proposal by Blubaugh and Govindjee of two sites of high-affinity bicarbonate binding per reaction center [5]. While the presence of a bicarbonate ion in the first binding site (A) is suggested to be a pre-requisite for keeping the reaction center in a functional configuration, the second binding site (B) is suggested to be involved in the protonation of Q_B^-. Upon donating a proton, the bicarbonate ion of this site should turn into a carbonate ion, CO_3^{2-}, and be exchanged with a new bicarbonate ion from the intrathylakoid HCO_3^- pool. Thus, the first bicarbonate effect on photo-inhibition could reflect an importance of a high concentration of bicarbonate ions in the medium for such an exchange with the bicarbonate of the site B. The second bicarbonate effect on photo-inhibition, induced by the formate treatment, could perhaps rather influence the functioning of the bicarbonate ions of site A.

The first bicarbonate effect could also explain the difference in sensitivity to photo-inhibition between aerobic and anaerobic conditions in terms of the presence or absence of around 0.130 mM bicarbonate in the medium. The sensitivity of photo-inhibition after N_2-flushing of the samples has been known since 1962 [3], but the mechanism for this phenomenon has remained unclear. Although not experimentally proven, it has generally been assumed that under aerobic conditions, oxygen acts as an electron acceptor in a Mehler reaction and thus protects the thylakoid membranes against photo-inhibition (see [8]). However, the re-addition of oxygen to the anaerobic sample in Fig. 3 clearly did not counteract photo-inhibition and thus, rather, than thinking of the aerobic case as a state where the thylakoid membranes are especially protected by oxygen or anything else, we may regard the anaerobic case as an especially sensitive state, due to the depletion of bicarbonate from the medium. In addition, no experimental evidence to support a Mehler type of protection has been seen in the experimental system used in this study in that no oxygen consumption was detectable during the course of a photo-inhibitory treatment, nor did the presence of the efficient electron acceptor PpBQ during the photo-inhibitory treatment reduce the degree of photo-inactivation (data not shown).

The bicarbonate effects on photo-inhibition of thylakoid membranes are important and new observations in themselves. They may be a new approach in investigations on the mechanism of photo-inhibition and the functioning of the reaction center of PS2. At this stage it is not clear how the bicarbonate effects on photo-inhibition presented here are related to the several observations of bicarbonate effects that have been made over the years (see [5]). For a thorough characterization of the bicarbonate effects on photo-inhibition, we are currently conducting a set of experiments where the content of oxygen and bicarbonate is carefully measured and varied systematically under controlled conditions.

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